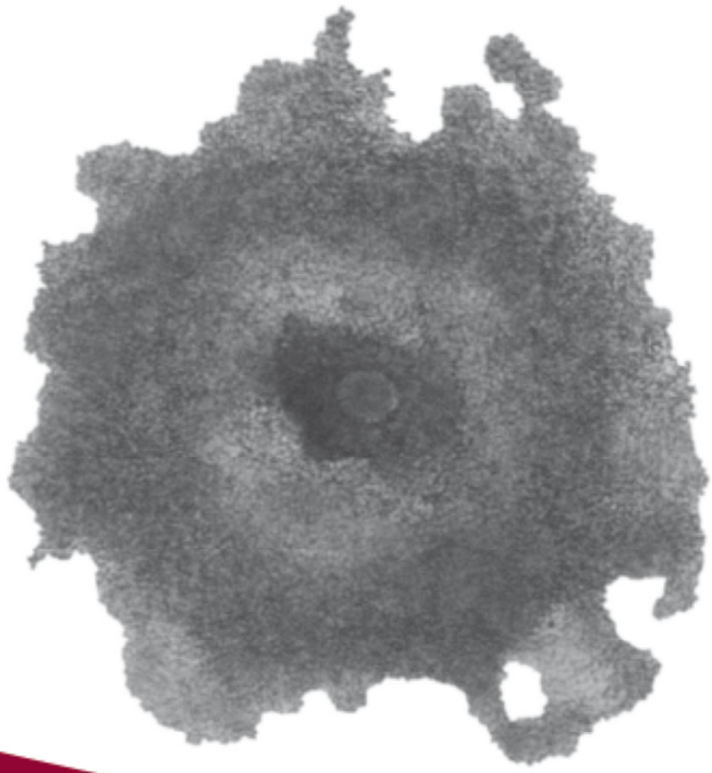
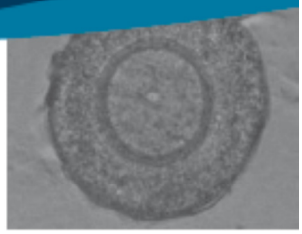
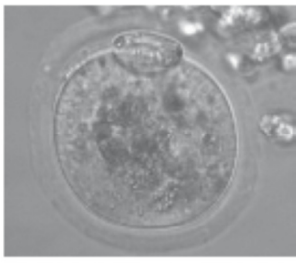
Maternal metabolic disorders and fertility: insights into the effect of a changed maternal micro-environment on follicular growth, the acquisition of oocyte developmental competence and subsequent embryo quality

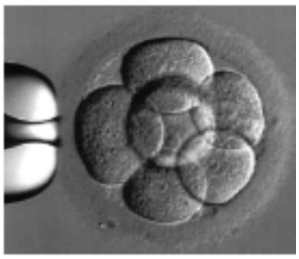
Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Diergeneeskundige Wetenschappen aan de Universiteit Antwerpen te verdedigen door

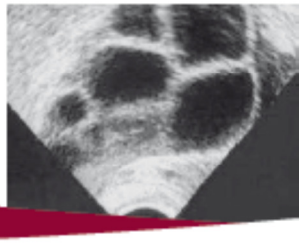
Sara Valckx











Supervisors
Prof. dr. Jo Leroy
Prof. dr. Peter Bols

Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen
Departement Diergeneeskundige Wetenschappen
Antwerpen 2015

Universiteit Antwerpen

It is the mark of an educated mind to be able to entertain a thought without accepting it.

Voor Jeroen en Jade
Bedankt om deze weg samen met mij te bewandelen.

ISBN: 9789057284724

(depotnummer D/2015/12.293/1)

Vormgeving cover: Sara Valckx en Anita Muys.

Dit onderzoek werd gesubsidieerd door een BOF-UA beurs van de Universiteit Antwerpen.

Het drukken van dit werk werd mede mogelijk gemaakt door middel van de financiële steun van de firma's medvet AML diergeneeskunde, Olympus, Fiers en De Bruyne Instruments.

Universiteit Antwerpen

Faculteit Biomedische, Farmaceutische en Diergeneeskundige Wetenschappen

Departement Diergeneeskundige Wetenschappen

Maternal metabolic disorders and fertility:

insights into the effect of a changed maternal micro-environment on follicular growth, the acquisition of oocyte developmental competence and subsequent embryo quality

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Diergeneeskundige Wetenschappen (PhD)
aan de Universiteit Antwerpen
te verdedigen door

Sara VALCKX

Supervisors
Prof. Dr. Jo L. M. R. Leroy
Prof. Dr. Peter E. J. Bols

Antwerpen, 2015

TABLE OF CONTENT

ABBREVIATIONS

A	Antral
AC	Adenylate cyclase 1
ACCA 1	Acetyl CoA carboxylase
AMH	Anti-Müllerian hormone
ANOVA	Analysis of variance
Apo	Apolipoprotein
AREG	Amphiregulin
ART	Artificial reproductive technology
ATP	Adenonisetriphosphate
BAX	Bcl-2 associated X protein
BCL2	B-cell lymphoma 2
BELRAP	Belgian register of assisted procreation
BMI	Body mass index
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BTC	Betacellulin
cAMP	cyclic Adenosine mono phosphate
CC	Cumulus cell
cGMP	cyclic Guanosine mono phosphate
CHE	Cholesteryl-ester
CLU	Clusterin
COC	Cumulus-oocyte complex
COX2	Cyclo-oxygenase 2
CRP	C-reactive protein
CPT1B/2	Carnitine palmitoyl transferase 1B/2
CYP19A1	Cytochrome P450 19A1, aromatase
D	Diffuse
D/A	Diffuse/antral
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DNMT3A 1	DNA (cytosine-5)-methyltransferase 3A1
DOHaD	Developmental origin of health and disease
E2	Estradiol
EGA	Embryonic genome activation
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERCC1	DNA excision repair protein ERCC1
EREG	Epiregulin
ERK	Extracellular signal-regulated kinase
F	Follicular
FA	Fatty acid
FABPpm/FATP	Plasma membrane associated fatty acid binding protein/fatty acid transport protein
FADH2	Flavin adenine dinucleotide
FAME	Fatty acid methyl ester
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FBS	Fetal bovine serum
F/D	Follicular/diffuse
FF	Follicular fluid
FSH	Follicle stimulating hormone
G6PD	Glucose-6-phosphate dehydrogenase
GADD45B	Growth arrest and DNA-damage-inducible beta
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATM	Glycine amidinotransferase, mitochondrial

GDF	Growth differentiation factor
GLUT 1/4	Glucose transporter type 1/4
GnRH	Gonadotrophine releasing hormone
GPX1	Glutathione peroxidase 1
HAS2	Hyaluronan synthase 2
HBP	Hexosamine biosynthetic pathway
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
GnRH	Gonadotropin releasing hormone
hCG	human Chorionic gonadotropin
hMG	human Menopausal gonadotropin
HSL	Hormone sensitive lipase
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
IGFBP	IGF-binding protein
IMM	Inner mitochondrial membrane
IRS-1	Insulin receptor substrate 1
ITS	Insulin-transferin-selenium
IU	International unit
IVF	*In vitro* fertilization
LDA	Linear discriminant analysis
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LHCGR	Luteinizing hormone/choriogonadotropin receptor
MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA
MAPK	Mitogen activated protein kinase
MCT1-4	Monocarboxylate transporter 1-4
MGC	Mural granulosa cell
mnSOD	manganese Superoxide dismutase
mRNA	messenger Ribonucleic Acid
NA	Not applicable
NAD+/NADH	Nicotinamide adenine dinucleotide
NADPH/NADP+	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid
NRF2	Nuclear factor like 2
NRP1	Neuropilin 1
NS	Not significant
OA	Oleic acid
OMM	Outer mitochondrial membrane
OPU	Oocyte pick-up
OxPh	Oxidative phosphorylation
P4	Progesterone
PA	Palmitic acid
PAPP-A	Pregnancy-associated plasma protein A
PCOS	Polycystic ovarian syndrome
PDE	Phosphodiesterase
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
PGE2	Prostaglandin E2
p.i.	post insemination
PKA	Protein kinase A
PL	Phospholipid
PN	Pronuclei
PPARGC1B	Peroxisome proliferator-activated receptor gamma coactivator 1 beta
PPP	Pentose phosphate pathway

PTGS2	Prostaglandin-endoperoxide synthase 2
PTX3	Pentraxin-related protein PTX3
PUFA	Poly-unsaturated fatty acid
PVP	Polyvinylpyrrolidinone
qRT-PCR	quantitative Real-time polymerase chain reaction
(r)QUICKI	(revised) Quantitative insulin sensitivity check index
REDOX	Reduction/Oxidation
RGS2	Regulators of G protein signalling 2
RIA	Radio immuno assay
ROS	Reactive oxygen species
rRNA	ribosomal Ribonucleic acid
SA	Stearic acid
SD	Standard deviation
SEM	Standard error of the mean
SHBG	Sex hormone binding globulin
SLC2A1-3,6,9,12	Solute carrier family 2, member 1-3, 6, 9, 12
SLC27A1	Long-chain fatty acid transport protein 1
SPE	Solid phase extraction
SOF	Synthetic oviductal fluid
TC	Theca cell
TCA	Tricarboxylic acid
TE	Trophectoderm
TG	Triglyceride
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TP53	Tumor protein 53
tRNA	transfer Ribonucleic acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPR	Unfolded protein response
ZP 2/3	Zona pellucida sperm-binding protein 2/3

CHAPTER 1: GENERAL INTRODUCTION

1. Facts and figures about subfertility and metabolic disorders

What's in a name? That which we call a rose, by any other name would smell as sweet
– William Shakespeare.

What does it mean to be fertile? Couples with a child wish would define it as getting pregnant and bringing the baby to term, while embryologists may say it is the oocyte's capacity to be fertilized and develop until the blastocyst stage. Others might state that it is only when live and healthy offspring are present, that a couple is fertile. In this thesis we look at fertility from the perspective of the oocyte that must grow and mature to a point where it has acquired developmental competence. Developmental competence of the oocyte, also termed oocyte quality, is defined as the oocyte's ability to resume meiosis, cleave after fertilization, develop to the blastocyst stage, induce a pregnancy, bring it to term and give rise to normal and healthy offspring (Sirard *et al.*, 2006).

However, many things can go wrong during the well-orchestrated process of oocyte growth and maturation, leading to an incompetent oocyte, which might lie at the origin of **infertility**. Infertility in women is defined as:

> *A disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2009).*

The World Health Organization states that the global burden of infertility has not increased since 1990 (Mascarenhas *et al.*, 2012). However, due to population growth, the absolute number of couples affected by infertility increased from 42.0 million in 1990 to 48.5 million in 2010 (Mascarenhas *et al.*, 2012), resulting in an increased need for fertility treatment. The following definition of fertility treatment, also termed artificial reproductive technology (ART), was put forward:

> *All treatments or procedures that include the in vitro handling of both human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy. This includes, but is not limited to, in vitro fertilization and embryo transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy. ART does not include assisted insemination (artificial insemination) using sperm from either a woman's partner or a sperm donor (Zegers-Hochschild et al., 2009).*

Our national registration system, called BELRAP (Belgian Register of Assisted Procreation), collects freely available data concerning fertility treatment in Belgium from 1989. Some interesting facts and figures from their last report (2011, revised in 2014) concerning fresh own cycles (embryo transfer without gamete donation or prior embryo cryopreservation), are listed below (http://www.belrap.be/Public/Reports.aspx):

- Indications for ART are female in 20%, male in 35% and both in 20% of the cases. The remaining part of infertility causes is unknown.
- Major female pathologies leading to infertility include tubal defects (40%), endometriosis (30%) and ovulatory disorders (40%). Factors like premature ovarian failure, genetic anomaly and immunological reactions account for a small amount of the explained female infertility causes.
- Male pathologies consist mainly of sperm abnormalities (98%), but genetic anomaly and immunological reactions have also been described.
- The major reason for no embryo transfer after ovum-pick up, is the lack of a transferrable embryo (non-viable embryo, 50%).
- In 40% of all the fresh own cycles, one or several embryos can be cryopreserved.
- Overall, 25% of the aspiration cycles (30% per embryo transfer) leads to a clinical pregnancy. On the other hand, only approximately 20% actually leads to a delivery.
- Mean implantation rates are 28% when the mother's age is below 35 and 17% when it is beyond 35 years of age. Birth rate decreases from 22% below 35 to 11% beyond 35 years of age.

Several risk factors for subfertility have been documented, such as advanced maternal age (Liu and Case, 2011), maternal metabolic disorders (obesity, diabetes type 2) (Pasquali *et al.*, 2007), endocrine disrupting chemicals (Marques-Pinto and Carvalho, 2013) and environmental pollution or smoking (The Practice Committee of the American Society for Reproductive Medicine, 2004, Buck Louis, 2014). **Obesity** is of special interest because it has become a

global epidemic, with 35% of all adults over 20 years of age being overweight and 11% obese, according to a worldwide survey in 2008 (World Health Organization, 2014). Generally, normal weight is defined as 18 ≤ body mass index (BMI, kg/m²) < 25, while an individual is overweight when 25.0 ≤ BMI < 30.0 and obese when BMI ≥ 30.0 (World Health Organization, 2014). Our national data from 2008 show that 47% of the adult population (≥ 18 years old) suffered from a too high body weight, of which 33% were overweight and 14% obese. The prevalence of overweight was higher in men (54%) than women (40%). No difference in obesity was found between men and women. Obesity is an important life-style disease, that can have a strong impact on the quality of life. Besides the increased risks for cardiovascular disease (mainly heart disease and stroke), diabetes, musculoskeletal disorders and some cancers, obesity is associated with an increased risk for reproductive failure (World Health Organization, 2014). More specifically, obese women present with an increased risk for irregular cycles, chronic anovulation, miscarriage and preterm delivery (Pasquali *et al.*, 2003, Jungheim *et al.*, 2013a). The time to pregnancy is increased in obese women, even if there are no cycle irregularities (Law *et al.*, 2007, Wise *et al.*, 2010). Furthermore, obese women require higher dosages of gonadotropins to stimulate the same number of follicles to grow during controlled ovarian hyperstimulation (Marci *et al.*, 2012) and have a reduced chance for success during ART (Luke *et al.*, 2011, Koning *et al.*, 2012). In this regard, mature oocytes from obese women have a lower potency to be fertilized, compared to the oocytes of normal weight women (Shah *et al.*, 2011), suggesting that the oocyte has not acquired developmental competence. Furthermore, obese women are less likely to achieve a clinical pregnancy after *in vitro* fertilization (IVF) (Luke *et al.*, 2011), are at higher risk of miscarriage after ART conception when BMI exceeds 35kg/m² (Thum *et al.*, 2007) and are less likely to bring the baby to term (live birth) (Luke *et al.*, 2011). Importantly, the costs of fertility treatment in obese women is higher, compared to normal weight women (Koning *et al.*, 2010), resulting in an increased economic burden. This, together with the known risk factors of obesity during ART and pregnancy, has led to a strong belief amongst physicians that fertility treatment should have restrictions based on body weight (Maheshwari *et al.*, 2009, Koning *et al.*, 2010, Pandey *et al.*, 2010, Harris *et al.*, 2011). However, many studies present conflicting results. There are studies that indeed show a detrimental effect of obesity on fertility and ART, while other studies are unable to find such an effect (reviewed by Koning *et al.,* 2012). This is why Koning *et al.* (2012) stated that overweight and obesity itself should not be a reason to withhold ART. However, obese women seeking ART should be made aware of all the known risk factors related to their metabolically compromised status. Even more so, modest weight loss, due to lifestyle intervention (healthy

diet, increase of physical activity and behavioural modification) is associated with restoration of ovulation in anovulatory women and improves the likelihood of a pregnancy (Clark *et al.*, 1995, Norman *et al.*, 2004). So, moderate weight loss should be encouraged.

Overall, the reproductive disorders caused by obesity originate from the effect of obesity on the whole body, including the reproductive system, comprising follicular recruitment and development, the acquisition of oocyte developmental competence, embryo development and implantation (Jungheim *et al.*, 2013a). Importantly, there is evidence, originating from the morphological grading of human oocytes and embryos and from *in vitro* animal studies, that obesity alters the **quality of oocytes and embryos** (Robker, 2008). Due to ethical restraints in using human material, many of these data are provided by animal models. Indeed, several animal models, like dietary induced obese mice, hypercholesterolemic rabbits and cows in negative energy balance, have been used to study the quality of oocytes and embryos in relation to maternal metabolic health (Leroy *et al.*, 2005, Igosheva *et al.*, 2010, Jungheim *et al.*, 2010, Picone *et al.*, 2011). Specific interest has been taken in dairy cows, because nutrient prioritizing towards milk production is associated with an altered metabolism and often results in a negative energy balance (Leroy *et al.*, 2008a). Such an altered metabolism in the dairy cow has been associated with infertility, due to defects in hormonal patterns, endocrine pathways, folliculogenesis and even oocyte and embryo quality (reviewed by Leroy *et al.*, 2008a, Leroy *et al.*, 2008b). Furthermore, the bovine model provided evidence that serum metabolic changes are reflected in the maternal micro-environment and such changes may affect oocyte developmental competence and subsequent embryo quality *in vitro* (Leroy *et al.*, 2004, Leroy *et al.*, 2005, Chagas *et al.*, 2007, Wathes *et al.*, 2007, Leroy *et al.*, 2012, Wathes *et al.*, 2013).

These data suggest that an altered maternal metabolism may have repercussions for the oocyte's micro-environment, potentially affecting oocyte developmental competence and subsequent embryo quality, which may contribute to the development of infertility in obese women. Therefore, the following hypothesis was put forward:

> **Maternal metabolic disorders, like obesity, are associated with changes in the serum profile, that are reflected in the maternal micro-environment of the pre-ovulatory follicle. These changes alter folliculogenesis and oocyte growth, ultimately leading to a reduced oocyte developmental competence and subsequent embryo quality.**

As this thesis looks at fertility from the perspective of the oocyte, it is important to keep in mind that a healthy oocyte is a prerequisite for optimal embryonic development and also the birth of healthy offspring. Put in other words, a healthy oocyte lies at the basis of being fertile.

Ex ovo Omnia, all things come from the egg – William Harvey.

2. Safeguarding the oocyte's developmental competence: folliculogenesis, oocyte growth and the interaction between the oocyte and the somatic cells of the follicle

In order to comprehend how maternal metabolic disorders may affect the complicated and well-orchestrated processes involved in oogenesis, folliculogenesis and the acquisition of oocyte developmental competence, a thorough understanding of follicular dynamics, follicular development and how the oocyte acquires developmental competence, is essential. Furthermore, knowledge about the processes involved in fertilization and early embryo development provides insight into the potential consequences of adverse maternal metabolic health on early embryo quality.

a) Follicle and oocyte development

Considerable similarities exist in most mammalian species and therefore animal models have been used to study human ovarian physiology and pathologies, including the regulation of follicular growth and the acquisition of oocyte developmental competence. The following description holds true for most mammalian species, but is specified for human.

Primordial follicles

During embryonic growth, primordial germ cells migrate to the forming gonad and become oogonia that proliferate by means of mitosis for several cell cycles. When **meiosis I** (Greek for diminution or lessening) is initiated, these cells are called primary oocytes. Primary oocytes are surrounded by one layer of flattened pre-granulosa cells and a basement membrane, together forming the gonadotropin-independent **primordial follicle** (for review, see Aerts and Bols, 2010, Collado-Fernandez *et al.*, 2012, **Figure 1**). In contrast to what was believed once, these primordial follicles are not completely quiescent. In the bovine, they express hundreds of genes that fulfil housekeeping and signalling functions (Scaramuzzi *et al.*, 2011). Because primordial follicles have numerous receptors and ligands for growth factors of the transforming growth factor (TGF)-β super family and estradiol, it is possible that several growth-promoting factors (both positive and negative) are involved in the initiation of follicular growth, a process that remains incompletely understood (Scaramuzzi *et al.*, 2011). Several factors have been identified that appear to act as repressors of follicle activation, for example anti-Müllarian hormone (AMH, Durlinger *et al.*, 2002, Fair, 2010). In the mammalian oocyte, during the *meiotic S phase*, chromosomes are replicated and are tightly bound together so that each chromosome consists of two sister chromatids at the start of *prophase I*.

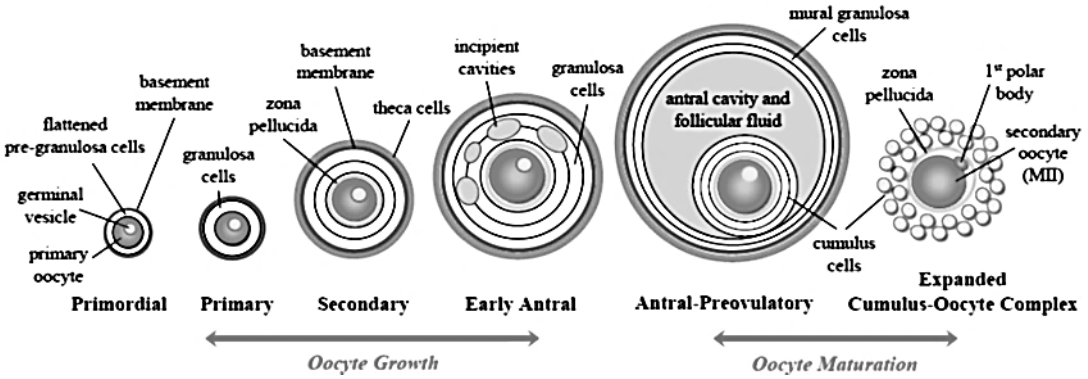


Figure 1: Stages of mammalian follicular and oocyte development (Collado-Fernandez *et al.*, 2012).

The duplicated paternal and maternal homologs (including two replicated sex chromosomes) pair up alongside each other and exchange genetic information through a process called genetic recombination. During the diplotene stage of *prophase I*, oocytes become arrested (germinal vesicle stage) until sexual maturity is achieved and a hormonal stimulus initiates the resumption of meiosis. However, the mammalian oocyte still grows and gradually differentiates during this period of time (Fair, 2003, Alberts *et al.*, 2008, Fair, 2010).

Primary and secondary follicles

Primordial follicle activation involves the stimulation of granulosa cells to resume mitosis and become cuboidal in shape. The oocyte's cytoplasmic organelles also start to proliferate and differentiate (Fair *et al.*, 1997, Fair, 2010). Follicles are now called **primary** follicles (**Figure 1**), which are characterized by a full layer of proliferative cuboidal granulosa cells (Picton, 2001). Interestingly, in mice it has been shown that the transition from primordial to primary follicle growth is accompanied by an upregulation of the expression of members of the TGF-β superfamily, including bone morphogenetic protein (BMP15), growth/differentiation factor (GDF) 9, BMP5, BMP6, TGF beta 2 and TGF beta 3, which have stimulatory effects on the primordial to primary follicle transition (Pan *et al.*, 2005). Granulosa cells continue to differentiate and theca cells start surrounding the follicle (Young and McNeilly, 2010). The oocyte also grows, with a major increase in the volume of smooth endoplasmic reticulum (ER), mitochondria, ribosomes and lipid droplets. Follicles are called **secondary** when the oocyte is surrounded by two or more layers of granulosa cells (**Figure 1**), a theca interna containing luteinizing hormone (LH) receptors and zona pellucida material (Aerts and Bols, 2010, Collado-Fernandez *et al.*, 2012). Cortical granules are synthesized within the oocyte's cytoplasm (Fair *et al.*, 1997, Fair, 2010). Also the presence of follicle stimulating hormone (FSH) receptors has been documented during this follicular stage in cattle and sheep (Tisdall *et al.*, 1995, Xu *et al.*, 1995), rendering these follicles gonadotropin-responsive (Fair, 2010).

Tertiary follicles

Mammalian follicles develop from the secondary stage to the **tertiary** stage, in which a follicular fluid filled antrum becomes the environment that sustains the growing oocyte with its surrounding granulosa cells (Sutton *et al.*, 2003, Aerts and Bols, 2010, Collado-Fernandez *et al.*, 2012, **Figure 1**). These follicles are gonadotropin-dependent, because inadequate support from gonadotropins leads to atresia. The period of transition from secondary to tertiary stages of oocyte growth is characterized by intensive mRNA and rRNA transcription and an almost quadrupling of oocyte volume and further proliferation of the oocyte organelles (Fair, 2003, Fair, 2010). The establishment of the antrum filled with follicular fluid is described in detail below (see: '*Follicular fluid formation'*). The follicular fluid separates two different types of granulosa cells, namely 1) the mural granulosa cells with a principal steroidogenic role, lining the follicular basement membrane and 2) the cumulus cells, directly surrounding the oocyte. At the end of this growth phase, the oocyte's transcriptome becomes quiescent, the nucleolus restructures to an inactive state and the cytoplasmic organelles migrate towards the oocyte cortex (Fair *et al.*, 1997). Transcriptional activity continues until the follicle reaches 2-3mm and the oocyte reaches a diameter of 100µm in the cow (Fair, 2003). Overall, chromosomes gradually develop to a condensed state that is associated with the repression of transcriptional activity and the gradual acquiring of developmental competence (Alberts *et al.*, 2008, Luciano *et al.*, 2014).

Follicular fluid formation

The formation of the follicular antrum and follicular fluid is based on a complex system involving both the function and replication of granulosa cells and an osmotic gradient drawling fluid from the thecal vasculature into the antral cavity (Rodgers and Irving-Rodgers, 2010). In order to reach the follicular antrum, fluid from the thecal capillaries needs to cross the endothelium, the subendothelial basal lamina, the thecal intersitium, the follicular basal lamina and the membrana granulosa. Capillaries are rare in the region of the ovarian cortex containing primordial follicles. As the follicle grows, a simple endothelial network provides blood flow to the follicle. The composition of the follicular fluid is similar to serum with respect to low-molecular-weight components, with most electrolytes present at the same concentration in follicular fluid and serum (Shalgi *et al.*, 1972, Gosden *et al.*, 1988, Rodgers and Irving-Rodgers, 2010). However, as molecular sizes reach 100 kDa, proteins are found at much lower concentrations in the follicular fluid than in plasma, which serves as evidence for the blood-follicle barrier. This barrier may prevent certain molecules above 100 kDa from entering the micro-environment of the follicle. The reverse is also possible, namely that large

molecules produced by oocytes and granulosa cells are unable to leave the follicular micro-environment, creating an osmotic gradient. Potential molecules contributing to the osmotic gradient, that is responsible for the accumulation of fluid within the follicle, are hyaluronan, chondroitin sulfate/dermatan sulfate and DNA (Rodgers and Irving-Rodgers, 2010). In rats, it has been suggested that granulosa cells may facilitate transcellular water transport by means of aquaporins (McConnell *et al.*, 2002). Interestingly, it has been proposed that the synthesis of osmotically active molecules can be regulated by endocrine and autocrine mechanisms (Rodgers and Irving-Rodgers, 2010). For example, *in vitro* rat granulosa cells increase the expression of versican, a chondroitin sulfate proteoglycan, in response to gonadotropins (Yanagishita *et al.*, 1981, Rodgers and Irving-Rodgers, 2010). So, it appears that both follicular growth and the formation of follicular fluid are influenced by the hormonal environment.

Interaction between granulosa cells and the oocyte

Granulosa and cumulus cells support oocyte development by providing metabolites and by regulating oocyte growth and maturation. In turn, oocyte derived factors regulate granulosa, cumulus and theca cell function (Su *et al.*, 2009, Binelli and Murphy, 2010). The metabolic coupling between the oocyte and granulosa cells and between granulosa-granulosa cells is accomplished by heterologous and homologous gap junctions, respectively. Gap junctions between the cumulus cells and the oocyte are located at the cumulus cell's trans-zonal cytoplasmic processes that penetrate through the zona pellucida and border the oolemma (Deloos *et al.*, 1991, Sutton *et al.*, 2003). These gap junctions allow free transit of small molecules (<1kDa), including ions, amino acids, pyruvate, glucose metabolites, nucleotides and other signalling molecules such as cyclic adenosine monophosphate (cAMP) and purines (Ackert *et al.*, 2001, Su *et al.*, 2009). They are essential for reproduction, because both folliculo- and oogenesis fail in mice homozygous null for either connexin-37 (protein building block of oocyte-cumulus cell gap junctions) or connexin-43 (gap junctions between granulosa cells) (Simon *et al.*, 1997, Ackert *et al.*, 2001, Sutton *et al.*, 2003). There is substantial evidence that the oocyte secretes growth factors, such as GDF9 and BMP15, that play a central role in the regulation of folliculogenesis, granulosa cell and cumulus cell differentiation, ovulation rate and even litter size (Gilchrist *et al.*, 2004, McNatty *et al.*, 2004, Scaramuzzi *et al.*, 2011). This increasing evidence is mainly obtained by studies relying on animal models using 1) granulosa or cumulus cells treated with oocyte secreted factors and 2) animals deficient in candidate molecules through genetic mutation or immunization (Scaramuzzi *et al.*, 2011). Oocyte-secreted factors regulate several important functions of granulosa or cumulus cells. This includes the regulation of cellular growth, enhancing cell survival, modulating

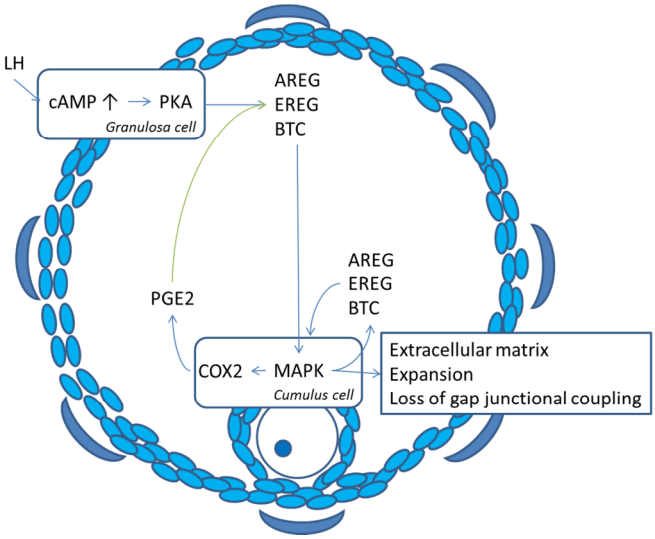
steroidogenesis, regulating cumulus expansion and metabolism (Eppig, 2001, Gilchrist *et al.*, 2004, Scaramuzzi *et al.*, 2011). For example, the murine oocyte actively directs the lineage of its surrounding granulosa cells towards the cumulus cell phenotype by inhibiting LH and FSH induced differentiation towards a luteinized phenotype (like in mural granulosa cells, Eppig *et al*., 1997). Overall, the oocyte is a major regulator of preantral and early antral follicular growth, affecting ovulation rate and the differentiation and metabolism of cumulus cells, including the capacity to expand, ultimately resulting in its own acquisition of developmental competence (Scaramuzzi *et al.*, 2011).

Pre-ovulatory follicle growth and nuclear oocyte maturation

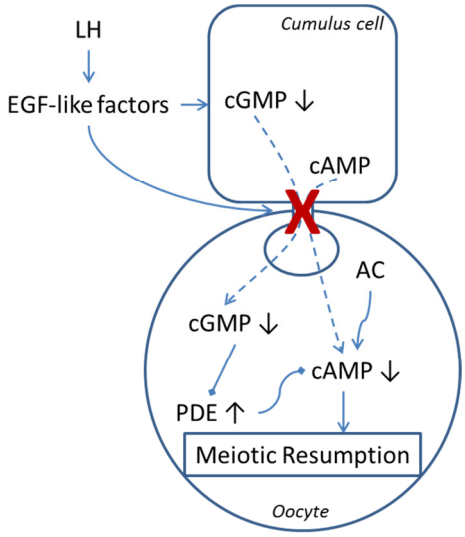
Under the influence of a surge of FSH, the growth of about 10-12 antral follicles is promoted (see: '*Hormonal regulation of follicular growth'*). In cycling women, usually one of these follicles becomes dominant and will give rise to the ovulation of an oocyte after the gonadotropin surge. The selection of the dominant follicle may be associated with an increased LH responsiveness (Fair, 2010), because LH targets its receptors in the external layers of mature follicles (mural granulosa cells, **Figure 2A**). In mice, binding of LH to its receptor in mural granulosa cells stimulates the production of cAMP through activation of adenylate cyclase, which in turn activates Protein Kinase A (PKA) that is responsible for the phosphorylation of transcription factors that start the ovulatory response in granulosa cells (Russell and Robker, 2007). It induces the production of peptides of the EGF family, the so called EGF-like factors Amphiregulin (AREG), Epiregulin (EREG) and Betacellulin (BTC) (Park *et al.*, 2004). These membrane bound EGF-like factors are shed by matrix metalloproteinases (Coticchio *et al.*, 2013), enabling them to reach their receptors that are located at the cumulus cells (Russell and Robker, 2007). EGF-like factors will activate a cascade of events mediated by the EGF receptor in both granulosa and cumulus cells. The subsequent activation of MAPK (ERK1/2) will generally cause changes in cumulus cell gene expression, production of extracellular matrix and expansion of the cumulus-oocyte complex (COC, Sutton *et al.*, 2003). More specifically, in mice it induces 1) an up-regulation of transcripts responsible of cumulus expansion (*HAS2*, *TNF alpha induced protein* and *PTX3*, 2), the production of prostaglandin-endoperoxide synthase 2 (PTGS2, or cyclooxygenase-2 – COX2) (Sanchez and Smitz, 2012), which enhances the response to the LH stimulus through the production of prostaglandin E2 (PGE2) and by stimulating the production of more EGF-like factors, increasing the subsequent reaction and 3) the closure of the gap junctions, which prevents the transfer of cGMP into the oocyte (Sanchez and Smitz, 2012). Within the mammalian oocyte, meiotic arrest is maintained by elevated levels of cAMP, originating from the cumulus cells (through gap junctions) and

endogenously produced (**Figure 2B**). cGMP, transferred from the cumulus cells through the gap junctions, prevents phosphodiesterase (PDE) activation within the oocyte and cAMP is then maintained at high levels (Sanchez and Smitz, 2012). The concentration of cAMP within the oocyte is regulated by its active production (adenylate cyclase) and its hydrolysis (PDE). At the time of ovulation, cAMP levels in the oocyte are decreased. This is established mainly by LH induced decreased cGMP (potent inhibitor of PDE) concentrations in cumulus cells and gap junctional loss, preventing cGMP from being transported to the oocyte. Decreased cAMP levels activate the pathway leading to meiotic resumption (for review, see Russell and Robker, 2007).

After the re-initiation of meiosis (germinal vesicle breakdown), during *metaphase I*, spindle fibres from the centriole attach to the centromeres of the chromosomes and they align at the equatorial plate. During *anaphase I*, the duplicated homologs are pulled apart and move towards opposite poles of the cell. The following *telophase I* results in the formation of two haploid daughter cells through cytokinesis. The cytoplasm divides asymmetrically, forming a polar body that is extruded and a secondary oocyte. In contrast to the situation for mitosis or meiosis I, no DNA replication occurs before the initiation of **meiosis II**. During *prophase II*, chromosomes condense and centrioles move to opposite poles of the cell. *Metaphase II* includes spindle fibres that attach to the centromeres of the chromosomes and chromosomes line up at the equator of the cell. At this time, the oocyte becomes arrested until it is fertilized (Alberts *et al.*, 2008). So, the oocyte ovulated from the dominant follicle, surrounded by cumulus cells, embedded in a hyaluronan-rich gel-like matrix, completes meiosis I and arrests again at metaphase II.



A



B

Figure 2: Schematic representation of the molecular pathways involved in oocyte maturation after the LH peak stimulus. A: follicular mechanisms, B: mechanisms within the cumulus-oocyte complex. LH: luteinizing hormone, PKA: protein kinase A, AREG: amphiregulin, EREG: epiregulin, BTC: betacellulin, MAPK: mitogen activated protein kinase, COX2: cyclooxygenase 2, PGE2: prostaglandin E2, AC: adenylate cyclase, PDE: phosphodiesterase, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate.

Cytoplasmic oocyte maturation

Besides nuclear oocyte maturation, consisting of the nuclear changes that are associated with the resumption of meiosis I, the extrusion of the first polar body and the progression to metaphase II (Tripathi *et al.*, 2010), the mammalian oocyte also undergoes cytoplasmic maturation. This includes structural and molecular changes occurring in the oocyte from the germinal vesicle stage to the end of metaphase II. It comprises amongst others the mobilization and relocation of macromolecules and organelles, reorganization of the ER (main calcium storage in the oocyte), protein synthesis, post-translational modifications of mRNAs accumulated during oogenesis, acquisition of mechanisms for sperm penetration and decondensation of the sperm chromatin. Overall, these processes of cytoplasmic maturation lead to the acquisition of mechanisms required for successful resumption and completion of meiosis, subsequent successful fertilization and early embryo development (Hyttel *et al.*, 1989, Ferreira *et al.*, 2009).

Hormonal regulation of follicular growth

Follicular growth and oocyte maturation are under the continuous influence of hormonal control. The arcuate nucleus of the hypothalamus synthesizes and releases gonadotropin-releasing hormone (GnRH) in rhythmic pulses, that stimulate FSH and LH secretion from the pituitary. The human ovarian cycle includes a follicular phase and a luteal phase, separated by ovulation (Boron and Boulpaep, 2005). The **follicular phase** begins with the initiation of menstruation and averages about 14 days in length. Increased FSH concentrations rescue a cohort of responsive antral follicles from atresia, a process called cyclic follicle recruitment (McGee and Hsueh, 2000). The granulosa cells of the follicles start producing estradiol, the main product of the follicular phase, which is important for the proliferative growth of the endometrium. More specifically, LH binds to its receptor on theca cells, stimulating the conversion of cholesterol to androstenedione. Because theca cells lack aromatase, androstenedione diffuses to the granulosa cells, whose aromatase activity has been stimulated by FSH and converts androstenedione to estradiol (Boron and Boulpaep, 2005). Granulosa cells also secrete activins and inhibins, that stimulate and inhibit FSH secretion at the level of the pituitary, respectively. However, inhibin does not only act as an endocrine regulator of FSH

secretion, inhibin also acts locally within the follicle to increase LH-stimulated production of androgens by theca cells and FSH-induced aromatase activity in granulosa cells (Scaramuzzi *et al.*, 2011). During the mid-follicular phase, estradiol acts together with inhibin to suppress FSH secretion, by a direct inhibitory action on the expression of the mRNA for FSHβ (Scaramuzzi *et al.*, 2011). Just before ovulation, the rise in estradiol secretions accelerates, triggering the LH surge through a positive feedback mechanisms at the level of the preoptic area of the hypothalamus (Boron and Boulpaep, 2005). This results in ovulation. After ovulation, during the **luteal phase**, the follicle transforms into a corpus luteum. The vascularization of the corpus luteum makes low-density lipoproteins (LDLs) available to granulosa cells, that use it to produce progesterone. With both theca and granulosa cells producing progesterone, it becomes the main secretion product of the corpus luteum (Boron and Boulpaep, 2005) In rats, it was shown that LH receptor expression was temporarilily decreased in granulosa cells after an ovulatory stimulus, while mRNA expression levels increased again during the formation of the corpus luteum (Peng *et al.*, 2013). This is indicative of the LH-stimulated take up and process of cholesterol during the luteal phase (Boron and Boulpaep, 2005). In women, the corpus luteum also produces significant amounts of estrogen and inhibin. Estrogens and progestins stimulate further endometrial growth and development. Importantly, the high levels of progesterone, estrogen and inhibin maximally suppress the hypothalamic-pituitary system, to cause a decrease in the frequency of the pituitary LH and FSH release, leading to LH and FSH levels to fall, which results in a lower production of estrogens and progestins. This has two effects: first, if no pregnancy is established, it induces a degeneration of the endometrium resulting in menstrual bleeding and second, it diminishes the negative feedback at the level of the hypothalamic-pituitary axis, which will ultimately result in the rise of gonadotropin levels and the start of a new cycle (Boron and Boulpaep, 2005).

Besides the systemic hormonal regulation of follicular growth, there is also an intra-ovarian regulation of both gonadotropin-responsive and gonadotropin-dependent follicles (Scaramuzzi *et al.*, 2011). In this regard, the **insulin-like growth factor (IGF) system** appears to be particularly relevant for folliculogenesis. IGF-1 increases the sensitivity of small follicles to gonadotropin stimulation and promotes their transition from the gonadotropin-responsive to the gonadotropin-dependent stage. Furthermore, in ovarian tissue *in vitro*, IGF-1 stimulates theca cell steroidogenesis and the proliferation/differentiation of granulosa cells (reviewed by Scaramuzzi *et al.* (2011)). Because of its potential effects, IGF-1 activity needs to be tightly controlled within the follicle. As most of the IGF-1 within the follicle is derived from the blood, intra-ovarian regulators are essential. IGF-binding proteins (IGFBP) inhibit IGF actions by

binding to IGF, preventing it from binding to its receptor. So, it is the bio-availability, rather than the actual concentration of IGF-1 that changes throughout folliculogenesis. Mostly, the concentrations of IGFBPs decrease as the follicle grows to pre-ovulatory sizes, due to increased rates of proteolytic degradation of IGFBPs by pregnancy-associated plasma protein-A (PAPP-A) (Mazerbourg *et al.*, 2003). It has even been suggested that gonadotropin-stimulated PAPP-A gene expression might be involved in the selection of the dominant follicle (Scaramuzzi *et al.*, 2011), thus highlighting the potential importance of the IGF-1 system in selection for dominance. Other metabolic and nutritional factors, like leptin and growth hormone, are also important for folliculogenesis. Noteworthy, the follicle has a complete and functional **glucose-insulin system** (insulin receptor, phosphatases, kinases and proteins involved in insulin action). The presence of glucose transporter type 4 (GLUT4) furthermore suggests a role of insulin-mediated glucose uptake in the follicle (Nishimoto *et al.*, 2006, Robker *et al.*, 2009, Scaramuzzi *et al.*, 2011). Besides the role of the glucose-insulin system in the maintenance of cellular health and integrity, as it does for all cells and tissues, there is also evidence that the glucose-insulin system has specific functions that affect granulosa and theca cells. For example, the deletion of the gene for IRS-2 (essential for insulin signalling), resulted in impaired folliculogenesis in mice (Neganova *et al.*, 2007, Scaramuzzi *et al.*, 2011). Insulin interacts with its own receptor and the IGF receptor type I and increases the sensitivity of the pituitary gonadotropes to GnRH action. It furthermore enhances the ovarian steroidogenic response to gonadotropins, an effect probably mediated by an increase of LH receptor number (Poretsky *et al.*, 1999).

From the above it is clear that follicular oocyte maturation up until the point of ovulation is a very well-balanced process, that may easily be tipped off by changes in circulating metabolites. But how may metabolic disorders, like obesity, affect this vulnerable balance and what may be the consequences for oocyte developmental competence?

b) Ovulation, fertilization and early embryo development

After the rupture of the ovarian follicle, the oocyte, surrounded by its granulosa cells and cumulus oophorus, is picked up by the fimbria of the oviduct. Shortly after ovulation, the movements of the cilia and the oviduct guide the COC towards the uterus. The oocyte usually comes into contact with spermatozoa in the ampullary region of the oviduct, where fertilization usually takes place several hours after ovulation (Boron and Boulpaep, 2005).

Approximately 300 000 000 spermatozoa are released from one ejaculate. In women, only 200 reach the site of fertilization in the oviduct (Alberts *et al.*, 2008), where the COC resides following ovulation. Both the oocyte and sperm do not survive if no fertilization occurs. Once a spermatozoon finds the oocyte, it must first find its way through the layers of granulosa cells, penetrate the zona pellucida and only then is the spermatozoon able to fuse with the oocyte's plasma membrane.

Capacitation of sperm

Ejaculated mammalian spermatozoa are initially not competent to fertilize the oocyte. Therefore capacitation occurs throughout the sperm's way through the female reproductive tract (Alberts *et al.*, 2008). After ejaculation, human spermatozoa are deposited into the anterior vagina, after which they proceed to the cervix. In addition to leakage as the major source of sperm loss, the cervical mucus filters out sperm with poor morphology and motility (Alberts *et al.,* 2008). This results in only a minority of the ejaculated spermatozoa entering the cervix. In the uterus, muscular contractions move the sperm cells through the uterine cavity (sperm swimming itself is slow-moving until they are capacitated). The spermatozoa then enter the utero-tubal junctions to reach the oviduct, where sperm are stored in a reservoir, or at least maintained in a fertile state, by interacting with oviductal epithelium. As the time of ovulation approaches, sperm become capacitated and hyperactive, which enables them to proceed towards the tubal ampulla (Suarez and Pacey, 2006). Sperm capacitation includes extensive biochemical and functional changes, such as an increased intracellular pH, tyrosine phosphorylation of various sperm proteins and the unmasking of cell-surface receptors that help bind the sperm to the zona pellucida. The spermatozoon's membrane also becomes hyperpolarized. These steps greatly increase the flagellum motility and make the sperm capable of undergoing the acrosome reaction. Of particular interest in sperm capacitation is the presence of albumin. Albumin helps extract cholesterol from the plasma membrane, increasing its ability to undergo the acrosome reaction (Boron and Boulpaep, 2005, Alberts *et al.*, 2008).

Fertilization

Spermatozoa must first make their way through different layers of granulosa cells, using a hyaluronidase enzyme on their surface. Hyaluronidase acts by degrading hyaluronan, which is present in high concentrations in the extracellular matrix of the COC, enabling the penetration of this layer by spermatozoa (Alberts *et al.*, 2008). After that, a spermatozoon is able to bind to the zona pellucida and the zona pellucida sperm-binding protein 3 (ZP3) glycoprotein of the

zona stimulates the sperm to undergo the acrosome reaction (release of the acrosome content). This is necessary for the sperm to get through the zona and for acquiring the ability to fuse with the plasma membrane of the oocyte (Boron and Boulpaep, 2005). The molecular basis of sperm–egg recognition is unknown, but is likely to require interactions between receptor proteins displayed on their surface. Izumo1 is an essential sperm cell-surface protein and Juno has recently been identified as the receptor for Izumo on the surface of the oocyte. The Izumo1–Juno interaction is conserved within several mammalian species, including humans and is essential for fertilization (Bianchi *et al.*, 2014). After fusion, all of the sperm content is drawn into the oocyte, which ultimately activates the oocyte by increasing Ca^{2+} in the cytosol, that spreads through the cell in a wave. The wave propagates by positive feedback: the increase in cytosolic Ca^{2+} causes Ca^{2+} channels to open, allowing more Ca^{2+} to enter the cytosol. The initial wave of Ca^{2+} release is usually followed within a few minutes by Ca^{2+} oscillations, which persist for several hours. This results in an increase of the concentration of inositol 1,4,5-trisphosphate, which releases Ca^{2+} from the endoplasmic reticulum and initiates the Ca^{2+} wave and oscillations (Alberts *et al.*, 2008). In contrast to Ca^{2+}, where increasing levels are required to induce oocyte activation pathways, Zn^{2+} levels decrease at oocyte activation. Upon fertilization, or parthenogenetic activation in the mouse, zinc is rapidly released from the oocyte in several exocytosis events (zinc sparks), that are possibly dependent on the Ca^{2+} signal. Even though the precise mechanisms are not fully elucidated yet, Zn^{2+} signalling is essential for the resumption of meiosis (Krauchunas and Wolfner, 2013).

The spermatozoon contributes not only its genome, but also its centrioles to the zygote (Alberts *et al.*, 2008). Interestingly, sperm mitochondria within the zygote are eliminated by a ubiquitin dependent mechanism (Sutovsky and Schatten, 2000). Polyspermy in mammals is prevented by multiple mechanisms. First, passage through the female reproductive tract drastically reduces the number of spermatozoa reaching the oviduct. Second, the cortical reaction, by which the content of the cortical granules is released by exocytosis, after a sperm-induced intracellular Ca^{2+} increase, changes the structure of the zona pellucida (inactivation of ZP3 and cleaving of ZP2). This reaction ensures that no other spermatozoa can bind or penetrate the zona pellucida (egg coat block to polyspermy). Third, there is the so called membrane block to polyspermy. In contrast to the situation in non-mammalian oocytes, where the membrane block to polyspermy is established by a rapid and transient depolarization of the oocyte's plasma membrane potential, the mechanisms leading to the membrane block to polyspermy in mammalian oocytes remain unclear (Gardner and Evans,

2006). However, very recent evidence has suggested that rapid shedding of Juno from the oolemma after fertilization, may be involved in the membrane block to polyspermy (Bianchi *et al.*, 2014).

Resumption of oocyte maturation

Upon fertilization, during *anaphase II*, spindle fibres contract, separating the chromosomes and moving them towards opposite poles of the cell. Finally, the *telophase II* results in chromosome de-condensation, nuclear membrane reformation and cytokinesis (again asymmetrically to produce the mature oocyte and a second polar body, both containing a haploid set of single chromosomes). This mature and fertilized oocyte is now called a totipotent zygote (Boron and Boulpaep, 2005, Alberts *et al.*, 2008).

Early embryo development

After fertilization, the haploid pronuclei of the two gametes come together to form the diploid nucleus of the zygote. Mammalian preimplantation embryo development is characterized by a series of cleavage divisions that subdivide the zygote into smaller and smaller compartments, followed by compaction at the morula stage (Latin for little mulberry) and cavitation when a blastula is formed. This blastula gives rise to a blastocyst, which consists of an outer epithelial trophectoderm (TE, progenitor of the placenta), encircling a small group of cells called inner cell mass (ICM, progenitor of the proper embryo and some extraembryonic tissues) and a large fluid-filled cavity or blastocoel. The blastocyst eventually hatches by breaking through the zona pellucida, which enables it to implant in the uterine wall. The oocyte is responsible for sustaining early embryo physiology, up until the embryo is able to produce its own gene transcripts at the time of the embryonic genome activation (EGA) (Niakan *et al.*, 2012). mRNA molecules, stored during oocyte growth, are normally inactive due to a short poly A tail. However, upon fertilization, cytoplasmic polyadenylation allows the activation of these mRNA molecules, that serve to sustain the first cleavage divisions of the embryo. At this time, both maternal and paternal DNA is highly methylated and packed with histones in a specific manner to initiate embryonic development (Li *et al.*, 2013). Upon EGA, maternal and paternal genomes are demethylated, allowing the embryo to provide its own transcript (Li *et al.*, 2013).

Again, this well-orchestrated sequence of events in development must be closely adhered to obtain a viable embryo. But how can maternal metabolic disorders, like obesity, affect fertilization and the growth of the early embryo?

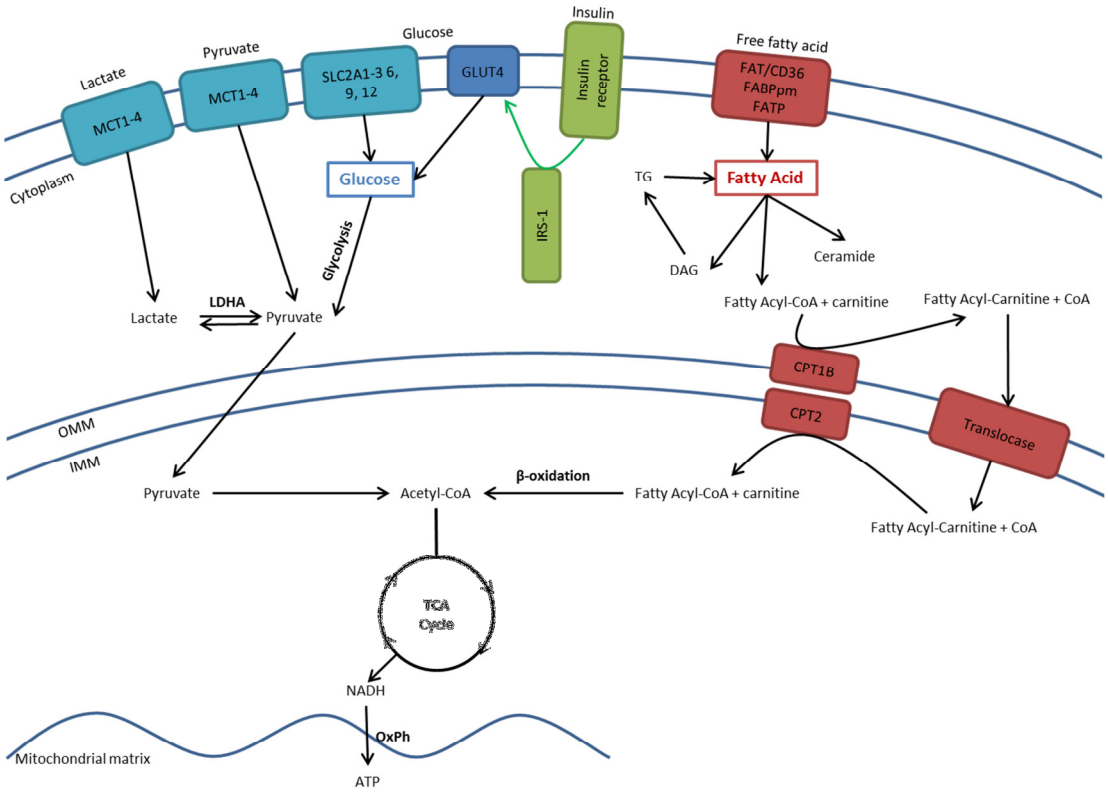
c) Metabolic requirements of follicles, oocytes and embryos

Follicles, oocytes and embryos can use a multitude of energy substrates, provided by the maternal micro-environment, to sustain growth and development. This suggests that the versatility of metabolic pathways used is key for high developmental potential (Collado-Fernandez *et al.*, 2012). **However, it also makes them vulnerable to any perturbation in the maternal metabolism, potentially changing the availability of nutrients or substrates or even jeopardizing cell survival by excess amounts of substrates**. This is why the metabolic requirements of follicles, oocytes and embryos, in normal circumstances, is described below.

Carbohydrate metabolism

Murine primordial follicles extract energy from glycolysis and from mitochondrial pyruvate oxidation (Harris *et al.*, 2009). Denuded murine oocytes, with a diameter approximately corresponding to the one in primordial follicles, do not use any glucose, but they do metabolize pyruvate *in vitro* (Eppig, 1976). Interestingly, pyruvate consumption was at a similar rate compared to that of murine primordial follicles, suggesting that the glucose consumption seen in cultured primordial follicles is due to the metabolism of the pre-granulosa cells, while the oocyte consumes pyruvate (Harris *et al.*, 2009). Pyruvate can be metabolized in the mitochondria through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, which produces most of the ATP (**Figure 3**). Lactate on the other hand, is oxidized to pyruvate by cytosolic lactate dehydrogenase (LDH) and is important for the regulation of the cytosolic redox state (**Figure 3**) (Sutton-McDowall *et al.*, 2010).

After the initiation of follicular growth, developing murine oocytes preferentially metabolize pyruvate over glucose, while the somatic cells of the follicle are more glycolytic (Harris *et al.*, 2009, Sutton-McDowall *et al.*, 2010, Collado-Fernandez *et al.*, 2012). However, a very intense metabolic relationship between the oocyte and its surrounding cumulus cells is pivotal for oocyte survival and developmental competence (Collado-Fernandez *et al.*, 2012), because cumulus cells provide glucose and pyruvate to the oocyte as energy substrate (Leese and Barton, 1985). Follicle glucose consumption and lactate production rates increase as murine follicles develop *in vitro* from primary/secondary stages to antral and ovulatory-like stages (Harris *et al.*, 2007, Collado-Fernandez *et al.*, 2012).



(Zhang et al., 2010; Dunning et al., 2010; Sutton-McDowall et al., 2012)

Figure 3: General intracellular metabolic pathways of glucose, lactate, pyruvate and free fatty acids. MCT1-4: MonoCarboxylate Transporter 1-4 (lactate and pyruvate transporters), GLUT4: glucose transporter type 4, SLC2A1-3, 6, 9, 12: solute carrier family 2, member 1-3, 6, 9, 12 (facilitated glucose transporters), FAT/CD36: fatty acid translocase/cluster of differentiation 36 (fatty acid transporter), FABPpm/FATP: plasma membrane associated fatty acid binding protein/fatty acid transport protein (fatty acid transporter), IRS-1: Insulin receptor substrate 1, TG: triglyceride, DAG: diacylglycerol, GAPDH : glyceraldehyde-3-phosphate dehydrogenase, PFK : phosphofructokinase, LDHA : lactate dehydrogenase A, PDH: pyruvate dehydrogenase, CPT 1B/2: carnitine palmitoyl transferase 1B/2, TCA: tricarboxyacid, ROS: reactive oxygen species, OxPh: oxidative phosphorylation, OMM: outer mitochondrial membrane, IMM: inner mitochondrial membrane.

Within the follicle, glucose may be metabolised through different pathways, providing the oocyte with substrates for different cellular processes through gap junctional coupling (Sutton-McDowall *et al.*, 2010, Collado-Fernandez *et al.*, 2012, Smith and Sturmey, 2013):

- **Glycolysis**, producing ATP, NADH and pyruvate or lactate;
- The **pentose phosphate pathway** (PPP), supplying the precursors for purine nucleotides (non-oxidative arm) and NADPH (oxidative arm) for biosynthetic pathways and antioxidant functions;
- The **hexosamine biosynthetic pathway** (HBP), where glucose and glutamine are involved in protein glycosylation and hyaluronic acid synthesis for cumulus expansion and
- The **polyol pathway**, providing sorbitol and fructose, whose roles remain largely unknown.

In the mouse, follicular glycolytic activity steeply increases at the time of the preovulatory surge of gonadotropins (Harris *et al.*, 2007). Furthermore, when the follicles reach the size at

which the supply from the theca capillaries is compromised, the follicular fluid serves as a source of oxygen and nutrients (Redding *et al.*, 2007). Oxygen is required for the mitochondrial oxidative metabolism, which is essential for oxidative phosphorylation, the synthesis of cholesterol and steroidogenesis in granulosa cells of the antral follicle and for the β-oxidation of intracellular lipid stores (Boland *et al.*, 1994, Collado-Fernandez *et al.*, 2012). An increase in oxidative metabolism at the time of the surge of gonadotropins provides the energy needed for the oocyte to resume meiosis. The most important substrates at this time are pyruvate, glutamine and glycine, which can be metabolized in the TCA cycle (Harris *et al.*, 2007). Lactate, can be oxidized to pyruvate (lactate dehydrogenase), but this lactate-derived pyruvate does not fuel the TCA cycle and oxidative phosphorylation. Instead, lactate is important for the regulation of cytosolic redox state (Dumollard *et al.*, 2007, Dumollard *et al.*, 2009). The preference of the oocyte for pyruvate persists into the zygote, but the embryo becomes more and more dependent on glucose as it develops to the blastocyst stage in all species studied, including human (for reviews, see Collado-Fernandez *et al.*, 2012, Leese, 2012). More specifically, the first cleavage stages *in vitro* mostly rely on pyruvate, lactate and some amino acids for energy provision (ATP) through oxidative phosphorylation (Sturmey *et al.*, 2009a). In Day 3 human embryos, most of the pyruvate is oxidized, but a significant part is converted to lactate to generate NAD+ as a redox regulator (Butcher *et al.*, 1998). On the other hand, in morula staged embryos, most of the glucose consumed is transformed into lactate. This phenomenon is called the 'Warburg effect' or the aerobic glycolysis, in which glucose is turned into pyruvate by glycolysis, after which the pyruvate in transformed into lactate with the formation of NAD+. Pyruvate is thus not oxidized in the TCA cycle for optimal ATP production, but rather it is kept as an intermediate for other cellular functions (PPP, HBP, polyol pathways) (Collado-Fernandez *et al.*, 2012, Krisher and Prather, 2012). A steep increase in glucose consumption is seen when the human embryo reaches the morula stage *in vitro* (Leese *et al.*, 1993). Much of the glucose is converted to lactate (aerobic glycolysis), which provides little ATP. However, converting glucose to lactate may enable the blastocyst to survive the hypoxia that occurs to a varying extent, depending on the species, at implantation (Leese, 2012). Glucose functions both as a cell signalling agent and as a potential substrate for energy (Leese, 2012). An overall increase in energy consumption (measured in nutrient uptake and oxygen consumption, Leese, 2008) around compaction of the embryo aims to meet the increased energy requirements of the embryo for protein synthesis, rapid cell divisions and ion pumping for blastocoel formation (Leese *et al.*, 2008).

Fatty acid metabolism

Fatty acid β-oxidation can generate high numbers of ATP molecules (up to 106 ATP from the complete oxidation of one molecule palmitate), which makes them highly efficient in producing energy for oocyte maturation and early embryo physiology (Sturmey *et al.*, 2009a). This as opposed to aerobic glucose metabolism, providing only approximately 30 ATP per glucose molecule (Sutton-McDowall *et al.*, 2012). Fatty acids, derived from triglycerides through the action of hormone sensitive lipase, are converted to acyl-CoA, bound to L-carnitine and translocated into the mitochondrial matrix by carnitine palmitoyl transferase 1B (CPT1B, **Figure 3**). Carnitine is then removed again by CPT2 and the fatty acid enters the β-oxidation spiral. This generates multiple acetyl-CoA molecules, which then serve as a substrate for the TCA cycle and oxidative phosphorylation (**Figure 3**). The rate limiting enzyme of the β-oxidation, when all substrates are available in abundance, is CPT1B and its essential co-factor L-carnitine (Sutton-McDowall *et al.*, 2012). During follicular development, lipid droplets (triglycerides) accumulate in the oocyte (Collado-Fernandez *et al.*, 2012). Variable amounts of intracellular stored fatty acids (triglycerides) are present in different species, with pig oocytes having high intracellular fat concentrations, followed by human and bovine oocytes (Sturmey *et al.*, 2009a). Mouse oocytes only contain little intracellular triglycerides, rendering them transparent under the microscope. Support for the use of these intracellular energy stores by oocytes and embryos comes from the observation that the triglyceride content of porcine and bovine oocytes decreased during *in vitro* maturation, as well as after fertilization in the cow (Sturmey and Leese, 2003, Ferguson and Leese, 2006). Furthermore, supplementing L-carnitine during *in vitro* murine follicle culture and oocyte maturation to improve β-oxidation, significantly enhanced oocyte developmental competence (Dunning *et al.*, 2010, Dunning *et al.*, 2011, Sutton-McDowall *et al.*, 2012). On the other hand, inhibiting β-oxidation during oocyte *in vitro* maturation prevented meiotic resumption in mouse oocytes (Downs *et al.*, 2009) and impaired oocyte developmental competence in the mouse, pig and cow (Ferguson and Leese, 2006, Sturmey *et al.*, 2006, Dunning *et al.*, 2010). Moreover, inhibition of β-oxidation during embryo development impaired blastocyst development in the mouse and the cow (Hewitson *et al.*, 1996, Ferguson and Leese, 2006, Dunning *et al.*, 2010). Overall, these data indicate that β-oxidation is an essential energy pathway for the acquisition of oocyte developmental competence and early embryo survival. In contrast, control bovine oocytes did not display differences in development after fertilization when β-oxidation was inhibited during oocyte maturation (Van Hoeck *et al.*, 2013), results similar to those found for porcine oocytes (Sturmey *et al.*, 2006), so adaptive alternative pathways may be activated.

Amino acid metabolism

Amino acids serve as substrates for the synthesis of proteins, nucleotides (glutamine, aspartate, glycine), glutathione (glutamate, cysteine, glycine), glycoproteins, hyaluronic acid (glutamine bound to glucose through the HBP) and signalling molecules such as nitric oxide (arginine). They are also important energy substrates (glutamine, glycine and others) and pH and osmolarity regulators (glycine, alanine, glutamine and others). Furthermore, they function as heavy metal chelators (glycine), donors of methyl groups (methionine) and they are important in the secretion of ammonia (alanine) and in anaplerosis (replenishing TCA cycle intermediates; aspartate, glutamate, leucine, lysine, isoleucine and others) (Dumollard *et al.*, 2007, Sturmey *et al.*, 2008a, Collado-Fernandez *et al.*, 2012). In comparison to carbohydrate metabolism, there is little information about amino acid uptake and metabolism by follicles and oocytes, especially at the earlier stages of folliculogenesis (Collado-Fernandez et al., 2012). However, significant levels of amino acid substrates are present in the follicular fluid and the reproductive tract (Harris *et al.*, 2007, Hugentobler *et al.*, 2007), potentially serving as substrates for multiple cellular processes during follicle and oocyte development. During oocyte growth, large amounts of RNA and proteins are synthesized (Picton *et al.*, 1998). Glutamine, aspartate, glycine and ribose-5-phosphate (product of glucose metabolism through the PPP) are required for *de novo* synthesis of purine and pyrimidine nucleotides for mRNA synthesis (Collado-Fernandez *et al.*, 2012) and thus essential for the acquisition of oocyte developmental competence. In this regard, it has been shown that the murine oocyte is capable of increasing the flux of specific amino acids, passed through to the oocyte, via cumulus cells, to meet its own requirements *in vitro* (Eppig *et al.*, 2005). Furthermore, some amino acids (glutamine and glycine) may be used for the purpose of ATP production through oxidative metabolism (Zuelke and Brackett, 1993, Rieger and Loskutoff, 1994). Even though efforts have been made to characterize amino acid metabolism in follicles, oocytes and embryos *in vitro* (Pelland *et al.*, 2009, Chand and Legge, 2011, Hemmings *et al.*, 2012), amino acid turnover is highly variable amongst species and in different culture conditions of oocytes and embryos.

Importantly, amino acid turnover as well as the rate of glucose and pyruvate consumption in early pre-implantation embryos have been proposed as non-invasive tools to predict embryo developmental competence (Leese, 2008, Sturmey *et al.*, 2008a) and even pregnancy outcome in women undergoing ART (Brison *et al.*, 2004, Gardner *et al.*, 2011). For example, bovine oocytes that failed to cleave depleted more glutamine, released more alanine and presented higher total amino acid depletion, appearance and turnover than oocytes that were able to

cleave (Hemmings *et al.*, 2012). Furthermore, higher bovine, porcine and human embryonic amino acid turnover has been shown to be positively correlated with DNA damage (Sturmey *et al.*, 2009b). These observations are in agreement with Leese's **quiet embryo hypothesis** that states that high embryo quality is associated with a relatively low level of metabolism, while an increased metabolic activity is proposed to be involved in increased energy needed to repair cellular injuries (Leese, 2002, Leese *et al.*, 2008). However, the translation of these findings to ART clinics has encountered some problems. In this regard, specialized spectroscopic analysis of culture medium droplets has been performed, but recent randomized controlled trials using these commercial instruments did not always show a consistent benefit in improving pregnancy rates when metabolomics is used as an adjunct to morphology (Sfontouris *et al.*, 2013, Uyar and Seli, 2014).

We may assume that any short or long term metabolic disturbance, leading to an alteration in the available substrates or nutrients for follicles and oocytes, may tip off the fragile balance of the well-orchestrated processes involved in the acquisition of oocyte developmental competence. This may result in a decreased oocyte developmental competence or the ovulation of a low quality oocyte that may lead to a low quality embryo with an altered metabolism. However, the mechanisms behind this remain incompletely understood.

3. General pathways leading to subfertility in metabolically compromised women

Lipolysis-linked metabolic disorders, like obesity, affect several organs that orchestrate fertility, like the hypothalamus, the ovary and ovarian follicle, but also the oocyte, the embryo and the uterine endometrium (Jungheim *et al.*, 2013a). Much information regarding the relationship between lipolysis-linked metabolic disorders and fertility has become available by studying reproductive performance in dairy cattle suffering from a negative energy balance. Even though the etiology of obesity in women and the negative energy balance in the dairy cow is not similar, they are both metabolic disorders associated with an increased lipolysis, resulting in apparent metabolic changes. As important aspects of regulating fertility are similar between women and cows, pathways leading to subfertility in metabolically compromised dairy cows are discussed briefly. Even though the focus of this review lies on the oocyte's acquisition of developmental competence, some general pathways that ultimately lead to reduced fertility in metabolically compromised women must be discussed as they mostly lead to anovulation or an incompetent oocyte.

a) The central nervous system

Lower **GnRH** concentrations have been observed in obese women and obese rodent models, potentially leading to abnormal GnRH pulsatility and subsequent oligo- or anovulation (Tortoriello *et al.*, 2004, Jain *et al.*, 2007, Jungheim *et al.*, 2013a). Furthermore, several, but not all, studies pointed out that **LH** and **FSH** concentrations were negatively associated with BMI (reviewed by Michalakis *et al.*, 2013). Obese women indeed exhibit decreased LH pulse amplitude (Jain *et al.*, 2007). In addition to causing anovulation, abnormal LH pulsatility may affect ovarian follicular steroidogenesis, leading to abnormal follicle recruitment and poor oocyte quality as well as an altered endometrial development. It could furthermore affect the function of the corpus luteum during the luteal phase (Jungheim et al., 2012). Much like in women, cows in a negative energy balance show a disturbed LH pulse frequency and amplitude, leading to prolonged intervals between calving and first ovulation (Lucy, 2003, Hunter *et al.*, 2004). Overall, a correct LH pulsatility is considered a keystone for optimal follicular development and thus for correct oocyte growth and maturation (Hyttel *et al.*, 1997).

b) Sex steroid balance

Obesity is associated with several abnormalities in the sex steroid balance. Such alterations involve both androgens and estrogens, as well as their main carrier protein, sex hormone binding globulin (SHBG) (Pasquali *et al.*, 2003). Obese women have lower **SHBG** concentrations, probably by the inhibition of SHBG synthesis in the liver due to elevated **insulin** concentrations (Gambineri *et al.*, 2002). Hyperinsulinemia in obese women originates from a decreased cellular response to normal insulin concentrations, leading to a higher secretion at the level of the pancreatic β-cells (Zhang *et al.*, 2010). But, obesity-related metabolites like NEFAs are also able to affect the secretion and the metabolism of insulin (Gambineri *et al.*, 2002, Zhang *et al.*, 2010). Moreover, insulin is able to inhibit both hepatic and ovarian **IGFBP1** synthesis, which binds and inactivates IGFs (Poretsky *et al.*, 1996). The insulin-induced decreased SHBG levels together with the higher bio-availability of IGF-1 leads to a higher concentration of free sex hormones at the level of the target tissues (Gambineri *et al.*, 2002). As sex hormones are preferentially stored in fat tissue and because a larger amount of fat is present in obese women, the steroid pool in obese individuals is greater than found in normal weight women (Azziz, 1989, Pasquali *et al.*, 2003). Additionally, visceral fat also represents a very active site of sex hormone metabolism and inter-conversion, because of the presence of steroidogenic enzymes, like 3β-dehydrogenase, 17β-hydroxydehydrogenase and the aromatase system (Azziz, 1989, Gambineri *et al.*, 2002, Pasquali *et al.*, 2003). Obesity is associated with hyperandrogenism, caused by a multitude of factors like an altered steroid metabolism in fat tissue, insulin-stimulated increased **androgen** production, reduced binding to SHBG etc. (Pasquali *et al.*, 2003). Circulating **estrogen** concentrations are also increased in obese women, due to increased aromatization of androgens in peripheral adipose tissue (Michalakis *et al.*, 2013) and decreased SHBG levels (Gambineri *et al.*, 2002). Importantly, hyperandrogenemia has been shown to cause granulosa cell apoptosis in multiple species, while peripheral conversion of androgens to estrogens in adipose tissue inhibits gonadotropin secretion (Billig *et al.*, 1996, Metwally *et al.*, 2007). Adipose tissue further influences reproductive function by the secretion of adipokines, such as leptin, adiponectin, ghrelin and resistin, which may impact on the hypothalamo-pituitary axis as well as on ovarian follicles and early embryos (for review, see Michalakis *et al.*, 2013). Overall, the major effect of obesity on steroid hormones is hyperandrogenism secondary to hyperinsulinemia (Metwally *et al.*, 2007).

Like in women, cows in negative energy balance show a disturbed metabolic profile. For example, lower insulin and IGF1, increased growth hormone and also reduced leptin concentrations. These alterations could indirectly (via lower LH concentrations and pulsatility) or directly (by altering the follicle's sensitivity to gonadotropins) affect follicular growth (Leroy

et al., 2008a). Lactating cows also present with deviating endocrine profiles, for example low estrogen concentrations, potentially impairing follicle and oocyte maturation (Kendrick *et al.*, 1999, Comin *et al.*, 2002, Leroy *et al.*, 2008a). For a more extensive review, see Wathes *et al.* (2003). Importantly, little research has so far focused on whether these endocrine imbalances have long term effects on the quality of an oocyte ovulated the next month.

c) The oviduct and the endometrium

The **oviduct** plays a pivotal role in fertilization, by serving as a sperm reservoir, functioning as the environment for sperm capacitation and directing the oocyte and the spermatozoa to one another. The oviduct furthermore provides the environment for early embryo development (Alberts *et al.*, 2008, Besenfelder *et al.*, 2012). As studying the physiology and dynamics of the oviduct in women is very difficult (both ethically and practically), researchers have to rely on animal models. Again, the cow provides a valuable model. Indeed, the oviduct of dairy cows has been used as a means to study the influence of lipolysis-linked maternal metabolic disorders on oviduct function. In a study of Rizos *et al*. (2010), *in vitro* produced embryos were transferred to the oviduct of non-lactating heifers and to the oviduct of post-partum cows. They recovered less embryos from cows, compared to heifers. Of those embryos recovered, less had reached the blastocyst stage on Day 7 of embryonic growth in cows, compared to heifers (Rizos *et al.*, 2010). Similar results were obtained when the sustaining ability of the oviduct of postpartum lactating versus postpartum non-lactating cows was investigated (Maillo *et al.*, 2012). Overall, these results indicate that the oviduct of metabolically compromised cows is less capable to sustain early embryo development, compared to normal, non-lactating cows of heifers (Rizos *et al.*, 2010, Maillo *et al.*, 2012).

Furthermore, the **endometrium** plays an essential role in establishing a successful pregnancy. Most, but not all studies report on reduced implantation and pregnancy rates and higher miscarriage rates in obese women when receiving donor oocytes (Bellver *et al.*, 2003, Bellver *et al.*, 2007, Luke *et al.*, 2011). Additionally, there are studies reporting that obesity changes endometrial gene expression patterns (Bellver *et al.*, 2011), confirming that it may indeed affect endometrial function. Further evidence for an impaired endometrial function is provided by mechanistic studies in women suffering from polycystic ovarian syndrome (PCOS). Polycystic ovarian syndrome (PCOS) is a common endocrine disorders characterized by for example oligo/anovulation, elevated levels of circulating androgens, insulin resistance and hyperinsulinemia. Interestingly, androgen receptors and steroid receptor co-activators are over-expressed in the endometrium of women with PCOS. Furthermore, biomarkers of

endometrial receptivity to embryonic implantation are decreased and epithelial estrogen receptor expression is altered in women with PCOS. The endometrium is not only responsive to steroid hormones, like estradiol, progesterone and androgens, it's function is also influenced by insulin, IGF1 and IGFBPs. Thus, elevated estrogen (without the counteracting effects of progesterone in the absence of ovulation), hyperinsulinemia, elevated free IGF1 and androgens, and obesity all likely contribute to endometrial dysfunction, infertility and increased miscarriage rate, common in women with PCOS (reviewed by Giudice, 2006).

d) Changes in the maternal micro-environment

Several studies aimed to investigate different parameters present in the follicular fluid and their potential impact on oocyte developmental competence. Numerous factors, like total protein, albumin (Butler, 2003, Melendez *et al.*, 2003, Iwata *et al.*, 2010), urea (De Wit *et al.*, 2001, Fahey *et al.*, 2001, Leroy *et al.*, 2004, Ferreira *et al.*, 2011), C-reactive protein (Levin *et al.*, 2007, Lamaita *et al.*, 2012) and levels of reactive oxygen species (ROS) (Attaran *et al.*, 2000, Das *et al.*, 2006, Oral *et al.*, 2006, Jana *et al.*, 2010) have been studied by using human and several animal models and different experimental designs. However, so far, no clear marker for oocyte quality has been found (Revelli *et al.*, 2009).

The importance of the maternal micro-environment for oocyte and embryo quality

There is much evidence that points to the importance of the maternal micro-environment for the acquisition of oocyte developmental competence and subsequent embryo development and quality. First, oocytes matured ***in vivo*** have been shown to be of superior developmental competence, compared to their ***in vitro*** couterparts (Rizos *et al.*, 2002a, c). In this regard, *in vitro* derived embryos present with a changed transcriptome (bovine) (Niemann and Wrenzycki, 2000, Rizos *et al.*, 2002b), reduced developmental kinetics (mice) (Bowman and McLaren, 1970, Harlow and Quinn, 1982) and a higher degree of apoptosis in the inner cell mass (bovine) (Gjorret *et al.*, 2003). Moreover, *in vivo* derived bovine blastocysts present with a more extensive diffusion of Lucifer Yellow, indicative for a better intercellular communication, compared to their *in vitro* counterparts (Boni et al., 1999). Also, rates of aerobic glycolysis and oxygen consumption were higher in bovine *in vitro* produced embryos (Khurana and Niemann, 2000). This is in agreement with the previously discussed quiet embryo hypothesis, stating that high embryo quality is related to a low energy metabolism (Leese, 2002). It furthermore shows that different environments (*in vitro* or *in vivo*), can have a very profound effect at the level of the embryo. Second, studies using donor oocytes provide us with the unique opportunity to study the effect of intrinsic oocyte quality on the further

development of the embryo. This **oocyte donation model** has been proposed as the best human model to discrimate effects of obesity on the oocyte and embryo as well as the endometrium and uterine receptivity (Bellver *et al.*, 2007). It has been reported that when using oocyte donation, implantation, clinical pregnancy, twin pregnancy and live-birth rates were significantly reduced as BMI increased in the recipient women (Bellver *et al.*, 2007, Bellver *et al.*, 2013), suggesting that the endometrial environment in obese women impairs development. However, studies are not always in agreement, as several studies report that use of donor oocytes overcomes the risk of failure to achieve a clinical pregnancy in obese women (Styne-Gross *et al.*, 2005, Luke *et al.*, 2011, Jungheim *et al.*, 2013b). This latter observation suggests that intrinsic oocyte and embryo quality are more important for ensuring continued embryonic growth and pregnancy, than the endometrial environment.

These sometimes conflicting results may originate from the embryo's ability to adapt to a new environment. Indeed, pre-implantation mammalian embryos have an impressive capacity to adapt to their environment (Leese *et al.*, 2008). The embryo can for example compensate for missing nutrients or growth factors and overcome the effect of hazardous components by changing its developmental characteristics (Fair *et al.*, 2004, Watson, 2006). However, it is important to keep in mind that such adaptations may persist into and affect postnatal health.

It is not the strongest or the most intelligent who will survive, but those who can best manage change - Charles Darwin (Origin of Species, 1859)

Maternal metabolic disorders and fertility: what can we learn from animal models?

Various researchers have used mice fed a high fat diet to mimic the effects of dietary-induced obesity and insulin resistance (Purcell and Moley, 2011). This generally results in weight gain, elevated insulin and NEFA concentrations, but not in severe hyperglycemia. **Dietary-induced obesity** in mice resulted in increased apoptosis of ovarian follicle cells and smaller as well as less mature oocytes (Jungheim *et al.*, 2010). In agreement, murine dietary-induced obesity, with accompanying hyperinsulinemia and dyslipidemia, resulted more often in anovulation, compared to normal weight mice (Minge *et al.*, 2008). Murine oocytes and zygotes exposed to maternal obesity preconceptionally, presented with altered mitochondrial function and a diminished blastocyst development (Igosheva et al., 2010). Additionally, *in vitro* embryo development was slower in embryos derived from oocytes out of obese mice and the inner cell mass to trophectoderm ratio was reduced, indicating that blastomeres were preferentially

allocated to placental precursors instead of to embryonic cells (Minge *et al.*, 2008). Finally, Jungheim *et al.* (2010) showed that at 13 weeks of age, pups delivered from obese mice were significantly larger, glucose intolerant and presented with increased cholesterol and body fat, compared to normal weight mice. All these results suggest that obesity during the pre- and periconceptional period may affect oocyte developmental competence and embryo quality, which may even have consequences for the offspring's health.

Even though hyperglycemia is not always present with obesity and insulin resistance, results from studies using the **hyperglycemic type I diabetic mouse** may be relevant to investigate the relation between diabetes/hyperglycemia and oocyte and embryo quality. Maternal type I diabetes caused smaller and less developed follicles with a higher degree of apoptosis in murine granulosa cells (Chang *et al.*, 2005). It was also shown that the gap junctional coupling between the murine oocyte and cumulus cells may be impaired by maternal diabetes (Ratchford *et al.*, 2008) and the communication between murine cumulus cells may be compromised as connexin-43 expression was found to be lower in diabetic samples (Chang *et al.*, 2005). Furthermore, mitochondrial dysfunction has been noted in the cumulus cells of type I diabetic mice, evidenced as decreased ATP and citrate, as well as increased apoptosis (Wang *et al.*, 2010). Oocytes from type I diabetic mice (induced by injection of streptozotocin/alloxan or by mutation) were smaller, showed a delayed maturation (Diamond *et al.*, 1989, Colton *et al.*, 2002, Chang *et al.*, 2005), spindle defects and chromosome misalignments during oocyte maturation (Chang *et al.*, 2005).

In women with obesity or type II diabetes **hyperglycaemic** insults may impair oocyte development through delayed nuclear maturation (Jungheim et al., 2010, Sutton-McDowall et al., 2010). Exposing embryos *in vitro* to high glucose concentrations is toxic for early embryo development (mouse) (Fraser *et al.*, 2007), decreases the number of cells per embryo and increases the frequency of apoptotic cells (bovine) (Jimenez *et al.*, 2003). Furthermore, amongst bovine embryos that did develop under hyperglycemic conditions *in vitro*, the proportion of female embryos was higher, which was proposed to be attributed to the higher production of X-linked inhibitor of apoptosis protein in female embryos (Jimenez *et al.*, 2003). Murine embryos from hyperglycemic mothers furthermore have increased concentrations ROS, lower levels of glutathione and an impaired mitochondrial function (reviewed by Amaral *et al.* (2008) and Grindler and Moley (2013)). On a molecular level, a micro-array analysis of bovine blastocysts exposed to high glucose concentrations during the early cleavage stages showed that pathways associated with diabetes and tumorigenesis through genes controlling

the Warburg effect (i.e. emphasis on use of anaerobic glycolysis rather than oxidative phosphorylation), were affected (Cagnone *et al.*, 2012). Additionally, **hypoglycaemia** in early postpartum dairy cows has an indirect negative effect on reproduction, by changing LH secretion or ovarian responsiveness to gonadotrophins (Rutter and Manns, 1987, Butler and Smith, 1989). Furthermore, hypoglycaemic conditions are reflected in the micro-environment of the bovine pre-ovulatory follicle (Leroy et al., 2004) and low glucose concentrations may hamper bovine oocyte development *in vitro* (Leroy et al., 2006).

Besides the previously described effects of insulin on the sex steroid balance, **hyperinsulinemia** may also directly affect oocytes and embryos. Indeed, feeding mice a high fat diet for 16 weeks led to weight gain and hyperinsulinemia, poor oocyte quality and impaired ovulation (Minge *et al.*, 2008). Even after removal from the maternal environment and fertilization and culture *in vitro*, oocytes from high fat fed mice had a reduced developmental competence and a decreased inner cell mass to trophoblast ratio (Minge *et al.*, 2008). Also, Adamiak *et al.* (2005) found a negative relationship between plasma insulin levels and blastocyst yield after bovine oocyte isolation and *in vitro* embryo culture. It has furthermore been suggested that elevated insulin or IGF1 *in vitro* can lead to apoptosis in murine blastocysts (Chi *et al.*, 2000).

Supplemental dietary fat in high yielding dairy cows has been shown to increase the size of the pre-ovulatory follicle and its production of estradiol (Beam and Butler, 1997, Moallem *et al.*, 2007, Zachut *et al.*, 2008). This is probably effectuated by the induction of **high cholesterol** concentrations, being the precursor for all steroid hormones (androgens, estrogens and progestins), in both follicular fluid and plasma. Hypercholesterolemia also enhances progesterone secretion, thus supporting early embryo developmental competence (Lammoglia *et al.*, 1996, McNamara *et al.*, 2003). However, serum from dietary-induced hyperlipidaemic cows, used during the *in vitro* maturation of bovine oocytes, resulted in reduced blastocyst formation, blastocyst cell number, mitotic index and hatching rate after vitrification (Leroy *et al.*, 2010). These embryos also presented with increased mRNA abundance of genes related to lipid metabolism and apoptosis. Furthermore, fat-rich diets have been shown to induce extensive and genome-wide DNA methylation changes in human skeletal muscle, which may contribute to the development of metabolic diseases (Jacobsen *et al.*, 2012). Long term maternal hypercholesterolemic conditions in the rabbit may eventually result in growth retardation of the offspring (Picone *et al.*, 2011).

From the above, it is clear that there is a link between fertility and maternal metabolic conditions (modulated by diet or not). More and more evidence points to the contribution of a reduced oocyte quality. As the oocyte is very vulnerable to changes in its micro-environment, the follicular fluid composition in metabolically compromised women, is of potential interest.

Follicular fluid as a link between maternal metabolic health and oocyte developmental competence

Previous studies in the cow, have shown that metabolic alterations in the serum compostion may have repercussions for the follicular micro-environment (Leroy *et al.*, 2004, Leroy *et al.*, 2012). Could the follicular fluid serve as a potential link between metabolic disorders and an impaired oocyte quality? Up until now, it remains unclear if the serum composition is reflected in the follicular fluid of women undergoing IVF treatment and if such a change in follicular fluid composition may affect oocyte developmental competence. Furthermore, it is unknown how this relation between serum and follicular fluid may be altered by metabolic disorders, like obesity. Therefore, a first research question is formulated (**Figure 4**):

How are serum metabolic changes, related to maternal metabolic disorders, like obesity, reflected in the ovarian follicular fluid and how may this affect oocyte developmental competence and subsequent embryo quality?

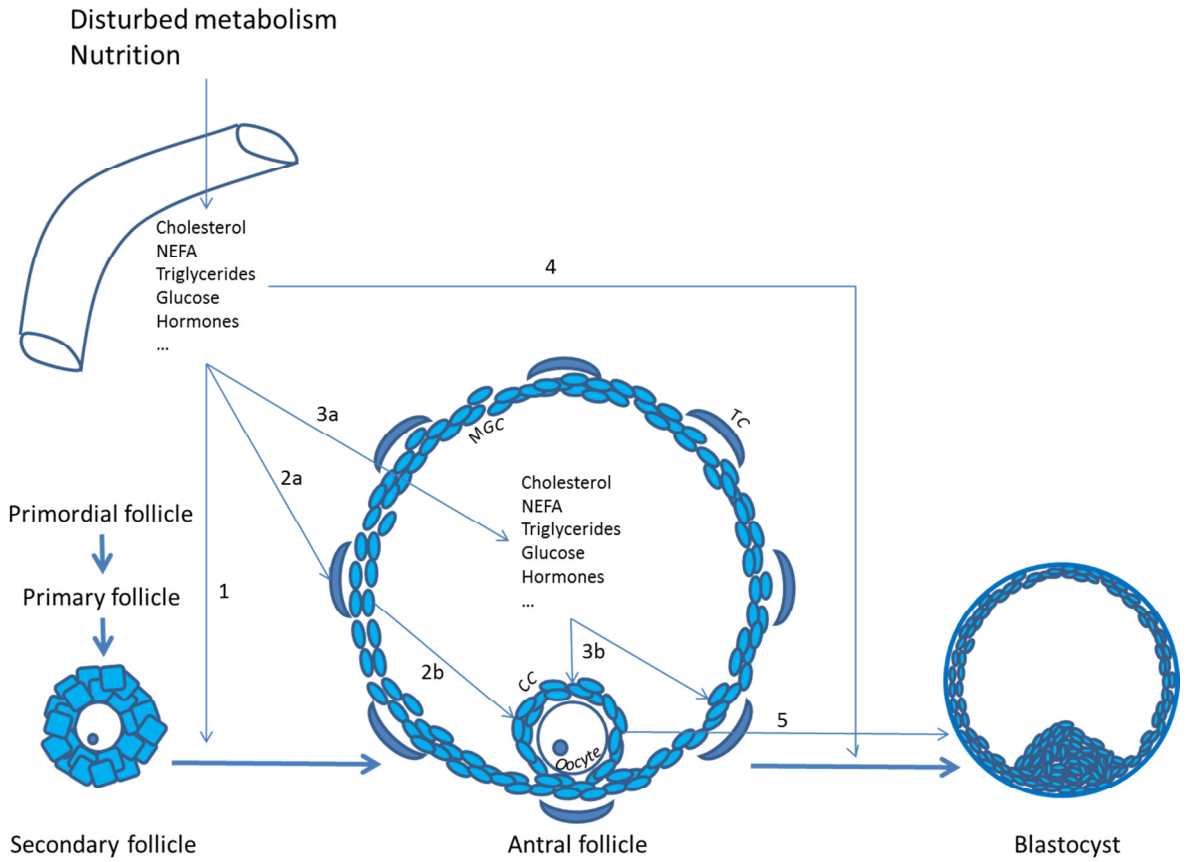


Figure 4: Pathways leading to subfertility in metabolically compromised women. A disturbed maternal metabolism or diet may influence the serum composition, by affecting for example cholesterol, triglyceride, NEFA, glucose and hormone concentrations. Such altered serum profile may directly affect follicular growth from the early pre-antral (primordial, primary, secondary) stage until the antral stage (**1**). Furthermore, serum alterations may affect theca and granulosa cell function (**2a**), that may in turn affect cumulus-oocyte complex function (**2b**). Serum changes may also be reflected in the follicular micro-environment (**3a**), where they can potentially alter granulosa and cumulus-oocyte complex function (**3b**). The serum composition may also alter the oviductal micro-environment, potentially influencing early embryo physiology (**4**). Ultimately, a changed cumulus-oocyte-complex physiology, through one of previously described pathways, may directly affect and pre-program early embryo development (**5**). CC: cumulus cell, MGC: mural granulosa cell, TC: theca cell.

4. The oocyte takes centre stage: a fatty acid rich pathway to subfertility

a) Fatty acids in the maternal micro-environment and their effect on reproductive function

From the preceeding review of literature, we have learned that the composition of the follicular fluid plays a pivotal role in the acquisition of oocyte developmental competence. Of particular importance in this micro-environment are fatty acids, because they are involved in numerous cellular processes and pathways. Under normal circumstances, fatty acids play an essential physiological role as a source of metabolic energy, as substrate for cell membrane biogenesis, and as precursor for many inter- and intracellular signalling molecules, such as prostaglandins, leukotrienes, thromboxanes, platelet-activating factors and others (McArthur *et al.*, 1999). Within the cell, exogenously provided fatty acids may be stored in lipid droplets (triglycerides), used for membrane biogenesis (phospholipids), cholesterol synthesis or energy provision through β-oxidation (NEFAs). So, fatty acids are esterified in cholesteryl-esters, triglycerides and phospholipids or free, mainly bound to albumin (McArthur *et al.*, 1999).

Importantly, the metabolic health of an individual, as indicated for example by body weight, can change the fatty acid composition in both serum and follicular fluid (Robker *et al.*, 2009, Jungheim *et al.*, 2011a), which could potentially affect oocyte developmental competence. Within the follicular fluid, fatty acids mainly serve as an essential energy source by following β-oxidation, the TCA cycle and oxidative phosphorylation (**Figure 3**) (Dunning *et al.*, 2010) and they can act as precursors for steroid synthesis and membrane biogenesis (McArthur *et al.*, 1999), which are essential in normal ovarian function. Studies in which β-oxidation is inhibited, provide support for the essential role of β-oxidation for the acquisition of developmental competence (Dunning *et al.*, 2010, Paczkowski *et al.*, 2013).

Much information regarding the effect of fatty acids on reproductive function is provided by studies relying on dietary fatty acid supplementation or fat feeding. These studies have shown that dietary fatty acids can impact on numerous levels of the reproductive axis, for example by changing ovarian activity, follicular growth, corpus luteum function and the uterine environment (for an extensive review, see Leroy *et al.*, 2014). However, a large number of studies also indicate that dietary fatty acid changes, can have repercussions for oocyte and embryo quality. In this regard, it has been shown that a diet rich in **n-3 poly-unsaturated fatty acids** (PUFAs) reduced ovulation rate and litter size in mice (Yi *et al.*, 2012) and increased the production of ROS and mitochondrial disfunction in murine oocytes (Wakefield *et al.*, 2008). In contrast, several studies revealed positive effects of n-3 fatty acids, like a favourable effect on

oocyte quality in sheep (Zeron et al., 2002), which was associated with changes in the fatty acid composition of the oocyte, resulting in a better membrane integrity following chilling. Also improved embryo morphology in women undergoing ART (Hammiche *et al.*, 2011) and embryo cleavage rate in bovine embryos (Zachut *et al.*, 2010) were documented in response to dietary n-3 intake. Likewise, *in vitro* bovine oocyte maturation in the presence of n-3 PUFAs improved nuclear oocyte maturation, but the presence of **n-6 PUFAs** reduced the resumption of meiosis (Marei *et al.*, 2009, Marei *et al.*, 2010). In agreement, feeding cows with linoleic acid (n-6) rich diets resulted in a lower oocyte develomental competence (Bilby *et al.*, 2006) and a significantly decreased cryotolerance (Guardieiro *et al.*, 2014). It is generally accepted that alterations in dietary fatty acid intake cause a similar shift in the fatty acid profile of bovine follicular fluid, cumulus cells and the oocyte (Wonnacott *et al.*, 2010, Zachut *et al.*, 2010). However, Fouladi-Nashta *et al.* (2009) showed that the ovary can protect the oocyte against major fluctuations in plasma n-3 and n-6 fatty acids, as they could not find significant effects on fatty acid composition in bovine granulosa cells or in the oocyte's developmental potential.

Even though much information is provided by studies investigating the effect of dietary fatty acid supplementation on reproductive function in general and oocyte and embryo more specifically, up until now, knowledge on the specific fatty acid composition of human follicular fluid in different lipid fractions is lacking. Importantly, the biological effect of a fatty acid at the cellular level may depend on the fraction it is esterified to. For example, phospholipid fatty acids mainly contribute to membrane integrity (Ibarguren *et al.*, 2014) and triglyceride fatty acids to the formation of lipid droplets (Watt and Hoy, 2012). Therefore a second research question is formulated:

How are fatty acids distributed in the ovarian follicular fluid and how do they associate with lipolytic conditions, like obesity?

b) Non-esterified fatty acids as an important link between maternal metabolic disorders and subfertility

Prolonged and increased mobilization of body fat (lipolysis), through an increased action of hormone sensitive lipase, typical in for example obese women or women with metabolic syndrome or type 2 diabetes, leads to chronic elevated NEFA concentrations in the serum. Elevated NEFA concentrations have known **cytotoxic effects** on several cell types *in vitro*, such as pancreatic β-cells (Cnop *et al.*, 2001), Leydig cells (Lu *et al.*, 2003), nerve growth factor differentiated cells (Ulloth *et al.*, 2003) and hepatocytes (Wu and Cederbaum, 2000). However, they can also exert a negative effect at the level of the **ovary** (Kruip and Kemp, 1999). Multiple *in vitro* studies have tried to elucidate the mechanisms by which elevated NEFA concentrations may affect granulosa (Vanholder *et al.*, 2005) and theca cell (Vanholder *et al.*, 2006) function, as well as oocyte developmental competence and embryo quality (Leroy *et al.*, 2005, Aardema *et al.*, 2011, Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a).

Pathways by which NEFAs may exert their negative effect

For NEFAs to exert a negative effect, they have to be taken up by cells and metabolized. Once a fatty acid is taken up by the cell (see McArtur *et al.* (1999) for specific mechanisms), fatty acids are targeted to a specific metabolic fate, like esterification to glycerides (phospholipids, triglycerides), mitochondrial oxidation and peroxisomal catabolism or formation and secretion of chylomicrons and very LDLs (McArthur *et al.*, 1999). So, long chain acyl CoAs (activated form of intracellular fatty acids) are substrates for multiple cellular pathways, including energy production through β-oxidation, phospholipid formation and triglyceride formation. In addition, there are also known pathways by which elevated NEFAs may lie at the origin of insulin resistance and type 2 diabetes.

Elevated NEFAs in relation to insulin resistance - Elevated NEFA concentrations contribute to the development of insulin resistance. More specifically, the **classic model of lipid-induced insulin resistance** (based on muscle cells) states that (Zhang *et al.*, 2010): Enhanced supply of fatty acid results in accumulation of lipid intermediates including triglycerides, diacylglycerol, long chain acyl CoA and ceramide. These intermediates interfere with the downstream signalling molecules along the insulin signalling pathway and cause a decrease in GLUT4 translocation. Consequently, less glucose is taken up by the cell. Furthermore, the same authors present an **alternative model for insulin resistance related to obesity**: Enhanced supply of fatty acids results in increased fatty acid uptake into the cell, which causes an increase in mitochondrial uptake of fatty acids in the form of long chain acyl CoA. As a result, β-oxidation of long chain acyl CoA is increased, to a degree that exceeds the capacity of the

TCA cycle and the electron transport chain to utilize β-oxidation products, also resulting in an build-up of products of incomplete β-oxidation. This leads to increased oxidative stress and ROS production, which in turn interferes with the downstream signalling molecules along the insulin signalling pathway, resulting in a decrease in GLUT4 translocation and consequently a decrease in glucose uptake by the cell.

Other pathways - Fatty acids are usually metabolized by mitochondrial β-oxidation for the purpose of **energy production**. Furthermore, the cytotoxic mechanism of elevated NEFA concentrations may also be brought about by the formation of **ceramides** (Maedler *et al.*, 2001, Lu *et al.*, 2003), as it has been shown that in response to an exposure to fatty acids, the islets cells of diabetic rats displayed increased incorporation of fatty acids into ceramide, which was accompanied by apoptosis (Shimabukuro *et al.*, 1998a, Shimabukuro *et al.*, 1998b). Exogenously provided NEFAs may also be stored within the cell as **triglycerides** (Aardema *et al.*, 2011), which has been proposed as a potential cytoprotective mechanism. Furthermore, fatty acids may influence cell membrane function by altering membrane **phospholipids**. It has indeed been shown that saturated fatty acids decrease and unsaturated fatty acids increase membrane fluidity (Macdonald and Macdonald, 1988). Elevated NEFAs may also exert their negative effect through increased **ER stress** (Kharroubi *et al.*, 2004, Cnop, 2008, Li *et al.*, 2010) and increased expression of **nitric oxide** synthase, enhancing subsequent nitric oxide-mediated β-cell apoptosis (Shimabukuro *et al.*, 1997, Van Hoeck *et al.*, 2014).

Elevated NEFA and reproductive cells

Somatic cells of the follicle - The somatic cells of the ovarian follicle sustain oocyte development up to a point where the oocyte has acquired developmental competence (Tanghe et al., 2002, Fair, 2003). It was previously shown that lipolysis linked elevated NEFA concentrations alter the steroidogenic profile and the viability of both granulosa and theca cells (Vanholder et al., 2005, Vanholder et al., 2006) (**Figure 5, pathway 2a**). In these studies, pathophysiological concentrations of palmitic, stearic and/or oleic acid were used, as they are the most predominant fatty acids in bovine follicular fluid (Leroy *et al.*, 2005). These elevated NEFA concentrations inhibited cell proliferation in bovine **granulosa cells** *in vitro*, which was partly due to the induction of apoptosis as determined by an annexin V-FITC/propidium iodide staining of the granulosa cells. Furthermore, estradiol production was stimulated by elevated NEFA concentrations (Vanholder et al., 2005). The authors suggested that apoptotic bovine granulosa cells can maintain steroidogenesis as long as the steroidogenic organelles remain intact (Amsterdam et al., 1997). A study in immortalized rat granulosa cells also showed that

these organelles cluster during the process of apoptosis (Kerental et al., 1995), which could create the image of a higher steroidogenic capacity, while it in fact is a consequence of apoptosis. However, Jorritsma *et al.* (2004) could not detect an effect of elevated NEFA (C18:1) concentrations on progesterone production by *in vitro* cultured bovine granulosa cells, even though they did document a negative effect on cell proliferation. It was also shown that saturated FAs (palmitic and stearic acid) induce apoptosis in human granulosa cells *in vitro* (Mu et al., 2001). Interestingly, arachidonic acid protected granulosa cells from palmitic acid and stearic acid induced apoptosis (Mu et al., 2001). *In vitro* bovine **theca cell** number was reduced due to exposure to a mixture of elevated NEFA concentrations. Furthermore, these elevated NEFA concentrations reduced progesterone production by *in vitro* bovine theca cells. This effect is most likely induced by the cytotoxic effect of elevated NEFAs, as the authors showed an increased percentage of early apoptotic and late apoptotic/necrotic cells (Vanholder et al., 2006). When bovine *in vitro* maturing COCs were exposed to elevated NEFA concentrations during the crucial final phase of maturation, remarkable differences in gene expression profiles in the **cumulus cells** surrounding the oocyte could be documented (Van Hoeck *et al.*, 2013a). In this regard, cumulus cells that were exposed to elevated NEFA concentrations presented with a decreased expression level of genes encoding enzymes involved in REDOX regulation: *GAPDH*, *GPX1*, *G6PD* and *LDHA* (Van Hoeck *et al.*, 2013a). Because cumulus cells are involved in safeguarding the oocyte from damage induced by ROS, likely by providing glutathione to the oocyte (Geshi et al., 2000), this altered gene expression profile could lead to an impaired oocyte quality. Furthermore, *in vitro* research showed that bovine cumulus cells presented with an increased rate of apoptosis and even necrosis after NEFA exposure (Leroy *et al.*, 2005).

Final oocyte maturation - The effect of elevated NEFA concentrations on oocyte (**Figure 5, pathway 3b**) and subsequent embryo quality has been studied extensively *in vitro*. Elevated NEFA concentrations have been shown to delay bovine oocyte maturation *in vitro* (Jorritsma *et al.*, 2004). Furthermore, Aardema *et al.* (2011), showed that oocytes actively take up and metabolize NEFAs out of their environment (store them in triglycerides, phospholipids and metabolize them), but that only a small fraction stayed in the oocyte in the NEFA fraction. In this study, palmitic and stearic acid had a negative effect on the amount of fat stored in intracellular lipid droplets. Additionally, there was a detrimental effect on oocyte developmental competence. On the contrary, oleic acid increased fat storage in lipid droplets and improved oocyte developmental competence. Also, the negative effects caused by palmitic and stearic acid could by counteracted by the addition of oleic acid to the treatment (Aardema et al., 2011). A similar protective effect was described in pancreatic islet cells, where

oleic acid was shown to reduce palmitate induced lipotoxicity, possibly by promoting triglyceride formation as a cytoprotective mechanism (Cnop et al., 2001). These studies further highlight the importance of fatty acid ratios and concentrations in maturing oocytes. Gene expression analysis in bovine oocytes after NEFA exposure showed that several genes related to REDOX regulation were differentially expressed (Van Hoeck *et al.*, 2013a). This, together with the previously described altered gene expression patterns in the surrounding cumulus cells, strongly suggests that elevated NEFA concentrations alter the REDOX status in COCs, which could affect early embryo physiology and quality.

The embryo - Alterations in the oocyte's metabolism, due to exposure to NEFAs during final oocyte maturation, could directly extend to the embryo as well (**Figure 5, pathway 5**). It was indeed shown that exposure of bovine oocytes to elevated NEFA concentrations during the crucial final phase of maturation, not only reduces oocyte developmental competence (Leroy *et al.*, 2005), but also alters the metabolism of the resultant embryos *in vitro* (Van Hoeck et al., 2011). Blastocysts, originating from NEFA exposed oocytes, presented with distinct differences

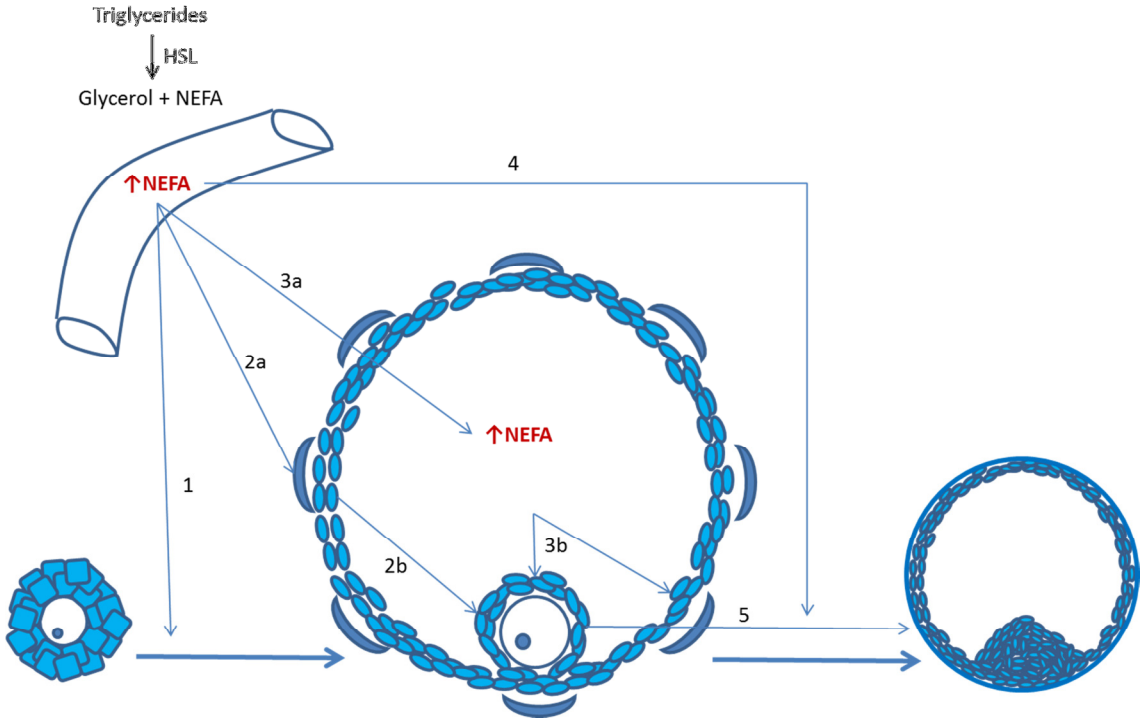


Figure 5: Pathways by which elevated NEFAs may exert their negative effect. Serum elevated NEFA concentrations may directly affect follicular growth (**1**). Furthermore, elevated NEFA concentrations may affect theca and granulosa cell function (**2a**), which may in turn affect cumulus oocyte function (**2b**). Serum elevated NEFA concentrations are also reflected in the follicular micro-environment (**3a**), where they can potentially alter granulosa and cumulus-oocyte complex function (**3b**). Elevated serum NEFA concentrations may also alter the oviductal micro-environment, potentially influencing early embryo physiology (**4**). Ultimately, a changed cumulus-oocyte-complex functionality, through one of previously described pathways, may directly affect early embryo development (**5**).

in gene expression patterns. By using qRT-PCR, genes involved in *de novo* DNA methylation, metabolism, fatty acid synthesis, mitochondrial biogenesis, oxidative stress and growth were identified as contributors to the effect of elevated NEFA concentrations during maturation on bovine blastocyst quality (Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a). Furthermore, micro-array approaches revealed that elevated NEFAs affected pathways involved in lipid metabolism, carbohydrate metabolism and cell death (Van Hoeck *et al.*, 2013b). Elevated NEFA concentrations also increased the overall turnover of amino acids by the bovine embryo (Van Hoeck *et al.*, 2011), which is predictive for a lower viability (Sturmey *et al.*, 2008a), compared to control counterparts. Also, in these embryos, oxygen, pyruvate and glucose consumption was decreased, while lactate consumption steeply increased (Van Hoeck *et al.*, 2011). These data thus indicate a reduced oxidative metabolism (reduced oxygen consumption) in bovine embryos originating from NEFA exposed oocytes. Together with the data on pyruvate and glucose consumption, this strongly suggests a drop in rate of oxidative phosphorylation (Van Hoeck *et al.*, 2011). But, if the exogenous NEFAs provided are not used for ATP production via β-oxidation, TCA cycle conversion and oxidative phosphorylation, then what are they used for? Interestingly and in accordance with the study of Aardema *et al.* (2011), embryos originating from NEFA exposed oocytes present with an increased intracellular lipid content, compared to embryos originating from oocytes only exposed to elevated stearic acid concentrations (Van Hoeck *et al.*, 2013b). This suggests that NEFAs may be stored in intracellular lipid droplets.

It is now also suggested that metabolic changes in pre-implantation embryos, may have repercussions for the metabolic health of the offspring (DOHaD principle). In this regard, it has been shown that elevated NEFA concentrations during oocyte maturation affected maternally imprinted IGF 2 receptor gene expression (Van Hoeck *et al.*, 2011). Furthermore, a study of Jungheim *et al.* (2011b) showed that pre-implantation exposure of mouse embryos *in vitro* to elevated palmitic acid concentrations resulted in fetal growth restriction followed by a catch-up growth in the offspring. These studies emphasize the ultimate importance of the maternal micro-environment in the earliest stages of life and for the normal and healthy development of the offspring.

When bovine embryos instead of oocytes were exposed to elevated NEFA concentrations during their *in vitro* development (**Figure 5, pathway 4**), embryo development was hampered and gene transcription changed. Transcriptome analyses showed that multiple pathways, mainly related to oxidative metabolism and ceramide formation, were involved in the negative effect of NEFA concentrations on embryo quality (Van Hoeck *et al.*, submitted).

Importantly, these experimental *in vitro* studies have mainly focused on the effect of **short term elevated NEFA** concentrations on somatic cells or gametes. Two other factors remain to be studied. First, Britt (Britt, 1992) hypothesized that the biochemical environment in which the follicle grows from the primordial to ovulatory stage can determine the steroidogenic capacity of the follicle and the subsequent developmental competence of the oocyte in high-yielding dairy cows. However, up until now, it remains unclear how **long term elevated NEFA** concentrations, as present in metabolically compromised (obese) women, may affect the acquisition of oocyte developmental competence during the very vulnerable processes of folliculogenesis and oocyte growth (**Figure 5, pathway 1**). Second, current research models have only focused on the effect of elevated NEFA concentrations during the *in vitro* maturation of COCs (**Figure 5, pathway 3b**), while *in vivo*, prolonged elevated NEFA concentrations affect the growing **follicle as a whole** throughout folliculogenesis and oocyte growth as previously described, which has never been studied.

Because of these knowledge 'gaps', the following third research question was formulated (**Figure 5, pathways 1, 2a, 2b and 5**):

> **How do prolonged elevated NEFA concentrations affect the growth and differentiation of the ovarian follicle as a whole and the maturing oocyte enclosed within? Furthermore, how are embryo development, quality and metabolism affected by adverse metabolic conditions during follicular development?**

5. Research models

Studying the relation between human infertility and metabolic disorders is irrevocably related to ethical restraints for the use of human material. Data from human fertility centres can provide us information regarding morphological grading and growth of oocytes and embryos, in relation to the metabolic health of the mother, but more in depth research often requires ethical approval. Therefore, researchers rely on animal models. The choice of animal model is extremely important to be able to make correct assumptions about the human situation, i.e. to extrapolate. Several animal models have been used to study the relation between metabolism and fertility, such as bovine, porcine, rodent and primate animal models. All of them have provided very useful information about specific pathways involved, but present species specific limitations in experimental design and extrapolating results (see also '*Maternal metabolic disorders and fertility: what can we learn from animal models?*').

a) Models used in this thesis

In the experimental studies described in this thesis, two animal models were used, namely a bovine and a murine model. The **bovine model** has been used substantially to study human (in)fertility, mainly because there are many similarities between bovine and human follicular physiology, oocyte maturation and early embryo development (for extensive review, see Menezo and Herubel, 2002 and Adams *et al.,* 2012). Using bovine oocytes retrieved from slaughterhouse ovaries, has furthermore provided many researchers with the necessary material to perform high-throughput *in vitro* research (*in vitro* embryo production). Moreover, relatively large volumes of follicular fluid for research purposes can be obtained through oocyte pick-up sessions. Also, high producing dairy cows often present with the metabolic challenging period of negative energy balance post-partum (Leroy *et al.*, 2008, Leroy *et al.*, 2008a), making these cows a valuable *in vivo* animal model to study the relation between lipolysis-linked maternal metabolic disorders and fertility. The **mouse model** has been used extensively for all kinds of *in vitro* and *in vivo* research. Mice are small and breed rapidly (short gestational period). However, important differences exist between human and rodent reproduction. For example the period of oocyte recruitment is much shorter in mice than in women and the mouse is a poly-ovulatory animal (Menezo and Herubel, 2002, Jungheim *et al.*, 2013a). Nevertheless, the murine genome and functional proteins are very similar to those of humans (Alberts *et al.*, 2008).

In this thesis, the bovine model was used to study the effect of potential stressors from the follicular fluid during the final phase of oocyte maturation (24h). However, the bovine model is limited to such a **short term exposure** during oocyte maturation, because only the routine *in vitro* production of bovine embryos, in which COCs are matured, fertilized and presumptive zygotes cultured until the blastocyst stage *in vitro*, is available for high-throughput research. Even though attempts are being made to optimize bovine *in vitro* follicle cultures, allowing prolonged *in vitro* follicle and oocyte growth (Jorssen *et al.*, 2014), no standardized and reproducible culture is currently available. *In vitro* maturation from pre-antral follicles, followed by oocyte fertilization and birth of offspring after embryo transfer has only been successful in mice (first time: Eppig and O'Brien (1996)). The murine model thus provides the unique opportunity to study follicular growth *in vitro*, including *in vitro* oocyte maturation and early embryo development (Cortvrindt and Smitz, 1998, Cortvrindt and Smitz, 2001, Cortvrindt and Smitz, 2002), by means of a well-established and validated follicle culture system. This culture system covers the processes of follicle growth from the early pre-antral until the antral stage *in vitro*, with the subsequent isolation and fertilization of the matured oocytes and *in vitro* embryo development until the blastocyst stage. The model presents two major advantages. Firstly, it allows a **long term exposure** to metabolic stressors during follicular growth from the early pre-antral until the antral stage *in vitro*, with subsequent oocyte isolation, fertilization and embryo development. Secondly, it allows the **exposure of the follicle as a biological unit**. The culture system has indeed been proven to have a functional interaction between theca cells, granulosa cells, cumulus cells and the oocyte, together forming a metabolic unit (Cortvrindt and Smitz, 2002). This also means that the effect of a potential stressor can be divided into **direct** effects at the level of the oocyte and **indirect** effects mediated through the altered function of theca, granulosa or cumulus cells. This is important, because in *in vitro* animal models like the bovine, only COCs are available, isolated from the follicle they once were part of. Effects of stressors may in this case only originate from effects at the level of the oocyte and/or the cumulus cells.

b) Animal models and experimental designs: how valid are they?

Extrapolating results from an in vitro mouse follicle culture – Even though the murine follicle culture system was chosen particularly because of the higher resemblance to the *in vivo* situation (long term exposure of the follicle as a whole), it is important to stress that *in vivo* conditions are not the same as *in vitro* conditions. For example, *in vitro* cultured cells are exposed to higher concentrations of oxygen, than *in vivo*, which might result in increased oxidative stress. Furthermore, factors like light, follicle/oocyte/embryo handling and overall metabolism of the follicles themselves may govern the generation of free radicals, potentially leading to impairment of cellular function *in vitro* (damaged lipids, proteins, nucleic acids, DNA and RNA) (Agarwal et al., 2008). Therefore, extrapolating results must be done with caution.

Embryo quality after in vitro follicle culture - The murine model applied in this thesis reproducibly provided embryonic growth until the blastocyst stage after follicular oocyte maturation. However, the quality of the resultant embryos might be impaired by the extensive culture period (up to 18 days). As it has been shown that bovine *in vivo* embryos are of superior quality than *in vitro* embryos (Rizos et al., 2002a), it might stand to reason that murine embryos originating from an extended follicle culture *in vitro*, might be of an even lower quality. This is evidenced for example by the decreased blastocyst rates from follicle cultured oocytes, compared to *in vitro* matured oocytes after *in vivo* follicle growth (personal observations). However, in depth studies into the viability and quality of the *in vitro* follicle culture derived embryos, compared to embryos originating from *in vivo* or *in vitro* matured oocytes, is currently lacking.

Limitations of in vitro follicle culture and embryo production - By using our murine model, we aimed to cover a large timeframe of follicular development. We were able, but also limited to culture the follicles from the early secondary stage onwards, which does not permit to evaluate the extremely important stages of 'inactive' primordial follicle growth, as well as initiation of growth and primary follicle growth (Fair, 2003, Fair, 2010). In this regard, culture systems still need further refinement and validation, to be able to culture follicles from a primordial stage up until the antral stage with the development of a competent oocyte in a standardized way. It is possible to culture mouse follicles from a primordial stage, which gives rise to mature oocytes that are able to undergo fertilization with the subsequent birth of live offspring (Eppig and O'Brien, 1996). However, oocyte developmental competence remains low.

In conclusion, the models used in this thesis were chosen particularly because of the highest resemblance to the human situation (bovine) and the possibility to study long term effects of the metabolic stressor on the follicular unit (murine).

6. Conclusions

Lipolysis-linked maternal metabolic disorders, like obesity, have been proposed as a major risk factor for fertility failure. An altered maternal micro-environment may lie at the origin of a changed follicular development, leading to an impaired oocyte developmental competence and an altered embryo metabolism.

Even though much information is available about the oocyte's micro-environment and potential effects for oocyte developmental competence, there remains a current lack of specific information regarding the composition of the follicular fluid in women undergoing IVF treatment and its relation to the maternal metabolic health-related serum composition. More specifically and in light of the pathophysiological importance of fatty acids, a description of the fatty acids in the follicular fluid, in different lipid fractions, is highly needed. It is furthermore unknown whether and how the composition of the follicular fluid might affect oocyte developmental competence, which can only be studied by uncoupling the follicular fluid factor from the factor of intrinsic oocyte quality. The effect of lipolysis-linked elevated NEFA concentrations on oocyte and embryo quality has been extensively reviewed. However, the consequences of elevated NEFA concentrations during the whole period of folliculogenesis on the follicle as a functional unit enclosing a growing oocyte, which is much more *in vivo* like, remains to be studied.

References

Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB and Gadella BM. Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biol Reprod* 2011; **85**: 62-69.

Ackert CL, Gittens JE, O'Brien MJ, Eppig JJ and Kidder GM. Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev Biol* 2001; **233**: 258-270.

Adamiak SJ, Mackie K, Watt RG, Webb R and Sinclair KD. Impact of nutrition on oocyte quality: cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. *Biol Reprod* 2005; **73**: 918-926.

Adams GP, Singh J and Baerwald AR. Large animal models for the study of ovarian follicular dynamics in women. *Theriogenology* 2012; **78**: 1733-1748.

Aerts JMJ and Bols PEJ. Ovarian Follicular Dynamics: A Review with Emphasis on the Bovine Species. Part I: Folliculogenesis and Pre-antral Follicle Development. *Reprod Domest Anim* 2010; **45**: 171-179.

Agarwal A, Gupta S, Sekhon L and Shah R. Redox considerations in female reproductive function and assisted reproduction: From molecular mechanisms to health implications. *Antioxidants & Redox Signaling* 2008; **10**: 1375-1403.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P. Development of multicellular organisms, In: Molecular Biology of the Cell, 2008. Garland Science, New York (USA).

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P. Sexual reproduction: meiosis, germ cells, and fertilization, In: Molecular Biology of the Cell. Fifth edition, 2008. Garland Science, New York (USA).

Amaral S, Oliveira PJ and Ramalho-Santos J. Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive oxygen species. *Curr Diabetes Rev* 2008; **4**: 46-54.

Amsterdam A, Dantes A, Selvaraj N and Aharoni D. Apoptosis in steroidogenic cells: Structure-function analysis. *Steroids* 1997; **62**: 207-211.

Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A and Sharma RK. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. *International journal of fertility and women's medicine* 2000; **45**: 314-320.

Azziz R. Reproductive endocrinologic alterations in female asymptomatic obesity. *Fertil Steril* 1989; **52**: 703-725.

Beam SW and Butler WR. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. *Biol Reprod* 1997; **56**: 133-142.

Bellver J, Martinez-Conejero JA, Labarta E, Alama P, Melo MA, Remohi J, Pellicer A and Horcajadas JA. Endometrial gene expression in the window of implantation is altered in obese women especially in association with polycystic ovary syndrome. *Fertil Steril* 2011; **95**: 2335-2341.

Bellver J, Melo MAB, Bosch E, Serra V, Remohi J and Pellicer A. Obesity and poor reproductive outcome: the potential role of the endometrium. *Fertil Steril* 2007; **88**: 446-451.

Bellver J, Pellicer A, Garcia-Velasco JA, Ballesteros A, Remohi J and Meseguer M. Obesity reduces uterine receptivity: clinical experience from 9,587 first cycles of ovum donation with normal weight donors. *Fertil Steril* 2013; **100**: 1050-1058.

Bellver J, Rossal LP, Bosch E, Zuniga A, Corona JT, Melendez F, Gomez E, Simon C, Remohi J and Pellicer A. Obesity and the risk of spontaneous abortion after oocyte donation. *Fertil Steril* 2003; **79**: 1136-1140.

Besenfelder U, Havlicek V and Brem G. Role of the oviduct in early embryo development. *Reprod Domest Anim* 2012; **47 Suppl 4**: 156-163.

Bianchi E, Doe B, Goulding D, Wright GJ. Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature* 2014; **508** (7497): 483-487

Bilby TR, Block J, do Amaral BC, Sa Filho O, Silvestre FT, Hansen PJ, Staples CR and Thatcher WW. Effects of dietary unsaturated fatty acids on oocyte quality and follicular development in lactating dairy cows in summer. *J Dairy Sci* 2006; **89**: 3891-3903.

Billig H, Chun SY, Eisenhauer K and Hsueh AJW. Gonadal cell apoptosis: Hormone-regulated cell demise. *Hum Reprod Update* 1996; **2**: 103-117.

Binelli M and Murphy BD. Coordinated regulation of follicle development by germ and somatic cells. *Reprod Fert Develop* 2010; **22**: 1-12.

Boland NI, Humpherson PG, Leese HJ and Gosden RG. Characterization of follicular energy metabolism. *Hum Reprod* 1994; **9**: 604-609.
Boni R, Tosti E, Roviello S and Dale B. Intercellular communication in in vivo- and in vitro-produced bovine embryos. *Biol Reprod* 1999; **61**: 1050-1055.
Boron W and Boulpaep E. Medical Physiology, 2005. Elsevier Saunders, USA.
Bowman P and McLaren A. Cleavage rate of mouse embryos in vivo and in vitro. *J Embryol Exp Morphol* 1970; **24**: 203-207.
Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod* 2004; **19**: 2319-2324.
Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. *Proceedings of the Annual Convention of the American Association of Bovine Practitioners* 1992; **24**: 39-43.
Buck Louis GM. Persistent environmental pollutants and couple fecundity: an overview. *Reproduction* 2014; **147**: R97-R104.
Butcher L, Coates A, Martin KL, Rutherford AJ and Leese HJ. Metabolism of pyruvate by the early human embryo. *Biol Reprod* 1998; **58**: 1054-1056.
Butler WR. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. *Livest Prod Sci* 2003; **83**: 211-218.
Butler WR and Smith RD. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. *J Dairy Sci* 1989; **72**: 767-783.
Cagnone GL, Dufort I, Vigneault C and Sirard MA. Differential gene expression profile in bovine blastocysts resulting from hyperglycemia exposure during early cleavage stages. *Biol Reprod* 2012; **86**: 50.
Chagas LM, Bass JJ, Blache D, Burke CR, Kay JK, Lindsay DR, Lucy MC, Martin GB, Meier S, Rhodes FM et al. New perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. *Journal of Dairy Science* 2007; **90**: 4022-4032.
Chand AL and Legge M. Amino acid transport system L activity in developing mouse ovarian follicles. *Hum Reprod* 2011; **26**: 3102-3108.
Chang AS, Dale AN and Moley KH. Maternal diabetes adversely affects preovulatory oocyte maturation, development, and granulosa cell apoptosis. *Endocrinology* 2005; **146**: 2445-2453.
Chi MM, Schlein AL and Moley KH. High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology* 2000; **141**: 4784-4792.
Clark AM, Ledger W, Galletly C, Tomlinson L, Blaney F, Wang X and Norman RJ. Weight-Loss Results in Significant Improvement in Pregnancy and Ovulation Rates in Anovulatory Obese Women. *Hum Reprod* 1995; **10**: 2705-2712.
Cnop M. Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes. *Biochem Soc Trans* 2008; **36**: 348-352.
Cnop M, Hannaert JC, Hoorens A, Eizirik DL and Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 2001; **50**: 1771-1777.
Collado-Fernandez E, Picton HM and Dumollard R. Metabolism throughout follicle and oocyte development in mammals. *The International journal of developmental biology* 2012; **56**: 799-808.
Colton SA, Pieper GM and Downs SM. Altered meiotic regulation in oocytes from diabetic mice. *Biol Reprod* 2002; **67**: 220-231.
Comin A, Gerin D, Cappa A, Marchi V, Renaville R, Motta M, Fazzini U and Prandi A. The effect of an acute energy deficit on the hormone profile of dominant follicles in dairy cows. *Theriogenology* 2002; **58**: 899-910.
Cortvrindt R and Smitz J. Early preantral mouse follicle in vitro maturation: Oocyte growth, meiotic maturation and granulosa-cell proliferation. *Theriogenology* 1998; **49**: 845-859.
Cortvrindt R and Smitz J. In vitro follicle growth: achievements in mammalian species. *Reprod Domest Anim* 2001; **36**: 3-9.
Cortvrindt RG and Smitz JEJ. Follicle culture in reproductive toxicology: a tool for in-vitro testing of ovarian function? *Hum Reprod Update* 2002; **8**: 243-254.
Coticchio G, Albertini DF and De Santis L. Oogenesis, 2013. Springer Verlag, London ; New York.

Das S, Chattopadhyay R, Ghosh S, Ghosh S, Goswami SK, Chakravarty BN and Chaudhury K. Reactive oxygen species level in follicular fluid--embryo quality marker in IVF? *Hum Reprod* 2006; **21**: 2403-2407.

De Wit AAC, Cesar MLF and Kruip TAM. Effect of urea during in vitro maturation on nuclear maturation and embryo development of bovine cumulus-oocyte-complexes. *Journal of Dairy Science* 2001; **84**: 1800-1804.

Deloos F, Kastrop P, Vanmaurik P, Vanbeneden TH and Kruip TAM. Heterologous Cell Contacts and Metabolic Coupling in Bovine Cumulus Oocyte Complexes. *Mol Reprod Dev* 1991; **28**: 255-259.

Diamond MP, Moley KH, Pellicer A, Vaughn WK and DeCherney AH. Effects of streptozotocin- and alloxan-induced diabetes mellitus on mouse follicular and early embryo development. *J Reprod Fertil* 1989; **86**: 1-10.

Downs SM, Mosey JL and Klinger J. Fatty Acid Oxidation and Meiotic Resumption in Mouse Oocytes. *Mol Reprod Dev* 2009; **76**: 844-853.

Dumollard R, Carroll J, Duchen MR, Campbell K and Swann K. Mitochondrial function and redox state in mammalian embryos. *Semin Cell Dev Biol* 2009; **20**: 346-353.

Dumollard R, Duchen M and Carroll J. The role of mitochondrial function in the oocyte and embryo. *Curr Top Dev Biol* 2007; **77**: 21-49.

Dunning KR, Akison LK, Russell DL, Norman RJ and Robker RL. Increased Beta-Oxidation and Improved Oocyte Developmental Competence in Response to L-Carnitine During Ovarian In Vitro Follicle Development in Mice. *Biol Reprod* 2011; **85**: 548-555.

Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ and Robker RL. Beta-Oxidation Is Essential for Mouse Oocyte Developmental Competence and Early Embryo Development. *Biol Reprod* 2010; **83**: 909-918.

Durlinger ALL, Visser JA and Themmen APN. Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction* 2002; **124**: 601-609.

Eppig JJ. Analysis of Mouse Oogenesis Invitro Oocyte Isolation and Utilization of Exogenous Energy-Sources by Growing Oocytes. *J Exp Zool* 1976; **198**: 375-381.

Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 2001; **122**: 829-838.

Eppig JJ and O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* 1996; **54**: 197-207.

Eppig JJ, Pendola FL, Wigglesworth K and Pendola JK. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: Amino acid transport. *Biol Reprod* 2005; **73**: 351-357.

Eppig JJ, Wigglesworth K, Pendola FL and Hirao Y. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol Reprod* 1997; **56**: 976-984

Fahey J, Boland MP and O'Callaghan D. The effects of dietary urea on embryo development in superovulated donor ewes and on early embryo survival and development in recipient ewes. *Anim Sci* 2001; **72**: 395-400.

Fair T. Follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci* 2003; **78**: 203-216.

Fair T. Mammalian oocyte development: checkpoints for competence. *Reprod Fert Develop* 2010; **22**: 13-20.

Fair T, Hulshof SC, Hyttel P, Greve T and Boland M. Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anat Embryol (Berl)* 1997; **195**: 327-336.

Fair T, Murphy M, Rizos D, Moss C, Martin F, Boland MP and Lonergan P. Analysis of differential maternal mRNA expression in developmentally competent and incompetent bovine two-cell embryos. *Mol Reprod Dev* 2004; **67**: 136-144.

Ferguson EM and Leese HJ. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. *Mol Reprod Dev* 2006; **73**: 1195-1201.

Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA and Navarro PA. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* 2009; **71**: 836-848.

Ferreira FA, Gomez RGG, Joaquim DC, Watanabe YF, Paula LADE, Binelli M and Rodrigues PHM. Short-term urea feeding decreases in vitro hatching of bovine blastocysts. *Theriogenology* 2011; **76**: 312-319.

Fouladi-Nashta AA, Wonnacott KE, Gutierrez CG, Gong JG, Sinclair KD, Garnsworthy PC and Webb R. Oocyte quality in lactating dairy cows fed on high levels of n-3 and n-6 fatty acids. *Reproduction* 2009; **138**: 771-781.

Fraser RB, Waite SL, Wood KA and Martin KL. Impact of hyperglycemia on early embryo development and embryopathy: in vitro experiments using a mouse model. *Hum Reprod* 2007; **22**: 3059-3068.

Gambineri A, Pelusi C, Vicennati V, Pagotto U and Pasquali R. Obesity and the polycystic ovary syndrome. *Int J Obesity* 2002; **26**: 883-896.

Gardner AJ and Evans JP. Mammalian membrane block to polyspermy: new insights into how mammalian eggs prevent fertilisation by multiple sperm. *Reprod Fert Develop* 2006; **18**: 53-61.

Gardner DK, Wale PL, Collins R and Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod* 2011; **26**: 1981-1986.

Geshi M, Takenouchi N, Yamauchi N and Nagai T. Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells. *Biol Reprod* 2000; **63**: 1730-1734.

Gilchrist RB, Ritter LJ and Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. *Anim Reprod Sci* 2004; **82-83**: 431-446.

Giudice LC. Endometrium in PCOS: Implantation and predisposition to endocrine CA. *Best practice & research Clinical endocrinology & metabolism* 2006; **20**: 235-244.

Gjorret JO, Knijn HM, Dieleman SJ, Avery B, Larsson LI and Maddox-Hyttel P. Chronology of apoptosis in bovine embryos produced in vivo and in vitro. *Biol Reprod* 2003; **69**: 1193-1200.

Gosden RG, Hunter RH, Telfer E, Torrance C, Brown N. Physiological factors underlying the formation of ovarian follicular fluid. *J Reprod Fertil* 1988; **82**: 813-825.

Grindler NM and Moley KH. Maternal obesity, infertility and mitochondrial dysfunction: potential mechanisms emerging from mouse model systems. *Mol Hum Reprod* 2013; **19**: 486-494.

Guardieiro MM, Machado GM, Bastos MR, Mourao GB, Carrijo LHD, Dode MAN, Leroy JLMR and Sartori R. A diet enriched in linoleic acid compromises the cryotolerance of embryos from superovulated beef heifers. *Reprod Fert Develop* 2014; **26**: 511-520.

Hammiche F, Vujkovic M, Wijburg W, de Vries JHM, Macklon NS, Laven JSE and Steegers-Theunissen RPM. Increased preconception omega-3 polyunsaturated fatty acid intake improves embryo morphology. *Fertil Steril* 2011; **95**: 1820-1823.

Harlow GM and Quinn P. Development of preimplantation mouse embryos in vivo and in vitro. *Aust J Biol Sci* 1982; **35**: 187-193.

Harris ID, Python J, Roth L, Alvero R, Murray S and Schlaff WD. Physicians' perspectives and practices regarding the fertility management of obese patients. *Fertil Steril* 2011; **96**: 991-992.

Harris SE, Adriaens I, Leese HJ, Gosden RG and Picton HM. Carbohydrate metabolism by murine ovarian follicles and oocytes grown in vitro. *Reproduction* 2007; **134**: 415-424.

Harris SE, Leese HJ, Gosden RG and Picton HM. Pyruvate and oxygen consumption throughout the growth and development of murine oocytes. *Mol Reprod Dev* 2009; **76**: 231-238.

Hemmings KE, Leese HJ and Picton HM. Amino acid turnover by bovine oocytes provides an index of oocyte developmental competence in vitro. *Biol Reprod* 2012; **86**: 165, 161-112.

Hewitson LC, Martin KL and Leese HJ. Effects of metabolic inhibitors on mouse preimplantation embryo development and the energy metabolism of isolated inner cell masses. *Mol Reprod Dev* 1996; **43**: 323-330.

Hugentobler SA, Diskin MG, Leese HJ, Humpherson PG, Watson T, Sreenan JM and Morris DG. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. *Mol Reprod Dev* 2007; **74**: 445-454.

Hunter MG, Robinson RS, Mann GE and Webb R. Endocrine and paracrine control of follicular development and ovulation rate in farm species. *Anim Reprod Sci* 2004; **82-3**: 461-477.

Hyttel P, Fair T, Callesen H and Greve T. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* 1997; **47**: 23-32.

Hyttel P, Greve T and Callesen H. Ultrastructural aspects of oocyte maturation and fertilization in cattle. *Journal of reproduction and fertility Supplement* 1989; **38**: 35-47.

Ibarguren M, Lopez DJ and Escriba PV. The effect of natural and synthetic fatty acids on membrane structure, microdomain organization, cellular functions and human health. *Biochim Biophys Acta* 2014; **1838**: 1518-1528.

Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR and McConnell J. Maternal Diet-Induced Obesity Alters Mitochondrial Activity and Redox Status in Mouse Oocytes and Zygotes. *Plos One* 2010; **5**: e10074.

Iwata H, Tanaka H, Kanke T, Sakaguchi Y, Shibano K, Kuwayama T and Monji Y. Follicle Growth and Oocyte Developmental Competence in Cows With Liver Damage. *Reprod Domest Anim* 2010; **45**: 888-895.

Jacobsen SC, Brons C, Bork-Jensen J, Ribel-Madsen R, Yang B, Lara E, Hall E, Calvanese V, Nilsson E, Jorgensen SW et al. Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men. *Diabetologia* 2012; **55**: 3341-3349.

Jain A, Polotsky AJ, Rochester D, Berga SL, Loucks T, Zeitlian G, Gibbs K, Polotsky HN, Feng S, Isaac B et al. Pulsatile luteinizing hormone amplitude and progesterone metabolite excretion are reduced in obese women. *J Clin Endocr Metab* 2007; **92**: 2468-2473.

Jana SK, K NB, Chattopadhyay R, Chakravarty B and Chaudhury K. Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. *Reprod Toxicol* 2010; **29**: 447-451.

Jimenez A, Madrid-Bury N, Fernandez R, Perez-Garnelo S, Moreira P, Pintado B, de la Fuente J and Gutierrez-Adan A. Hyperglycemia-induced apoptosis affects sex ratio of bovine and murine preimplantation embryos. *Mol Reprod Dev* 2003; **65**: 180-187.

Jorritsma R, Cesar ML, Hermans JT, Kruitwagen CLJJ, Vos PLAM and Kruip TAM. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. *Anim Reprod Sci* 2004; **81**: 225-235.

Jorssen EPA, Langbeen A, Fransen E, Martinez EL, Leroy JLMR and Bols PEJ. Monitoring preantral follicle survival and growth in bovine ovarian biopsies by repeated use of neutral red and cultured in vitro under low and high oxygen tension. *Theriogenology* 2014; **82**: 387-395.

Jungheim ES, Louden ED, Chi MM, Frolova AI, Riley JK and Moley KH. Preimplantation exposure of mouse embryos to palmitic acid results in fetal growth restriction followed by catch-up growth in the offspring. *Biol Reprod* 2011b; **85**: 678-683.

Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS and Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. *Fertil Steril* 2011a; **95**: 1970-1974.

Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE and Moley KH. Diet-Induced Obesity Model: Abnormal Oocytes and Persistent Growth Abnormalities in the Offspring. *Endocrinology* 2010; **151**: 4039-4046.

Jungheim ES, Schon SB, Schulte MB, DeUgarte DA, Fowler SA and Tuuli MG. IVF outcomes in obese donor oocyte recipients: a systematic review and meta-analysis. *Hum Reprod* 2013b; **28**: 2720-2727.

Jungheim ES, Travieso JL, Carson KR and Moley KH. Obesity and reproductive function. *Obstet Gynecol Clin North Am* 2012; **39**: 479-493.

Jungheim ES, Travieso JL and Hopeman MM. Weighing the impact of obesity on female reproductive function and fertility. *Nutr Rev* 2013a; **71**: S3-S8.

Kendrick KW, Bailey TL, Garst AS, Pryor AW, Ahmadzadeh A, Akers RM, Eyestone WE, Pearson RE and Gwazdauskas FC. Effects of energy balance on hormones, ovarian activity, and recovered oocytes in lactating Holstein cows using transvaginal follicular aspiration. *Journal of Dairy Science* 1999; **82**: 1731-1741.

Kerental I, Suh BS, Dantes A, Lindner S, Oren M and Amsterdam A. Involvement of P53 Expression in Camp-Mediated Apoptosis in Immortalized Granulosa-Cells. *Experimental cell research* 1995; **218**: 283-295.

Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M and Eizirik DL. Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. *Endocrinology* 2004; **145**: 5087-5096.

Khurana NK and Niemann H. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. *Biol Reprod* 2000; **62**: 847-856.

Koning AMH, Kuchenbecker WKH, Groen H, Hoek A, Land JA, Khan KS and Mol BWJ. Economic consequences of overweight and obesity in infertility: a framework for evaluating the costs and outcomes of fertility care. *Hum Reprod Update* 2010; **16**: 246-254.

Koning AMH, Mutsaerts MAQ, Kuchenbecher WKH, Broekmans FJ, Land JA, Mol BW and Hoek A. Complications and outcome of assisted reproduction technologies in overweight and obese women. *Hum Reprod* 2012; **27**: 457-467.

Krauchunas AR, Wolfner MF. Molecular changes during egg activation. *Curr Top Dev Biol* 2013; **102**: 267-292.

Krisher RL and Prather RS. A role for the Warburg effect in preimplantation embryo development: metabolic modification to support rapid cell proliferation. *Mol Reprod Dev* 2012; **79**: 311-320.

Kruip TA and Kemp B. Voeding en vruchtbaarheid bij landbouwhuisideren. *Tijdschr Diergeneesk* 1999; **124**: 462-467.

Lamaita RM, Pontes A, Belo AV, Caetano JP, Andrade SP, Candido EB, Trainman P, Carneiro MM and Silva-Filho AL. Inflammatory response patterns in ICSI patients: a comparative study between chronic anovulating and normally ovulating women. *Reprod Sci* 2012; **19**: 704-711.

Lammoglia MA, Willard ST, Oldham JR and Randel RD. Effects of dietary fat and season on steroid hormonal profiles before parturition and on hormonal, cholesterol, triglycerides, follicular patterns, and postpartum reproduction in Brahman cows. *J Anim Sci* 1996; **74**: 2253-2262.

Law DCG, Maclehose RF and Longnecker MP. Obesity and time to pregnancy. *Hum Reprod* 2007; **22**: 414-420.

Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* 2002; **24**: 845-849.

Leese HJ. Metabolism of the preimplantation embryo: 40 years on. *Reproduction* 2012; **143**: 417-427.

Leese HJ and Barton AM. Production of Pyruvate by Isolated Mouse Cumulus Cells. *J Exp Zool* 1985; **234**: 231-236.

Leese HJ, Baumann CG, Brison DR, McEvoy TG and Sturmey RG. Metabolism of the viable mammalian embryo: quietness revisited. *Mol Hum Reprod* 2008; **14**: 667-672.

Leese HJ, Conaghan J, Martin KL and Hardy K. Early human embryo metabolism. *Bioessays* 1993; **15**: 259-264.

Leroy J, Sturmey R, Van Hoeck V, De Bie J, McKeegan P and Bols P. Dietary fat supplementation and the consequences for oocyte and embryo quality: hype or significant benefit for dairy cow reproduction? *Reprod Domest Anim* 2014; **49**: 353-361.

Leroy JL, Opsomer G, Van Soom A, Goovaerts IG and Bols PE. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part I. The importance of negative energy balance and altered corpus luteum function to the reduction of oocyte and embryo quality in high-yielding dairy cows. *Reprod Domest Anim* 2008a; **43**: 612-622.

Leroy JL, Van Hoeck V, Clemente M, Rizos D, Gutierrez-Adan A, Van Soom A, Uytterhoeven M and Bols PE. The effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality. *Hum Reprod* 2010; **25**: 768-778.

Leroy JL, Van Soom A, Opsomer G, Goovaerts IG and Bols PE. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part II. Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows. *Reprod Domest Anim* 2008b; **43**: 623-632.

Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G and Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 2005; **130**: 485-495.

Leroy JLMR, Rizos D, Sturmey R, Bossaert P, Gutierrez-Adan A, Van Hoeck V, Valckx S and Bols PEJ. Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: a focus on dairy cow fertility. *Reprod Fert Develop* 2012; **24**: 1-12.

Leroy JLMR, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PEJ, Dewulf J and de Kruif A. Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. *Theriogenology* 2004; **62**: 1131-1143.

Leroy JLMR, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G and Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 2005; **130**: 485-495.

Leroy JLMR, Vanholder T, Opsomer G, Van Soom A and de Kruif A. The In vitro development of bovine oocytes after maturation in glucose and beta-hydroxybutyrate concentrations associated with negative energy balance in dairy cows. *Reprod Domest Anim* 2006; **41**: 119-123.

Levin I, Gamzu R, Mashiach R, Lessing JB, Amit A and Almog B. Higher C-reactive protein levels during IVF stimulation are associated with ART failure. *Journal of Reproductive Immunology* 2007; **75**: 141-144.
Li L, Lu X and Dean J. The maternal to zygotic transition in mammals. *Mol Aspects Med* 2013; **34**: 919-938.
Li LO, Klett EL and Coleman RA. Acyl-CoA synthesis, lipid metabolism and lipotoxicity. *Biochim Biophys Acta* 2010; **1801**: 246-251.
Liu K and Case A. Advanced reproductive age and fertility. *J Obstet Gynaecol Can* 2011; **33**: 1165-1175.
Lu ZH, Mu YM, Wang BA, Li XL, Lu JM, Li JY, Pan CY, Yanase T and Nawata H. Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. *Biochem Bioph Res Co* 2003; **303**: 1002-1007.
Luciano AM, Franciosi F, Dieci C, Lodde V. Changes in large-scale chromatin structure and function during oogenesis: a journey in company with follicular cells. *Anim Reprod Sci* 2014; **149**: 3-10.
Lucy MC. Mechanisms linking nutrition and reproduction in postpartum cows. *Reprod Suppl* 2003; **61**: 415-427.
Luke B, Brown MB, Stern JE, Missmer SA, Fujimoto VY, Leach R and Grp ASW. Female obesity adversely affects assisted reproductive technology (ART) pregnancy and live birth rates. *Hum Reprod* 2011; **26**: 245-252.
Macdonald RC and Macdonald RI. Membrane-Surface Pressure Can Account for Differential Activities of Membrane-Penetrating Molecules. *Journal of Biological Chemistry* 1988; **263**: 10052-10055.
Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N and Donath MY. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes* 2001; **50**: 69-76.
Maheshwari A, Scotland G, Bell J, McTavish A, Hamilton M and Bhattacharya S. The direct health services costs of providing assisted reproduction services in overweight or obese women: a retrospective cross-sectional analysis. *Hum Reprod* 2009; **24**: 633-639.
Maillo V, Rizos D, Besenfelder U, Havlicek V, Kelly AK, Garrett M and Lonergan P. Influence of lactation on metabolic characteristics and embryo development in postpartum Holstein dairy cows. *J Dairy Sci* 2012; **95**: 3865-3876.
Marci R, Lisi F, Soave I, Lo Monte G, Patella A, Caserta D and Moscarini M. Ovarian stimulation in women with high and normal body mass index: GnRH agonist versus GnRH antagonist. *Gynecol Endocrinol* 2012; **28**: 792-795.
Marei WF, Wathes DC and Fouladi-Nashta AA. The Effect of Linolenic Acid on Bovine Oocyte Maturation and Development. *Biol Reprod* 2009; **81**: 1064-1072.
Marei WF, Wathes DC and Fouladi-Nashta AA. Impact of linoleic acid on bovine oocyte maturation and embryo development. *Reproduction* 2010; **139**: 979-988.
Marques-Pinto A and Carvalho D. Human infertility: are endocrine disruptors to blame? *Endocr Connect* 2013; **2**: R15-29.
Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S and Stevens GA. National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. *Plos Med* 2012; **9**: e1001356.
Mazerbourg S, Bondy CA, Zhou J and Monget P. The insulin-like growth factor system: a key determinant role in the growth and selection of ovarian follicles? a comparative species study. *Reprod Domest Anim* 2003; **38**: 247-258.
McArthur MJ, Atshaves BP, Frolov A, Foxworth WD, Kier AB and Schroeder F. Cellular uptake and intracellular trafficking of long chain fatty acids. *J Lipid Res* 1999; **40**: 1371-1383.
McConnell NA, Yunus RS, Gross SA, Bost KL, Clemens MG and Hughes FM. Water permeability of an ovarian antral follicle is predominantly transcellular and mediated by aquaporins. *Endocrinology* 2002; **143**: 2905-2912.
McGee EA and Hsueh AJW. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* 2000; **21**: 200-214.
McNamara S, O'Mara FP, Rath M and Murphy JJ. Effects of different transition diets on dry matter intake, milk production, and milk composition in dairy cows. *J Dairy Sci* 2003; **86**: 2397-2408.
McNatty KP, Moore LG, Hudson NL, Quirke LD, Lawrence SB, Reader K, Hanrahan JP, Smith P, Groome NP, Laitinen M et al. The oocyte and its role in regulating ovulation rate: a new paradigm in reproductive biology. *Reproduction* 2004; **128**: 379-386.

Melendez P, Donovan A, Hernandez J, Bartolome J, Risco CA, Staples C and Thatcher WW. Milk, plasma, and blood urea nitrogen concentrations, dietary protein, and fertility in dairy cattle. *Journal of the American Veterinary Medical Association* 2003; **223**: 628-634.

Menezo YJ and Herubel F. Mouse and bovine models for human IVF. *Reprod Biomed Online* 2002; **4**: 170-175.

Metwally M, Li TC and Ledger WL. The impact of obesity on female reproductive function. *Obes Rev* 2007; **8**: 515-523.

Michalakis K, Mintziori G, Kaprara A, Tarlatzis BC and Goulis DG. The complex interaction between obesity, metabolic syndrome and reproductive axis: a narrative review. *Metabolism* 2013; **62**: 457-478.

Minge CE, Bennett BD, Norman RJ and Robker RL. Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone reverses the adverse effects of diet-induced obesity on oocyte quality. *Endocrinology* 2008; **149**: 2646-2656.

Moallem U, Katz M, Lehrer H, Livshitz L and Yakoby S. Role of peripartum dietary propylene glycol or protected fats on metabolism and early postpartum ovarian follicles. *Journal of Dairy Science* 2007; **90**: 1243-1254.

Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K et al. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. *Endocrinology* 2001; **142**: 3590-3597.

Neganova I, Al-Qassab H, Heffron H, Selman C, Choudhury AI, Lingard SJ, Diakonov I, Patterson M, Ghatei M, Bloom SR et al. Role of central nervous system and ovarian insulin receptor substrate 2 signaling in female reproductive function in the mouse. *Biol Reprod* 2007; **76**: 1045-1053.

Niakan KK, Han J, Pedersen RA, Simon C and Pera RA. Human pre-implantation embryo development. *Development* 2012; **139**: 829-841.

Niemann H and Wrenzycki C. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: Implications for subsequent development. *Theriogenology* 2000; **53**: 21-34.

Nishimoto H, Matsutani R, Yamamoto S, Takahashi T, Hayashi KG, Miyamoto A, Hamano S and Tetsuka M. Gene expression of glucose transporter (GLUT) 1, 3 and 4 in bovine follicle and corpus luteum. *J Endocrinol* 2006; **188**: 111-119.

Norman RJ, Noakes M, Wu RJ, Davies MJ, Moran L and Wang JX. Improving reproductive performance in overweight/obese women with effective weight management. *Hum Reprod Update* 2004; **10**: 267-280.

Oral O, Kutlu T, Aksoy E, Ficicioglu C, Uslu H and Tugrul S. The effects of oxidative stress on outcomes of assisted reproductive techniques. *J Assist Reprod Genet* 2006; **23**: 81-85.

Paczkowski M, Silva E, Schoolcraft WB and Krisher RL. Comparative Importance of Fatty Acid Beta-Oxidation to Nuclear Maturation, Gene Expression, and Glucose Metabolism in Mouse, Bovine, and Porcine Cumulus Oocyte Complexes. *Biol Reprod* 2013; **88**: 111.

Pan H, O'Brien MJ, Wigglesworth K, Eppig JJ and Schultz RM. Transcript profiling during mouse oocyte development and the effect of gonadotropin priming and development in vitro. *Developmental Biology* 2005; **286**: 493-506.

Pandey S, Maheshwari A and Bhattacharya S. Should access to fertility treatment be determined by female body mass index? *Hum Reprod* 2010; **25**: 815-820.

Park JY, Su YQ, Ariga M, Law E, Jin SLC and Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 2004; **303**: 682-684.

Pasquali R, Patton L and Gambineri A. Obesity and infertility. *Curr Opin Endocrinol Diabetes Obes* 2007; **14**: 482-487.

Pasquali R, Pelusi C, Genghini S, Cacciari M and Gambineri A. Obesity and reproductive disorders in women. *Hum Reprod Update* 2003; **9**: 359-372.

Pelland AM, Corbett HE and Baltz JM. Amino Acid transport mechanisms in mouse oocytes during growth and meiotic maturation. *Biol Reprod* 2009; **81**: 1041-1054.

Peng XR, Hsueh AJ, LaPolt PS, Bjersing L, Ny T. Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. *Endocrinology* 1991; **129**: 3200-3207.

Picone O, Laigre P, Fortun-Lamothe L, Archilla C, Peynot N, Ponter AA, Berthelot V, Cordier AG, Duranthon V and Chavatte-Palmer P. Hyperlipidic hypercholesterolemic diet in prepubertal

rabbits affects gene expression in the embryo, restricts fetal growth and increases offspring susceptibility to obesity. *Theriogenology* 2011; **75**: 287-299.
Picton H, Briggs D and Gosden R. The molecular basis of oocyte growth and development. *Mol Cell Endocrinol* 1998; **145**: 27-37.
Picton HM. Activation of follicle development: The primordial follicle. *Theriogenology* 2001; **55**: 1193-1210.
Poretsky L, Cataldo NA, Rosenwaks Z and Giudice LC. The insulin-related ovarian regulatory system in health and disease. *Endocr Rev* 1999; **20**: 535-582.
Poretsky L, Chandrasekher YA, Bai C, Liu HC, Rosenwaks Z and Giudice L. Insulin receptor mediates inhibitory effect of insulin, but not of insulin-like growth factor (IGF)-I, on IGF binding protein 1 (IGFBP-1) production in human granulosa cells. *J Clin Endocrinol Metab* 1996; **81**: 493-496.
Purcell SH and Moley KH. The impact of obesity on egg quality. *J Assist Reprod Genet* 2011; **28**: 517-524.
Ratchford AM, Esguerra CR and Moley KH. Decreased oocyte-granulosa cell gap junction communication and connexin expression in a type 1 diabetic mouse model. *Mol Endocrinol* 2008; **22**: 2643-2654.
Redding GP, Bronlund JE and Hart AL. Mathematical modelling of oxygen transport-limited follicle growth. *Reproduction* 2007; **133**: 1095-1106.
Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M and Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrin* 2009; **4**: 40.
Rieger D and Loskutoff NM. Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro. *J Reprod Fertil* 1994; **100**: 257-262.
Rizos D, Carter F, Besenfelder U, Havlicek V and Lonergan P. Contribution of the female reproductive tract to low fertility in postpartum lactating dairy cows. *Journal of Dairy Science* 2010; **93**: 1022-1029.
Rizos D, Fair T, Papadopoulos S, Boland MP and Lonergan P. Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. *Mol Reprod Dev* 2002a; **62**: 320-327.
Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, de la Fuente J and Gutierrez-Adan A. Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: Implications for blastocyst quality. *Biol Reprod* 2002b; **66**: 589-595.
Rizos D, Ward F, Duffy P, Boland MP and Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev* 2002c; **61**: 234-248.
Robker RL. Evidence that obesity alters the quality of oocytes and embryos. *Pathophysiology* 2008; **15**: 115-121.
Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M and Norman RJ. Obese Women Exhibit Differences in Ovarian Metabolites, Hormones, and Gene Expression Compared with Moderate-Weight Women. *J Clin Endocr Metab* 2009; **94**: 1533-1540.
Rodgers RJ and Irving-Rodgers HF. Formation of the Ovarian Follicular Antrum and Follicular Fluid. *Biol Reprod* 2010; **82**: 1021-1029.
Russell DL and Robker RL. Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Hum Reprod Update* 2007; **13**: 289-312.
Rutter LM and Manns JG. Hypoglycemia alters pulsatile luteinizing hormone secretion in the postpartum beef cow. *J Anim Sci* 1987; **64**: 479-488.
Sanchez F and Smitz J. Molecular control of oogenesis. *Biochim Biophys Acta* 2012; **1822**: 1896-1912.
Scaramuzzi RJ, Baird DT, Campbell BK, Driancourt MA, Dupont J, Fortune JE, Gilchrist RB, Martin GB, McNatty KP, McNeilly AS et al. Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reprod Fert Develop* 2011; **23**: 444-467.
Sfontouris IA, Lainas GT, Sakkas D, Zorzovilis IZ, Petsas GK and Lainas TG. Non-invasive metabolomic analysis using a commercial NIR instrument for embryo selection. *Journal of human reproductive sciences* 2013; **6**: 133-139.
Shah DK, Missmer SA, Berry KF, Racowsky C and Ginsburg ES. Effect of Obesity on Oocyte and Embryo Quality in Women Undergoing In Vitro Fertilization. *Obstet Gynecol* 2011; **118**: 63-70.
Shalgi R, Kraicer PF, Soferman N. Gases and electrolytes of human follicular fluid. *J Reprod Fertil* 1972; **28**: 335-340.

Shimabukuro M, Higa M, Zhou YT, Wang MY, Newgard CB and Unger RH. Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression. *J Biol Chem* 1998a; **273**: 32487-32490.

Shimabukuro M, Ohneda M, Lee Y and Unger RH. Role of nitric oxide in obesity-induced beta cell disease. *J Clin Invest* 1997; **100**: 290-295.

Shimabukuro M, Zhou YT, Levi M and Unger RH. Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 1998b; **95**: 2498-2502.

Simon AM, Goodenough DA, Li E and Paul DL. Female infertility in mice lacking connexin 37. *Nature* 1997; **385**: 525-529.

Sirard MA, Richard F, Blondin P and Robert C. Contribution of the oocyte to embryo quality. *Theriogenology* 2006; **65**: 126-136.

Smith DG and Sturmey RG. Parallels between embryo and cancer cell metabolism. *Biochem Soc Trans* 2013; **41**: 664-669.

Sturmey RG, Brison DR and Leese HJ. Assessing embryo viability by measurement of amino acid turnover. *Reprod Biomed Online* 2008a; **17**: 486-496.

Sturmey RG, Hawkhead JA, Barker EA and Leese HJ. DNA damage and metabolic activity in the preimplantation embryo. *Hum Reprod* 2009b; **24**: 81-91.

Sturmey RG and Leese HJ. Energy metabolism in pig oocytes and early embryos. *Reproduction* 2003; **126**: 197-204.

Sturmey RG, O'Toole PJ and Leese HJ. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. *Reproduction* 2006; **132**: 829-837.

Sturmey RG, Reis A, Leese HJ and McEvoy TG. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod Domest Anim* 2009a; **44 Suppl 3**: 50-58.

Sturmey RG, O'Toole PJ, Leese HJ. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the procine oocyte. *Reproduction* 2006; **132**: 829-837.

Styne-Gross A, Elkind-Hirsch K and Scott RT, Jr. Obesity does not impact implantation rates or pregnancy outcome in women attempting conception through oocyte donation. *Fertil Steril* 2005; **83**: 1629-1634.

Su YQ, Sugiura K and Eppig JJ. Mouse Oocyte Control of Granulosa Cell Development and Function: Paracrine Regulation of Cumulus Cell Metabolism. *Semin Reprod Med* 2009; **27**: 32-42.

Suarez SS and Pacey AA. Sperm transport in the female reproductive tract. *Hum Reprod Update* 2006; **12**: 23-37.

Sutovsky P and Schatten G. Paternal contributions to the mammalian zygote: fertilization after sperm-egg fusion. *Int Rev Cytol* 2000; **195**: 1-65.

Sutton-McDowall ML, Feil D, Robker RL, Thompson JG and Dunning KR. Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. *Theriogenology* 2012; **77**: 1632-1641.

Sutton-McDowall ML, Gilchrist RB and Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 2010; **139**: 685-695.

Sutton ML, Gilchrist RB and Thompson JG. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update* 2003; **9**: 35-48.

Tanghe S, Van Soom A, Nauwynck H, Coryn M and De Kruif A. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* 2002; **61**: 414-424.

The Practice Committee of the American Society for Reproductive Medicine. Smoking and infertility. *Fertil Steril* 2004; **82**: S62-S67.

Thum MY, El-Sheikhah A, Faris R, Parikh J, Wren M, Ogunyemi T, Gafar A and Abdalla H. The influence of body mass index to in-vitro fertilisation treatment outcome, risk of miscarriage and pregnancy outcome. *J Obstet Gynaecol* 2007; **27**: 699-702.

Tisdall DJ, Watanabe K, Hudson NL, Smith P and McNatty KP. FSH receptor gene expression during ovarian follicle development in sheep. *J Mol Endocrinol* 1995; **15**: 273-281.

Tortoriello DV, McMinn J and Chua SC. Dietary-induced obesity and hypothalamic infertility in female DBA/2J mice. *Endocrinology* 2004; **145**: 1238-1247.

Tripathi A, Kumar KV and Chaube SK. Meiotic cell cycle arrest in mammalian oocytes. *J Cell Physiol* 2010; **223**: 592-600.

Ulloth JE, Casiano CA and De Leon M. Palmitic and stearic fatty acids induce caspase-dependent and -independent cell death in nerve growth factor differentiated PC12 cells. *Journal of neurochemistry* 2003; **84**: 655-668.

Uyar A and Seli E. Metabolomic Assessment of Embryo Viability. *Semin Reprod Med* 2014; **32**: 141-151.

Van Hoeck V, Bols PE, Binelli M and Leroy JL. Reduced oocyte and embryo quality in response to elevated non-esterified fatty acid concentrations: A possible pathway to subfertility? *Anim Reprod Sci* 2014; **149**: 19-29.

Van Hoeck V, De Bie J, Ramos P, Rizos D, Gutierrez-Adan A, Dufort I, Sirard MA, Merckx E, Andries S, Bols PEJ et al. Elevated concentrations of non-esterified fatty acids during bovine embryo culture compromise pre-implantation embryo development and influence gene transcription. *Reproduction* submitted.

Van Hoeck V, Leroy JLMR, Arias-Alvarez M, Rizos D, Gutierrez-Adan A, Schnorbusch K, Bols PEJ, Leese HJ and Sturmey RG. Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: mechanistic insights. *Reproduction* 2013a; **145**: 33-44.

Van Hoeck V, Rizos D, Gutierrez-Adan A, Pintelon I, Jorssen E, Dufort I, Sirard MA, Verlaet A, Hermans N, Bols PE et al. Interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations. *Reprod Fertil Dev* 2013b: [Epub ahead of print].

Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE and Leroy JL. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *Plos One* 2011; **6**: e23183.

Vanholder T, Leroy JL, Soom AV, Opsomer G, Maes D, Coryn M and de Kruif A. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci* 2005; **87**: 33-44.

Vanholder T, Leroy JL, Van Soom A, Maes D, Coryn A, Fiers T, de Kruif A and Opsomer G. Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci* 2006; **92**: 51-63.

Wakefield SL, Lane M, Schulz SJ, Hebart ML, Thompson JG and Mitchell M. Maternal supply of omega-3 polyunsaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse. *Am J Physiol-Endoc M* 2008; **294**: E425-E434.

Wang Q, Frolova AI, Purcell S, Adastra K, Schoeller E, Chi MM, Schedl T and Moley KH. Mitochondrial dysfunction and apoptosis in cumulus cells of type I diabetic mice. *Plos One* 2010; **5**: e15901.

Wathes DC, Clempson AM and Pollott GE. Associations between lipid metabolism and fertility in the dairy cow. *Reprod Fert Develop* 2013; **25**: 48-61.

Wathes DC, Fenwick M, Cheng Z, Bourne N, Llewellyn S, Morris DG, Kenny D, Murphy J and Fitzpatrick R. Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. *Theriogenology* 2007; **68**: S232-S241.

Wathes DC, Taylor VJ, Cheng Z and Mann GE. Follicle growth, corpus luteum function and their effects on embryo development in postpartum dairy cows. *Reprod Suppl* 2003; **61**: 219-237.

Watson A. Oocyte cytoplasmic maturation: A key mediator of both oocyte and embryo developmental competence. *J Anim Sci* 2006; **84**: 1-1.

Watt MJ and Hoy AJ. Lipid metabolism in skeletal muscle: generation of adaptive and maladaptive intracellular signals for cellular function. *American journal of physiology Endocrinology and metabolism* 2012; **302**: E1315-1328.

Wise LA, Rothman KJ, Mikkelsen EM, Sorensen HT, Riis A and Hatch EE. An internet-based prospective study of body size and time-to-pregnancy. *Hum Reprod* 2010; **25**: 253-264.

Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC and Sinclair KD. Dietary omega-3 and-6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. *Reproduction* 2010; **139**: 57-69.

World Health Organization.Obesity and overweight. Fact sheet N° 311 (http://www.who.int/mediacentre/factsheets/fs311/en/index.html).

Wu D and Cederbaum AI. Ethanol and arachidonic acid produce toxicity in hepatocytes from pyrazole-treated rats with high levels of CYP2E1. *Molecular and cellular biochemistry* 2000; **204**: 157-167.

Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA and Youngquist RS. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biol Reprod* 1995; **53**: 951-957.

Yanagishita M, Hascall VC, Rodbard D. Biosynthesis of proteoglycans by rat granulosa cells cultured in vitro: moduclation by gonadotropins, steroid hormones, prostaglandins, and a cyclic nucleotide. *Endocrinology* 1981; **109**: 1641-1649.

Yi D, Zeng S and Guo Y. A diet rich in n-3 polyunsaturated fatty acids reduced prostaglandin biosynthesis, ovulation rate, and litter size in mice. *Theriogenology* 2012; **78**: 28-38.

Young JM and McNeilly AS. Theca: the forgotten cell of the ovarian follicle. *Reproduction* 2010; **140**: 489-504.

Zachut M, Arieli A, Lehrer H, Argov N and Moallem U. Dietary unsaturated fatty acids influence preovulatory follicle characteristics in dairy cows. *Reproduction* 2008; **135**: 683-692.

Zachut M, Dekel I, Lehrer H, Arieli A, Arav A, Livshitz L, Yakoby S and Moallem U. Effects of dietary fats differing in n-6:n-3 ratio fed to high-yielding dairy cows on fatty acid composition of ovarian compartments, follicular status, and oocyte quality. *Journal of Dairy Science* 2010; **93**: 529-545.

Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, Sullivan E and van der Poel S. The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology, 2009. *Hum Reprod* 2009; **24**: 2683-2687.

Zeron Y, Sklan D and Arav A. Effect of polyunsaturated fatty acid supplementation on biophysical parameters and chilling sensitivity of ewe oocytes. *Mol Reprod Dev* 2002; **61**: 271-278.

Zhang LY, Keung W, Samokhvalov V, Wang W and Lopaschuk GD. Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. *Bba-Mol Cell Biol L* 2010; **1801**: 1-22.

Zuelke KA and Brackett BG. Increased glutamine metabolism in bovine cumulus cell-enclosed and denuded oocytes after in vitro maturation with luteinizing hormone. *Biol Reprod* 1993; **48**: 815-820.

CHAPTER 2: HYPOTHESIS AND AIMS

Obesity has been recognized as a major risk factor for subfertility. Both human research and studies relying on animal models have focused on the relation between lipolysis-linked maternal metabolic disorders, the maternal micro-environment and/or the acquisition of oocyte developmental competence. Overall, it is accepted that adverse maternal metabolic health may jeopardize the oocyte's developmental competence. It has even been proposed that if the oocyte acquires competence and the embryo survives, embryo quality, metabolism and gene expression patterns may be altered or pre-programmed by adverse metabolic conditions in the oocyte's micro-environment. Particular attention has been paid to the role of fatty acids in the maternal micro-environment, because of their pathological relevance in both the origin and the manifestation of metabolic disorders. However, several knowledge gaps were identified throughout the introduction, formulated as research questions. Based on these research questions, the following **general hypothesis** was put forward:

> **Maternal metabolic disorders, like obesity, are associated with changes in the serum profile, that are reflected in the maternal micro-environment of the pre-ovulatory follicle. These changes alter folliculogenesis and oocyte growth, ultimately leading to a reduced oocyte developmental competence and subsequent embryo quality.**

The identified research questions (see general introduction) and the corresponding specific aims to address them are presented below.

- **How are serum metabolic changes, related to maternal metabolic disorders, like obesity, reflected in the ovarian follicular fluid and how may this affect oocyte developmental competence and subsequent embryo quality?**

  Aim 1: To investigate the relationship between a woman's BMI, the serum composition and the follicular fluid composition and how this may be associated with oocyte developmental competence (Chapter 3)

  Aim 2: To study more specifically the effect of the follicular fluid composition on oocyte developmental competence and embryo quality (Chapter 4)

- **How are fatty acids distributed in the ovarian follicular fluid and how do they associate with lipolytic conditions, like obesity?**

  Aim: To investigate how BMI is associated with the follicular fluid fatty acid composition in each specific lipid fractions (Chapter 5)

- **How do prolonged elevated NEFA concentrations affect the growth and differentiation of the ovarian follicle as a whole and the maturing oocyte enclosed within? Furthermore, how are embryo development, quality and metabolism affected by adverse metabolic conditions during follicular development?**

  Aim 1: To investigate the effect of long term elevated NEFA concentrations on follicular growth, follicle quality and oocyte developmental competence in a murine in vitro preantral follicle culture model (Chapter 6)

  Aim 2: To investigate the effect of elevated NEFA concentrations during murine preantral follicular and oocyte growth in vitro on subsequent embryo quality and metabolism (Chapter 7)

CHAPTER 3: BMI RELATED METABOLIC COMPOSITION OF THE FOLLICULAR FLUID OF WOMEN UNDERGOING ASSISTED REPRODUCTIVE TREATMENT AND THE CONSEQUENCES FOR OOCYTE AND EMBRYO QUALITY

Published in Human Reproduction. Valckx SDM, De Pauw I, De Neubourg D, Inion I, Berth M, Fransen E, Bols PEJ, Leroy JLMR (2012): BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. Human Reproduction 27(12): 3531-3539

Abstract

Background: Many studies have focused on the effect of metabolic disorders, such as obesity and type 2 diabetes, on assisted reproduction outcomes. There are, however, only few studies focusing on the importance of the correlation between serum and follicular fluid (FF) compositions and the composition of the FF as the oocyte's micro-environment, affecting its development and subsequent embryo quality. Therefore, we hypothesized that the metabolic composition of the FF of women undergoing assisted reproductive treatment is related to the serum composition and BMI and that this is associated with oocyte and embryo quality.

Methods: In this prospective cohort study, patient information, fertility treatment outcome data, FF and serum were obtained from women undergoing assisted reproductive treatment. Patients were categorized according to their body mass index (BMI, kg/m²) as normal (n = 60), overweight (n = 26) or obese (n = 20). Serum and FF samples were analysed for urea, total protein, albumin, cholesterol, HDL cholesterol, triglycerides, non-esterified fatty acids, apolipoprotein A1, apolipoprotein B, glucose, lactate, C-reactive protein (CRP), insulin-like growth factor -1 (IGF-1), IGF binding protein 3 (only in FF), free carnitine and total carnitine. Metabolite concentrations in serum and FF samples were correlated and were associated with BMI and fertility treatment outcome.

Results: Most serum metabolite differences between patients were well reflected in the FF ($P < 0.05$). Follicular fluid apolipoprotein A1 and FF total protein concentrations negatively affected oocyte quality parameters ($P < 0.05$). However, overall BMI-related associations were poor.

Conclusions: We showed that metabolic alterations in the serum are reflected in the FF and that some of these alterations may affect oocyte quality, irrespective of BMI.

Introduction

Obesity has been associated with an increased risk of reproductive failure (Pasquali *et al.*, 2007, Lash and Armstrong, 2009), with changes in the menstrual pattern, anovulatory cycles, delayed conception and multiple complications during pregnancy (Sebire *et al.*, 2001). Besides the lower chance of a spontaneous pregnancy, obesity has also been stated to impact on the overall success as well as on the costs of fertility treatments (Koning *et al.*, 2010). Even though the majority of BMI related fertility studies report a negative effect of BMI on the success rate of fertility treatments (Sebire *et al.*, 2001, Fedorcsak *et al.*, 2004), there are multiple studies that were unable to show an effect of BMI on assisted reproductive outcome (Lashen *et al.*, 1999, Dechaud *et al.*, 2006, Martinuzzi *et al.*, 2008). This controversy illustrates that the effect of obesity or other maternal metabolic disorders on oocyte quality and fertility outcome is complex and of a multifactorial kind.

Overweight and obese women display serum metabolic alterations, leading to high serum cholesterol and non-esterified fatty acid (NEFA) concentrations, hyperglycemia and insulin resistance (Lebovitz, 2006). Using repeated ovum pick-up over time in the bovine animal model, we showed that serum metabolic changes are reflected in the follicular fluid (FF) (Leroy *et al.*, 2004, Leroy *et al.*, 2005). Knowing that both the oocyte and the embryo are very vulnerable to changes in their micro-environment (Gilchrist *et al.*, 2004, Leroy *et al.*, 2012), recent research has been focusing on the follicular micro-environment of women undergoing assisted reproductive treatment (Robker *et al.*, 2009, Jungheim *et al.*, 2011a). An alteration in the composition of this micro-environment might impact on oocyte and cumulus cell quality (Leroy *et al.*, 2005, Robker *et al.*, 2009, Jungheim *et al.*, 2011a) and may in turn hamper embryo physiology, characterized for example by glucose intolerance and altered gene expression patterns in the pre-implantation embryo, possibly resulting in a reduced implantation rate and subsequent developmental abnormalities (Robker, 2008, Brewer and Balen, 2010, Van Hoeck *et al.*, 2011). However, only few studies focused on how such differences in metabolically important serum parameters are reflected in the FF and how this may affect the oocyte's developmental capacity in normal, overweight and obese women. Therefore, we hypothesized that metabolic changes in the serum of women undergoing assisted reproductive treatment are reflected in the FF of the pre-ovulatory follicle and that such metabolic changes influence the oocyte's developmental capacity. We chose to study body mass index (BMI) as a grouping variable, because of its known involvement in metabolic disorders and its potential role in subfertility. The specific aims of this study were: 1) to study if the blood metabolic profile in women, subjected to an *in vitro* fertilization (IVF) treatment, is

reflected in the FF, 2) to investigate whether the maternal BMI is related to FF metabolite concentrations and 3) to explore if the metabolic composition of the FF affects fertility treatment outcome parameters in a BMI dependent way.

Material and methods

a) Patients and IVF data

This study was approved by the ethical committee of the University of Antwerp (UA A09-04) and the ZNA Middelheim hospital, Antwerp (EC 3352). During the sampling period, every patient seeking assisted reproductive services, was asked to participate in the study. Patients who chose to enter the study signed an informed consent form. The participating women (n = 106) were treated with a long agonist protocol, in which desensitization was initiated with Busereline Nasal spray (Suprefact ®, Sanofi-aventis, Frankfurt, Germany). Follicular stimulation was induced by human menopausal gonadotropin (hMG, Menopur ®, Ferring Pharmaceuticals, Copenhagen, Denmark) or recombinant follicle stimulating hormone (rFSH, Gonal-F ®, Merck Serono, Geneva, Switserland or Puregon ®, MSD, Oss, The Netherlands). When 3 or more follicles reached a diameter of ≥ 18 mm, human chorionic gonadotropin (hCG, Pregnyl ®, MSD) was administered. Transvaginal, ultrasound-guided oocyte retrieval was performed 37 h later. Standard IVF/ICSI procedures were used as described by Van Royen et al. (1999). Briefly, oocytes were fertilized by either insemination or intra-cytoplasmatic sperm injection (ICSI) on Day 0. On Day 1, oocytes were evaluated for the presence of both pronuclei (2PN). The evaluation and grading of embryos was based on a morphological assessment of cell cleavage, cell fragmentation and the presence of multinucleated blastomeres. A top quality embryo was defined as an embryo with (1) four or five blastomeres on Day 2, and seven or more on Day 3; (2) 20% fragmentation or less on Day 3 and (3) no multinucleated blastomeres ever (Van Royen *et al.*, 1999). All embryo transfers were performed on Day 3.

Height (cm) and body weight (kg) of the patients were used to calculate the BMI score. While normal weight was defined as 18,5 ≤ BMI ≤ 24.9 kg/m² and overweight as 25.0 ≤ BMI ≤ 29.9 kg/m², patients were considered obese when the BMI exceeded 30 kg/m² (WHO, 2011). Underweight (BMI < 18.50) patients were excluded from the study. Patient specific data included age (years), IVF attempt number, dose of gonadotropins (IU) used, causes of female infertility (poly-cystic ovarian syndrome or PCOS, endometriosis, immunological subfertility, tubo-peritoneal and tubal abnormalities) and male infertility (azo-obstructive, andrologic and immunologic infertility) or idiopathic infertility. Further patient specific data revealed: serum estradiol concentrations on the day of hCG administration, total number of oocytes retrieved,

number of fertilized oocytes (number of zygotes with 2PN) following IVF or ICSI, total number of embryos, total number of top quality embryos, number of embryos transferred, fertility treatment outcome (no conception, biochemical pregnancy, clinical miscarriage, full term pregnancy) and date of birth. Based on these data, we defined different parameters as markers for oocyte quality. These include fertilization rate (number of 2PNs/number of oocytes), percentage of embryos (number of embryos/number of oocytes), percentage of top quality embryos (number of top quality embryos/number of oocytes), percentage of embryos following fertilization (number of embryos/number of 2PNs) and the percentage of top quality embryos following fertilization (number of top quality embryos/number of 2PNs).

b) Sampling and biochemical analysis of blood and follicular fluid

The follicular fluid of the largest, first punctured follicle was collected during the oocyte retrieval procedure (transvaginal follicular aspiration). Although most antral follicles > 15 mm were punctured, for the study only single follicles ≥ 18 mm were included. Only aspirates free of blood and flushing medium were used for analysis. Two blood samples were taken directly following ovum pick-up (OPU), for the retrieval of serum (Serum-gel with clotting activator, Sarstedt Monovette, Essen, Belgium) and plasma (Glucose determination with fluoride, Sarstedt Monovette, Essen, Belgium). All samples were kept at 4°C and transported to the laboratory on melting ice. Both FF (1500 g, 10 min) and blood samples (1400 g, 30 min) were centrifuged (Sigma 2-16, Qlab, Vilvoorde, Belgium) and subsequently, the supernatant was aliquoted within 2 h after sampling. All samples were stored at -80°C until analysed for standard biochemical parameters [urea, total protein, albumin and C-reactive protein (CRP)], parameters involved in fat metabolism [cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides (TG), non-esterified fatty acids (NEFAs), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), total carnitine and free carnitine], carbohydrate metabolism parameters (glucose and lactate) and important hormones and related parameters [insulin, insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 3 (IGFBP3, only on FF samples)]. Serum glucose, insulin and NEFA concentrations were used to calculate the quantitative insulin sensitivity check index (QUICKI) and the revised QUICKI (rQUICKI), which indicate the degree of insulin sensitivity (Perseghin *et al.*, 2001).

$$QUICKI = 1 / [\log (I_0) + \log (G_0)]$$

$$RQUICKI = 1 / [\log (I_0) + \log (G_0) + \log (NEFA_0)]$$

I_0: serum insulin values (mU/l), G_0: serum glucose values (mg/dl), $NEFA_0$: serum free fatty acid concentration (mmol/l).

Within and between assays variations are indicated respectively in brackets as percentages. Urea (1.7, 2.2), total protein (0.7, 2.3), albumin (0.9, 2.4), cholesterol (1.1, 1.4), HDL cholesterol (1.9, 3.8), TG (1.3, 2.3), glucose (1.1, 1.3), lactate (1.8, 1.9), ApoA1 (0.9, 2.9) and ApoB (1.0, 3.5) concentrations were determined using a Modular P system (Roche diagnostics) according to the manufacturer's instructions. NEFAs (2.5, 4.9), total carnitine (2.4, 3.5) and free carnitine (3.0, 4.4) were measured using RX Daytona (Randox Laboratories). Radio-immunoassay (RIA) kits were used to measure insulin (2.1, 6.5), IGF-1 (9.8, 10.4) and IGFBP3 (4.0, 5.6) concentrations (Biosource and Diagnostic Systems Laboratories and Diasource, respectively). High sensitivity CRP (3.1, 5.9) measurements were performed by immunonephelometry on BNII (Siemens Diagnostics).

c) Statistical analysis

All statistical analyses were performed with either PASW statistics 18 (for Windows, Chicago, IL, USA) or R 2.13.1 (www.r-project.org). Several statistical hypothesis tests were carried out to study the relation between patient information and reproductive outcome parameters, with the type of statistical test depending on the nature of the outcome parameter. The effect of ICSI/IVF on the success of the resulting pregnancy was tested using a chisquare test. A T-test was used to investigate whether the BMI was different in the group of patients treated with IVF compared to the patients treated with ICSI. Counted numbers were analysed using quasipoisson regression, with an F-test as described by Agresti (2002). Numeric variables were analysed using linear regression and binary outcomes were analysed using logistic regression. The number of embryos transferred was associated with BMI by means of a Kruskal Wasllis test. Pairwise correlation coefficients between FF and serum values were calculated with a Spearman correlation analysis. Associations between (1) FF and serum metabolites and BMI and (2) FF metabolites and fertility outcome parameters were determined based on linear regression. Linear discriminant analysis (LDA) followed by logistic regression analyses was used to investigate differences in serum and FF parameters related to female infertility etiologies. First, LDA was performed using all FF or SR compounds as predictors, and the etiology as outcome. A robust form of LDA was performed, as described by Venables and Ripley (2003). This LDA was run several times, each time contrasting one etiology versus all others combined. The importance of the individual compounds in predicting the etiology was scored using the loadings of the compounds in the LDA function. Subsequently, for the parameters with the highest loadings, logistic regression was performed with the parameter as input variable, and membership to the infertility etiology as outcome. This regression was carried out separately for each infertility cause. For these analyses, women of couples with only a male cause for

infertility were considered 'normal', rather than 'idiopathic'. The only couple with an immunological cause of infertility was excluded, due to a lack of statistical power of 1 observation. Follicular fluid and serum parameters were presented as means ± standard deviation (SD). In case the FF or serum parameters were not normally distributed, regression was performed on the logtransformed parameters. The threshold for statistical significance was set at $P < 0.05$.

Results

a) Study population

There was no difference in average age of the patients (n = 106) between BMI groups (**Table 1**). Patient information showed that 56.6% of the women had a normal body weight, 24.5% was overweight while 18.9% was obese. Body weight associated significantly with BMI (r = 0.911, $P < 0.01$) but not with height. QUICKI ($P < 0.01$) and rQUICKI ($P < 0.01$) values decreased with rising BMI, indicating a greater chance of insulin resistance in the overweight and obese group. The reason for fertility treatment was female subfertility in 13.2% of the couples, male infertility in 51.9% and combined male/female infertility in 16.0% of the couples. A total of 18.9% of the couples suffered from an unexplained infertility cause. Increasing BMI did not associate with male or female indications of subfertility. There was no difference in success rates between IVF and ICSI treatments, nor was there a statistical significant difference in the use of IVF/ICSI between BMI classes. The mean number of embryos transferred did not differ significantly between BMI groups. Linear regression showed that the IVF attempt number increased as BMI increased ($P < 0.01$). Also the gonadotropin dose increased as BMI increased ($P < 0.01$).

Table 1: Patient characteristics and reproductive outcome according to BMI with the statistical method used and the subsequent *P*-values.

	BMI				
	18.5 - 24.9 (n = 60)	**25.0-29.9** (n = 26)	**≥ 30.0** (n = 20)	**Statistical method***	***P*-value**
Age (years)	34 ± 4.7	32 ± 4.6	35 ± 6.1	Linear regression	NS
BMI (kg/m²)	21.4 ± 1.7	27.4 ± 1.5	33.0 ± 2.3	NA	NA
Infertility cause (n)				NA	NA
Idiopathic	44	17	15		
PCOS	2	4	3		
Endometriosis	8	2	0		
Immunological	1	0	0		
Tubo-peritoneal	2	1	0		
Tubal	3	2	2		
Mean IVF attempt number	1.6 ± 0.9	2.0 ± 1.2	2.4 ± 1.6	Quasipoisson regression	< 0.01
hMG / rFSH administered (IU)	1870 ± 609	2230 ± 850	2460 ± 853	Linear regression	< 0.01
Ratio IVF/ICSI	2.3	3.2	1.2	Student's t-test	NS
n. oocytes	9.6 ± 4.7	8.7 ± 3.4	6.1 ± 3.9	Quasipoisson regression	< 0.01
n. embryos	2.6 ± 2.0	2.0 ± 1.2	1.8 ± 0.6	Quasipoisson regression	0.02
n. embryos/n. oocytes (%)	31 ± 20.4	24 ± 13.0	43 ± 28.7	Logistic regression	NS
n. embryos/n. 2PN (%)	49 ± 29.8	35 ± 17.4	59 ± 29.3	Logistic regression	NS
n. top quality embryos/n. oocytes (%)	22 ± 20.9	17 ± 17.0	20 ± 25.6	Logistic regression	NS
n. top quality embryos/n. 2PN (%)	30 ± 27.9	26 ± 23.9	27 ± 28.1	Logistic regression	0.08
n. embryos transferred	1.2 ± 0.5	1.2 ± 0.5	1.5 ± 0.6	Quasipoisson regression	NS
Live births (%)	40	31	35	Logistic regression	NS
Maximal estradiol values (pg/ml)	1920 ± 942	2100 ± 1156	1310 ± 973	Linear regression	0.06
QUICKI	0.36 ± 0.02	0.34 ± 0.03	0.33 ± 0.03	Linear regression	< 0.01
rQUICKI	0.40 ± 0.05	0.36 ± 0.04	0.36 ± 0.03	Linear regression	< 0.01

Percentages are calculated within groups. Data are presented as means ± standard deviations. PCOS: poly cycstic ovarian syndrome, IVF: *in vitro* fertilization, hMG: human menopausal gonadotropin, rFSH: recombinant follicle stimulating hormone, ICSI: intra-cytoplasmatic sperm injection, 2PN: zygotes presenting with 2 pronuclei, (r)QUICKI: (revised) quantitative insulin sensitivity check index, NS: not significant, NA: not applicable.

*For all statistical tests, BMI was entered as a continuous variable. In all tests except the student's T-test to study the difference in BMI between IVF and ICSI treated patients (Ratio IVF/ICSI), BMI was the independent variable.

b) Follicular fluid and serum metabolites

Mean serum and FF concentrations of the parameters are presented in **Table 2**. All serum concentrations were significantly higher than their FF counterparts, except for urea, lactate and carnitine, where concentrations were lower ($P < 0.05$). ApoB was not detected in the FF. A statistically significant Spearman correlation coefficient between FF and serum parameter concentrations could be found for urea, total protein, albumin, cholesterol, HDL cholesterol, NEFA, Apo A1, CRP, insulin, IGF-1 and free and total carnitine, whereas no such significance could be found for TG, glucose and lactate (**Table 2**).

Table 2: Concentrations of the assessed parameters in serum and follicular fluid (FF) samples with Spearman correlation coefficients.

Subgroups of parameters	Parameter*	Serum	FF	Spearman (P)
Standard biochemical	Urea (mg/dl)	23 ± 6.0	25 ± 9.1	0.763 (< 0.01)
	Total protein (g/dl)	6.7 ± 0.4	5.6 ± 0.5	0.605 (< 0.01)
	Albumin (g/dl)	4.4 ± 0.3	4.0 ± 0.3	0.578 (< 0.01)
	CRP (mg/l)	3 ± 3.4	2 ± 2.1	0.946 (< 0.01)
Fat metabolism	Cholesterol (mg/dl)	164 ± 25	31 ± 7	0.249 (0.013)
	HDL cholesterol (mg/dl)	61 ± 12.5	29 ± 7.1	0.628 (< 0.01)
	Triglycerides (mg/dl)	96 ± 49.3	9 ± 4.6	0.168 (0.098)
	NEFA (mmol/l)	0.6 ± 0.2	0.3 ± 0.1	0.262 (0.009)
	Apolipoprotein A1 (mg/dl)	160 ± 25	100 ± 17	0.683 (< 0.01)
	Apolipoprotein B (mg/dl)	61 ± 15.6	-	-
	Free carnitine (µmol/l)	24 ± 6.1	28 ± 6.9	0.778 (< 0.01)
	Total carnitine (µmol/l)	35 ± 6.2	36 ± 7.2	0.913 (< 0.01)
Carbohydrate metabolism	Glucose (mg/dl)	75 ± 7.7	56 ± 15.2	-0.045 (0.655)
	Lactate (mg/dl)	11 ± 3.7	36 ± 11.3	0.146 (0.148)
Hormonal	Insulin (mU/l)	12 ± 7.8	9 ± 6.0	0.396 (< 0.01)
	IGF-1 (ng/ml)	180 ± 58	125 ± 42	0.807 (< 0.01)
	IGFBP3 (ng/ml)	-	4100 ± 838	-

Data are presented as means ± standard deviations. *Every parameter's concentration differed significantly ($P < 0.05$) between serum and follicular fluid. CRP: C-reactive protein, HDL: high density lipoprotein, NEFA: non-esterified fatty acid, IGF-1: insulin-like growth factor 1, IGFBP3: IGF binding protein 3.

c) Subfertility etiologies

To analyse whether the cause for infertility was related to the serum and FF composition, we investigated the distribution of the parameters according to these etiologies in two different ways. First, we used LDA to explore which of the FF/serum parameters were most determining to belong to a particular subfertility etiology group. To formally test whether one single parameter was associated with one particular subfertility etiology, we selected the compounds that had the highest loading in the previously calculated LDA, and tested these for association with the etiology using logistic regression. Not all parameters selected using the LDA showed a significant association using logistic regression: the discriminant analysis proposed that women with endometriosis had higher levels of FF and serum NEFA, and lower levels of FF total protein, for which logistic regression showed *P*-

values of 0.13, 0.84 and 0.53, respectively. Women with tubal infertility would have higher levels of FF albumin and FF insulin according to LDA, with logistic regression showing *P*-values of 0.03 and 0.94, respectively. PCOS patients were proposed to have elevated levels of FF insulin, lower levels of FF total protein and FF NEFA ($P < 0.01$, 0.34 and 0.81 in logistic regression). Women with a tubo-peritoneal infertility cause were thought to present with lower FF insulin ($P = 0.51$) and FF/serum albumin ($P = 0.34$, $P = 0.04$ respectively) levels and higher FF NEFA ($P = 0.02$) concentrations.

Several factors may explain the non-significance of the logistic regression results. The LDA was used in an exploratory way and does not test for significance. In case only a few individuals belong to a particular etiological group, the regression analysis lacks power and won't show a significant *P*-value. Moreover, the LDA accounts for complex and nonlinear effects of a particular parameter, whereas the logistic regression tests one single parameter assuming a linear effect. So, the non-significant association between a parameter and an etiology does not exclude that parameter from a possible role in the etiology.

d) Effect of follicular fluid metabolites on fertility treatment outcome and the importance of BMI

Mean concentrations of the metabolites in the serum and FF, according to the different BMI classes, are provided in **Table 3a** and **3b**, respectively. In the FF, only CRP ($P < 0.01$), TG ($P = 0.03$), insulin ($P < 0.01$) and IGF-1 ($P = 0.04$) were significantly associated with BMI.

The number of collected oocytes was significantly associated with BMI (**Table 1**), indicating that a lower number of oocytes was aspirated in obese women ($P < 0.01$). The number of embryos was negatively associated with BMI ($P = 0.02$). There was a trend for an association between the ratio 'number of top quality embryo/number of 2PNs' and BMI that narrowly failed to achieve statistical significance ($P = 0.08$), as well as between BMI and maximal estradiol values ($P = 0.06$) (**Table 1**). Further association studies between FF metabolites and fertility treatment outcome parameters were conducted, taking into account the consequences of multiple testing. All FF metabolites were tested for association with treatment outcome parameters and oocyte quality parameters. Only few associations could be considered significant. Maximal estradiol values associated negatively with FF albumin ($P = 0.05$) and FF total carnitine ($P < 0.01$) concentrations, whereas the number of embryos associated negatively with FF total protein ($P = 0.03$) levels. The ratio 'number of top quality embryos/number of oocytes' showed a negative association with FF ApoA1 ($P < 0.01$). Finally, the ratio 'number of top quality embryos/number of 2PNs' tended to be negatively associated with FF ApoA1 ($P = 0.09$). At the end of the study, 36.8% of the treated women had a live-birth, while 63.2% either miscarried or failed to conceive. No significant difference in the establishment of a successful pregnancy could be detected between BMI groups.

Table 3a: Serum composition according to BMI class, including *P*-values of the linear regression analysis.

Serum Metabolite	**18.5 ≤ BMI ≤ 24.9** (n = 60)	**25.0 ≤ BMI ≤ 29.9** (n = 26)	**BMI ≥ 30.0** (n = 20)	***P*-value**
Urea (mg/dl)	23 ± 4.7	23 ± 5.5	25 ± 9.5	NS
Total protein (g/dl)	6 ± 0.4	7 ± 0.4	7 ± 0.5	NS
Albumin (g/dl)	4 ± 0.6	4 ± 0.2	4 ± 0.3	NS
CRP (mg/l)	2 ± 3.3	4 ± 3.5	4 ± 3.6	< 0.01
Cholesterol (mg/dl)	160 ± 21.6	180 ± 25.4	170 ± 23.5	0.01
HDL cholesterol (mg/dl)	63 ± 10.7	56 ± 12.5	58 ± 10.3	< 0.01
Triglycerides (mg/dl)	85 ± 39.9	124 ± 64.8	104 ± 50.6	0.03
NEFA (mmol/l)	0.6 ± 0.2	0.7 ± 0.23	0.7 ± 0.20	NS
Apo A1 (mg/dl)	160 ± 23.1	160 ± 26.1	150 ± 21.0	0.04
Apo B (mg/dl)	56 ± 13.3	72 ± 14.2	65 ± 17.4	NS
Free carnitine (µmol/l)	24 ± 5.5	24 ± 6.3	26 ± 6.3	NS
Total carnitine (µmol/l)	35 ± 5.7	34 ± 6.5	36 ± 6.3	NS
Glucose (mg/dl)	74 ± 7.1	75 ± 7.7	78 ± 7.0	NS
Lactate (mg/dl)	11 ± 3.3	12 ± 3.7	13 ± 4.1	0.01
Insulin (mU/l)	9 ± 3.8	16 ± 11.3	16 ± 9.1	< 0.01
IGF-1 (ng/ml)	185 ± 59.7	190 ± 57.0	150 ± 48.3	0.03

Data are presented as means ± standard deviation. NS: not significant, $P > 0.05$. CRP: C-reactive protein, HDL: high density lipoprotein, NEFA: non-esterified fatty acid, IGF-1: insulin-like growth factor 1, IGFBP3: IGF binding protein 3.

Table 3b: Follicular fluid composition according to BMI class, including *P*-values of the linear regression analysis.

Follicular Fluid Metabolite	**18.5 ≤ BMI ≤ 24.9** (n = 60)	**25.0 ≤ BMI ≤ 29.9** (n = 26)	**BMI ≥ 30.0** (n = 20)	***P*-value**
Urea (mg/dl)	25 ± 6.1	25 ± 6.9	29 ± 16.4	NS
Total protein (g/dl)	6 ± 0.5	6 ± 0.3	6 ± 0.6	NS
Albumin (g/dl)	4 ± 0.3	4 ± 0.2	4 ± 0.4	NS
CRP (mg/l)	2 ± 2.6	2 ± 1.9	2 ± 1.8	< 0.01
Cholesterol (mg/dl)	31 ± 7.1	30 ± 6.6	32 ± 7.6	NS
HDL cholesterol (mg/dl)	28 ± 6.9	28 ± 6.4	30 ± 7.3	NS
Triglycerides (mg/dl)	10 ± 3.5	11 ± 5.9	11 ± 3.5	0.03
NEFA (mmol/l)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	NS
Apo A1 (mg/dl)	100 ± 16.5	110 ± 15.2	105 ± 15.2	NS
Free carnitine (µmol/l)	29 ± 6.6	27 ± 6.1	29 ± 5.6	NS
Total carnitine (µmol/l)	37 ± 6.2	33 ± 9.1	37 ± 6.3	NS
Glucose (mg/dl)	55 ± 14.6	54 ± 14.7	58 ± 17.6	NS
Lactate (mg/dl)	35 ± 11.3	38 ± 10.7	38 ± 12.2	NS
Insulin (mU/l)	8 ± 5.3	11 ± 6.2	12 ± 6.7	< 0.01
IGF-1 (ng/ml)	128 ± 42.4	134 ± 43.4	101 ± 39.1	0.04
IGF-BP3 (ng/ml)	4100 ± 619.0	4200 ± 673.2	4050 ± 1414.4	NS

Data are presented as means ± standard deviation. NS: not significant, $P > 0.05$. CRP: C-reactive protein, HDL: high density lipoprotein, NEFA: non-esterified fatty acid, IGF-1: insulin-like growth factor 1, IGFBP3: IGF binding protein 3.

Discussion

In the present study, we investigated if alterations in the blood composition of overweight and obese women, are reflected in the FF and if that might harm the oocyte's developmental competence, subsequent embryo quality and pregnancy rate. We showed that, with increasing BMI, significantly fewer oocytes were obtained and although not statistically significant, fertilized oocytes (2PN) tended to have a reduced chance to develop into a morphological top quality embryo. This could not be linked to BMI associated differences in FF concentrations of the parameters analysed. We showed, however, that irrespective of BMI, differences in serum parameters between patients are partly reflected in the FF and that FF ApoA1 and FF total protein were negatively associated with the chance of an oocyte to develop into a top quality embryo and with the number of formed embryos, respectively.

In line with previous findings, our data show that obese women have lower peak estradiol levels (Lashen *et al.*, 1999, Dechaud *et al.*, 2006), a lower number of oocytes aspirated and a lower number of embryos originating from these oocytes (Metwally *et al.*, 2007, Robker *et al.*, 2009). However, in this study, there was no significant difference in the chance for a successful pregnancy between obese and normal weight women.

It is well known that the oocyte is vulnerable to changes in its micro-environment and that many factors may affect its crucial final maturation, leading to a developmentally competent oocyte (Revelli *et al.*, 2009, Leroy *et al.*, 2012). It is, however, not known to what extent BMI related changes in the FF may affect oocyte quality. Therefore, we chose to study the presence of standard biochemical, fat metabolism related, carbohydrate metabolism related and hormonal parameters in the serum and FF. Our analyses revealed that most FF metabolite concentrations (except for urea, lactate and carnitine) are lower than serum concentrations, confirming a modulating role for the blood-FF barrier and/or a substantial contribution of oocyte, granulosa and cumulus cell metabolism (Webb *et al.*, 2004, Rodgers and Irving-Rodgers, 2010). Because most serum and FF metabolite concentrations correlated significantly, we conclude that serum metabolite concentrations are partly reflected in the FF, potentially directly influencing cumulus-oocyte complex (COC) quality. However, a significant effect of BMI could be detected only for FF CRP, insulin, IGF-1 and TG, and apparently none of these parameters were associated with any reproductive outcome in our study. The concentration of FF total protein, regardless of BMI, associated with the number of embryos, which is consistent with the findings of Iwata et al. (2010) who showed that oocytes from cows with higher FF concentrations of total protein and lower concentrations of albumin are impaired in their developmental capacity (Iwata *et al.*, 2010). Furthermore, our data show that an alteration in serum total protein concentration, is mimicked in the FF, potentially directly influencing oocyte quality.

Nutritionally induced hypercholesterolemic conditions during early embryo development have been shown to affect oocyte developmental competence and embryo quality in terms of cell number, apoptotic cell index and gene expression patterns of metabolically and developmentally important genes (Leroy *et al.*, 2010) and may result in growth retardation (Picone *et al.*, 2011). The BMI dependent concentration of serum HDL cholesterol in our study associated significantly with FF concentrations, indicating that FF HDL cholesterol is vulnerable to serum cholesterol changes and could therefore influence COC quality. The negative association between FF ApoA1 concentrations and (1) the ratio 'number of top quality embryos/number of oocytes' and (2) the ratio 'number of top quality embryos/number of 2PNs' (trend) substantiates this, suggesting that this surrogate marker of the number of HDL particles in the FF could have a negative impact on the oocyte's developmental capacity, even if fertilization occurred normally. Interestingly, it has been proposed that the combined effect of FF HDL cholesterol and FF ApoA1 plays a protective role for the health of the oocyte and subsequent embryo development through a reduction in embryo fragmentation (Browne *et al.*, 2008). However, Browne *et al*. (2008) also showed that an increase in either FF HDL cholesterol or in FF ApoA1, caused a slight reduction in the 'protective' effect of FF ApoA1 and FF HDL cholesterol, respectively, on embryo grading (Browne *et al.*, 2008), suggesting that a change of the ratio HDL cholesterol / ApoA1 in our study could result in the documented negative effects of ApoA1 on oocyte quality. Carnitine showed a good correlation between serum and FF samples, with the highest concentrations in the FF. Increased carnitine levels have been proposed to increase β-oxidation and improve oocyte developmental competence (Dunning *et al.*, 2011). Our data suggest that inadequate levels of carnitine in the blood are reflected in the FF and could thus potentially harm oocyte quality. Carnitine in the FF is also associated with maximal estradiol values, suggesting a role for carnitine in the ovarian response to gonadotropin stimulation and maturation of the oocyte. Moreover, these high carnitine concentrations have an antioxidant function, possibly protecting the COC from excess oxidative stress, caused by other parameters present in the FF (Dunning *et al.*, 2011). The latter is substantiated by the complex non-linear interaction between FF compounds we propose in the association study between infertility etiologies and FF compound concentrations.

Insulin showed an elevated concentration in both serum and FF of obese women and correlated well between serum and FF samples. This is in agreement with our (r)QUICKI data, showing a higher chance for insulin resistance when being obese. Also, women with tubal infertility and PCOS presented with elevated FF insulin levels. Insulin has a growth/anti-apoptotic effect and has the metabolic ability to increase glucose uptake in the COC (Purcell and Moley, 2011). It exerts its effect in various cell types and is involved in multiple (steroidogenic) pathways within the follicular unit, with both elevated and decreased levels potentially altering oocyte maturation and quality (Adamiak *et al.*, 2005, Garnsworthy *et al.*, 2009, Vlaisavljevic *et al.*, 2009, Sanchez *et al.*, 2011). IGF-1

concentrations correlated well between serum and FF samples, indicating that changes in serum IGF-1 concentrations are mimicked in the FF. Interestingly, FF IGFBP3 concentrations did not associate with BMI, while IGF-1 concentrations did (in both serum and FF). Differences in FF IGFBPs and interaction with locally produced IGF-II may affect the IGF-I bioavailability and this has been implicated in pathways of anovulatory ovarian pathologies (Amato *et al.*, 1999, Silva *et al.*, 2009). Furthermore, IGF-1 influences the oocyte's developmental competence (Silva *et al.*, 2009), as for example early pre-antral mouse follicles, cultured in IGF-1 rich conditions, give rise to oocytes which develop up to blastocysts with higher cell numbers, at a higher rate (Demeestere *et al.*, 2004). The concentrations of urea in the serum and FF correlated well, which indicates that an elevated concentration of urea in the blood is directly reflected in the FF. Uremic conditions may therefore directly affect oocyte quality and subsequent *in vitro* hatching of blastocysts (De Wit *et al.*, 2001, Ferreira *et al.*, 2011). A strong correlation could also be found between serum and FF CRP levels and higher BMI was accompanied by elevated CRP concentrations in both serum and FF. Elevated FF and serum CRP concentrations have previously been associated with chronic anovulation and no conception in women undergoing fertility treatment (Levin *et al.*, 2007, Lamaita *et al.*, 2012). Such high CRP levels are characteristic for increased inflammation (Das, 2001) and are proposed to be involved in oxidative stress and reactive oxygen species mechanisms, which are both very topical in research concerning oocyte developmental competence and subsequent embryo quality (Agarwal *et al.*, 2008, Combelles *et al.*, 2009).

Unexpectedly, we found only little significant correlations between both BMI and BMI related parameter concentrations, and oocyte quality parameters. For example, we could not find a significant association between fertility treatment outcome and glycemia or NEFA concentrations. Both hyperglycemia (Purcell and Moley, 2011) and elevated NEFA (Leroy *et al.*, 2005, Van Hoeck *et al.*, 2011, Jungheim *et al.*, 2011a) concentrations have previously been linked to impaired COC morphology and oocyte developmental competence. NEFAs, which correlated significantly between serum and FF samples, have been suggested as an interesting metabolic key in the pathogenesis of subfertility in overweight and obese women (Mu *et al.*, 2001). Also, research done in our laboratory has mimicked hyperlipolytic conditions associated with high FF NEFA conditions during bovine *in vitro* maturation and found that blastocysts originating from these exposed oocytes present with a lower cell count, an increased apoptotic cell ratio and altered gene expression profiles of key metabolic genes (Leroy *et al.*, 2005, Van Hoeck *et al.*, 2011).

In our study, we chose to solely analyse the composition of the FF of the largest, first punctured follicle in order to reduce the confounding effects of follicle size on FF composition. In doing so, we were unable to account for differences between follicles of the same patient. One major limitation of this study is that the number of women enlisted in each BMI category was different, skewing BMI

distribution and although characteristic for a standard population in the West European region, possibly masking significant associations. Other researchers have chosen to balance out the number of patients in each group (Robker *et al.*, 2009) or were inconclusive on the effect of BMI on overall pregnancy outcome, as some studies report for example a lower proportion of embryos, reduced implantation rates and reduced pregnancy rates in obese women, while other studies (Lashen *et al.*, 1999, Dechaud *et al.*, 2006), including our own, were unable to confirm this. Furthermore, the BMI spread in the obese group in the present study was low, because extremely obese women were firstly advised to lose weight before starting assisted reproductive treatment. However, even within our study cohort, with only moderate obese women, we identified several FF parameters possibly affecting oocyte quality, independent of the patient's BMI. So, although our findings do not fully support the common belief concerning the negative impact of obesity on assisted reproductive treatment, we are confident that the data we present are useful in the study of potential pathways determining oocyte quality.

In conclusion, we described the presence of metabolically important parameters in the FF and serum of women undergoing assisted reproduction and we showed that serum metabolite changes are partly reflected in the follicular micro-environment of the maturing oocyte. Follicular fluid ApoA1 and total protein concentrations were negatively linked with oocyte quality and differences in BMI could not explain this observation. Our data stress the involvement of metabolic aberrations in the FF rather than BMI related changes as having potential hazardous effects on oocyte quality. Further research into the underlying mechanisms responsible for poor reproductive outcomes in women with metabolic disorders will help to identify the particular metabolic conditions, which pose a woman at risk for poor reproductive performance.

References

Adamiak SJ, Mackie K, Watt RG, Webb R and Sinclair KD. Impact of nutrition on oocyte quality: cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. *Biol Reprod* 2005; **73**: 918-926.

Agarwal A, Gupta S, Sekhon L and Shah R. Redox considerations in female reproductive function and assisted reproduction: From molecular mechanisms to health implications. *Antioxidants & Redox Signaling* 2008; **10**: 1375-1403.

Agresti A. Categorical Data Analysis. 2nd edn, 2002. Wiley-Interscience, New York, USA.

Amato G, Izzo A, Tucker AT and Bellastella A. Lack of insulin-like growth factor binding protein-3 variation after follicle-stimulating hormone stimulation in women with polycystic ovary syndrome undergoing in vitro fertilization. *Fertil Steril* 1999; **72**: 454-457.

Brewer CJ and Balen AH. The adverse effects of obesity on conception and implantation. *Reproduction* 2010; **140**: 347-364.

Browne RW, Shelly WB, Bloom MS, Ocque AJ, Sandler JR, Huddleston HG and Fujimoto VY. Distributions of high-density lipoprotein particle components in human follicular fluid and sera and their associations with embryo morphology parameters during IVF. *Hum Reprod* 2008; **23**: 1884-1894.

Combelles CMH, Gupta S and Agarwal A. Could oxidative stress influence the in-vitro maturation of oocytes? *Reprod Biomed Online* 2009; **18**: 864-880.

Das UN. Is obesity an inflammatory condition? *Nutrition* 2001; **17**: 953-966.

De Wit AAC, Cesar MLF and Kruip TAM. Effect of urea during in vitro maturation on nuclear maturation and embryo development of bovine cumulus-oocyte-complexes. *Journal of Dairy Science* 2001; **84**: 1800-1804.

Dechaud H, Anahory T, Reyftmann L, Loup V, Hamamah S and Hedon B. Obesity does not adversely affect results in patients who are undergoing in vitro fertilization and embryo transfer. *Eur J Obstet Gynecol Reprod Biol* 2006; **127**: 88-93.

Demeestere I, Gervy C, Centner J, Devreker F, Englert Y and Delbaere A. Effect of insulin-like growth factor-I during preantral follicular culture on steroidogenesis, in vitro oocyte maturation, and embryo development in mice. *Biol Reprod* 2004; **70**: 1664-1669.

Dunning KR, Akison LK, Russell DL, Norman RJ and Robker RL. Increased beta-oxidation and improved oocyte developmental competence in response to l-carnitine during ovarian in vitro follicle development in mice. *Biol Reprod* 2011; **85**: 548-555.

Fedorcsak P, Dale PO, Storeng R, Ertzeid G, Bjercke S, Oldereid N, Omland AK, Abyholm T and Tanbo T. Impact of overweight and underweight on assisted reproduction treatment. *Hum Reprod* 2004; **19**: 2523-2528.

Ferreira FA, Gomez RGG, Joaquim DC, Watanabe YF, Paula LADE, Binelli M and Rodrigues PHM. Short-term urea feeding decreases in vitro hatching of bovine blastocysts. *Theriogenology* 2011; **76**: 312-319.

Garnsworthy PC, Fouladi-Nashta AA, Mann GE, Sinclair KD and Webb R. Effect of dietary-induced changes in plasma insulin concentrations during the early post partum period on pregnancy rate in dairy cows. *Reproduction* 2009; **137**: 759-768.

Gilchrist RB, Ritter LJ and Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. *Anim Reprod Sci* 2004; **82-83**: 431-446.

Iwata H, Tanaka H, Kanke T, Sakaguchi Y, Shibano K, Kuwayama T and Monji Y. Follicle Growth and Oocyte Developmental Competence in Cows With Liver Damage. *Reprod Domest Anim* 2010; **45**: 888-895.

Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS and Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. *Fertil Steril* 2011a; **95**: 1970-1974.

Koning AMH, Kuchenbecker WKH, Groen H, Hoek A, Land JA, Khan KS and Mol BWJ. Economic consequences of overweight and obesity in infertility: a framework for evaluating the costs and outcomes of fertility care. *Hum Reprod Update* 2010; **16**: 246-254.

Lamaita RM, Pontes A, Belo AV, Caetano JP, Andrade SP, Candido EB, Trainman P, Carneiro MM and Silva-Filho AL. Inflammatory response patterns in ICSI patients: a comparative study between chronic anovulating and normally ovulating women. *Reprod Sci* 2012; **19**: 704-711.

Lash MM and Armstrong A. Impact of obesity on women's health. *Fertil Steril* 2009; **91**: 1712-1716.

Lashen H, Ledger W, Bernal AL and Barlow D. Extremes of body mass do not adversely affect the outcome of superovulation and in-vitro fertilization. *Hum Reprod* 1999; **14**: 712-715.

Lebovitz HE. Insulin resistance--a common link between type 2 diabetes and cardiovascular disease. *Diabetes Obes Metab* 2006; **8**: 237-249.

Leroy JLMR, Rizos D, Sturmey R, Bossaert P, Gutierrez-Adan A, Van Hoeck V, Valckx S and Bols PEJ. Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: a focus on dairy cow fertility. *Reprod Fert Develop* 2012; **24**: 1-12.

Leroy JLMR, Van Hoeck V, Clemente M, Rizos D, Gutierrez-Adan A, Van Soom A, Uytterhoeven M and Bols PEJ. The effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality. *Hum Reprod* 2010; **25**: 768-778.

Leroy JLMR, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PEJ, Dewulf J and de Kruif A. Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. *Theriogenology* 2004; **62**: 1131-1143.

Leroy JLMR, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G and Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 2005; **130**: 485-495.

Levin I, Gamzu R, Mashiach R, Lessing JB, Amit A and Almog B. Higher C-reactive protein levels during IVF stimulation are associated with ART failure. *Journal of Reproductive Immunology* 2007; **75**: 141-144.

Martinuzzi K, Ryan S, Luna M and Copperman AB. Elevated body mass index (BMI) does not adversely affect in vitro fertilization outcome in young women. *Journal of Assisted Reproduction and Genetics* 2008; **25**: 169-175.

Metwally M, Li TC and Ledger WL. The impact of obesity on female reproductive function. *Obes Rev* 2007; **8**: 515-523.

Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K et al. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. *Endocrinology* 2001; **142**: 3590-3597.

Pasquali R, Patton L and Gambineri A. Obesity and infertility. *Curr Opin Endocrinol Diabetes Obes* 2007; **14**: 482-487.

Perseghin G, Caumo A, Caloni M, Testolin G and Luzi L. Incorporation of the fasting plasma FFA concentration into QUICKI improves its association with insulin sensitivity in nonobese individuals. *J Clin Endocr Metab* 2001; **86**: 4776-4781.

Picone O, Laigre P, Fortun-Lamothe L, Archilla C, Peynot N, Ponter AA, Berthelot V, Cordier AG, Duranthon V and Chavatte-Palmer P. Hyperlipidic hypercholesterolemic diet in prepubertal rabbits affects gene expression in the embryo, restricts fetal growth and increases offspring susceptibility to obesity. *Theriogenology* 2011; **75**: 287-299.

Purcell SH and Moley KH. The impact of obesity on egg quality. *J Assist Reprod Genet* 2011; **28**: 517-524.

Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M and Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrin* 2009; **4**: 40.

Robker RL. Evidence that obesity alters the quality of oocytes and embryos. *Pathophysiology* 2008; **15**: 115-121.

Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M and Norman RJ. Obese Women Exhibit Differences in Ovarian Metabolites, Hormones, and Gene Expression Compared with Moderate-Weight Women. *J Clin Endocr Metab* 2009; **94**: 1533-1540.

Rodgers RJ and Irving-Rodgers HF. Formation of the Ovarian Follicular Antrum and Follicular Fluid. *Biol Reprod* 2010; **82**: 1021-1029.

Sanchez F, Romero S and Smitz J. Oocyte and cumulus cell transcripts from cultured mouse follicles are induced to deviate from normal in vivo conditions by combinations of insulin, follicle-stimulating hormone, and human chorionic gonadotropin. *Biol Reprod* 2011; **85**: 565-574.

Sebire NJ, Jolly M, Harris JP, Wadsworth J, Joffe M, Beard RW, Regan L and Robinson S. Maternal obesity and pregnancy outcome: a study of 287 213 pregnancies in London. *Int J Obesity* 2001; **25**: 1175-1182.

Silva JRV, Figueiredo JR and van den Hurk R. Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. *Theriogenology* 2009; **71**: 1193-1208.

Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE and Leroy JL. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *Plos One* 2011; **6**: e23183.

Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, Eestermans W and Gerris J. Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod* 1999; **14**: 2345-2349.

Venables WN and Ripley BD. Modern applied statistics with S. 4th edn, 2003. Springer, New York, USA.

Vlaisavljevic V, Kovac V and Sajko MC. Impact of insulin resistance on the developmental potential of immature oocytes retrieved from human chorionic gonadotropin-primed women with polycystic ovary syndrome undergoing in vitro maturation. *Fertil Steril* 2009; **91**: 957-959.

Webb R, Garnsworthy PC, Gong JG and Armstrong DG. Control of follicular growth: local interactions and nutritional influences. *J Anim Sci* 2004; **82 E-Suppl**: E63-74.

WHO.Obesity and overweight. Fact sheet N° 311.

CHAPTER 4: THE EFFECT OF HUMAN FOLLICULAR FLUID ON BOVINE OOCYTE DEVELOPMENTAL COMPETENCE AND EMBRYO QUALITY

Published in Reproductive BioMedicine Online. Valckx SDM, De Bie J, Michiels EDG, Goovaerts IGF, Punjabi U, Ramos-Ibeas P, Gutierrez-Adan A, Bols PEJ, Leroy JLMR (2014): The effect of human follicular fluid on bovine oocyte developmental competence and embryo quality. *In press.*

Abstract

Background: It is well known that the oocyte and embryo are very vulnerable to changes in their micro-environment. Many studies have focused on the composition of the follicular fluid (FF), but it remains unclear whether and how the composition of the pre-ovulatory FF may affect the enclosed oocyte's developmental competence after *in vitro* fertilization. Therefore, we hypothesized that the FF composition during final maturation *in vivo* might influence the quality of embryos generated during human *in vitro* fertilization (IVF) procedures.

Methods: To investigate this, FF from women with obesity (OBESE) and a POSITIVE or NEGATIVE IVF outcome was added during the *in vitro* maturation of bovine oocytes. Subsequently, bovine maturation rate, fertilization rate, cleavage rate, blastocyst development, hatching rate, blastocyst cell number and apoptotic cell index were investigated. Furthermore, gene expression patterns of developmentally important genes in Day 7.5 bovine blastocysts were studied.

Results: NEGATIVE and OBESE FF reduced bovine embryo development, compared to the lab-control ($P < 0.05$ or $P < 0.1$). Adding FF also altered bovine blastocyst gene expression. Additionally, *LDHA* and *PPARGC1B* gene expression differed between FF groups.

Conclusions: Our data suggest that pre-ovulatory FF can potentially affect oocyte developmental competence and embryo quality. Furthermore, the bovine model may be used as a screening tool.

Introduction

Maternal metabolic disorders, like obesity and type 2 diabetes, are associated with hormonal and biochemical changes in the serum, which are reflected in the micro-environment of the maturing oocyte (Leroy et al., 2012, Valckx et al., 2012), the follicular fluid (FF). Such changes, like high FF free fatty acid (FFA) concentrations, hamper *in vitro* bovine oocyte developmental competence as well as embryo quality and metabolism (Van Hoeck et al., 2011). Moreover, adding human FF high in triglycerides and FFAs from obese patients impaired murine oocyte maturation and caused endoplasmic reticulum stress (Yang et al., 2012). Furthermore, adding bovine FF with high concentrations of FFA during bovine *in vitro* cumulus-oocyte complex (COC) maturation, resulted in a massive intracellular lipid storage in the cumulus cells, which may be seen as a protective mechanism (Aardema et al., 2013). Other parameters such as insulin, cholesterol and total protein in the FF have also been studied (Valckx et al., 2012), but it remains unclear whether and how the composition of the pre-ovulatory FF may influence the enclosed oocyte's developmental success during *in vitro* embryo growth. Therefore, we hypothesized that the FF composition during final maturation *in vivo* might influence the quality of embryos generated during human *in vitro* fertilization (IVF) procedures. To investigate this, it was essential to uncouple intrinsic oocyte quality from potential effects caused by the FF. Therefore, we used human FF in an entirely independent *in vitro* setting, where we aimed to study the effect of exposing bovine *in vitro* maturing oocytes to human FF from women with differential IVF outcomes or obesity, on bovine oocyte developmental competence and subsequent embryo quality. We chose the bovine model because of multiple similarities between human and bovine ovarian physiology, oocyte maturation and early embryo development (Menezo and Herubel, 2002) and we aimed to implement this model for the first time as a potential screening tool to study the effect of the composition of pre-ovulatory FF on oocyte and embryo quality.

Material and methods

a) Collection and selection of FF samples

Samples were collected as previously described by Valckx *et al.* (2012), with ethical approval (FER-P0905/F18). Because numerous variables may influence IVF outcome, we adopted very strict exclusion criteria to eliminate known confounders: age > 38 years, body mass index (BMI) < 18kg/m², polycystic ovarian syndrome, blood sample contamination, < 6 oocytes aspirated, < 50% successfully fertilized oocytes and the male factors cryptozo- or azoospermia. Selected samples consequently originated from women with a good response to ovarian stimulation, with the exclusion of major male and female subfertility risk factors. Using specific inclusion criteria, 3 groups were defined:

(1) NEGATIVE (n = 6): ≤ 30% Day 3 good quality embryos (criteria as described in Valckx *et al*., 2012) originating from that ovum pick-up (OPU) session, BMI < 30kg/m² and the oocyte originating from the FF sample did not develop into a good quality embryo;

(2) POSITIVE (n = 6): ≥ 50% Day 3 good quality embryos originating from that OPU session, BMI < 30kg/m² and the oocyte originating from the FF sample developed into a good quality embryo;

(3) OBESE (n = 6): BMI > 30kg/m².

Following these criteria, 6 samples per treatment group, originating from 15 women, were selected out of a total of 120 samples from 70 women.

b) Bovine *in vitro* embryo production

Immature bovine COCs were isolated from slaughterhouse ovaries and matured in groups of 50 for 24h in 500µl serum-free maturation medium, supplemented with 25% heat-inactivated FF pools from the different treatments. A routine serum/FF free lab-control was run in parallel. Oocytes were fertilized and presumptive zygotes cultured in groups of 25-30 embryos (50µl serum free SOF medium droplets under oil), following routine laboratory procedures as in Van Hoeck *et al.* (2011) with minor modifications.

c) Bovine oocyte and embryo quality parameters (4 replicates)

In each replicate, 100 COCs were cultured in each treatment group. Five or 6 oocytes and presumptive zygotes per treatment and per replicate were stained in 5µg/ml DAPI (5min, Sigma-Aldrich, Bornem, Belgium), to determine maturation and fertilization rate, respectively. Denuded mature oocytes were collected after 24h *in vitro* maturation (10 min vortexing to remove cumulus cells) and presumptive zygotes were collected after fertilization (3 min vortexing). Cleavage rate and blastocyst formation, for the remaining presumptive zygotes, were documented on Days 2 and 8 post insemination (p.i.), respectively. Six or 7 Day 8 expanded blastocysts, per treatment and per replicate were stained with DAPI and TUNEL (Roche Diagnostics, Vilvoorde, Belgium) as previously described (Van Hoeck *et al.*, 2011) to study total cell number and apoptotic cell ratio, respectively.

d) Gene expression (4 replicates)

An equal number of normal and expanded blastocysts for each treatment was snap frozen (in groups of 10) on Day 7.5 of culture and analysed for the transcript abundance of the genes presented in **Figure 1**. These genes were particularly chosen for their relevance in embryo development, quality and metabolism. mRNA isolation, retrotranscription and real-time PCR quantification were performed as described by Van Hoeck *et al.*(2011), with minor modifications.

e) Statistical analyses (PASW 18.0 for Windows, Chicago, IL, USA)

Binary outcomes (oocyte/embryo development) were analysed with binary logistic regression, continuous variables (total cell number/apoptotic cell index) with a mixed model ANOVA and post-hoc Sheffé tests. Treatment was entered as a fixed factor and replicate as random factor. Insignificant interaction terms were omitted from the model. mRNA abundance was studied with a One Way ANOVA and post-hoc Student-Newman-Keuls tests. Statistical significance and trends were set at $P < 0.05$ and $P < 0.1$, respectively.

Results

The results showed no significant difference in maturation rate, fertilization rate, blastocyst hatching rate, total cell number and apoptotic cell index. However, cleavage rate was reduced for the NEGATIVE treatment, compared with the Lab-control ($P < 0.05$). Furthermore, there was a trend for a reduced blastocyst formation for NEG FF and OBESE embryos, compared with the lab-control and a trend for a reduced blastocyst formation from cleaved zygotes in OBESE embryos, compared with lab-control embryos ($P < 0.1$, **Table 1**).

Table 1: Oocyte and embryo outcome parameters.

	Lab-control	NEGATIVE	POSITIVE	OBESE
Maturation rate (%)	74 (14/19)	92 (22/24)	78 (18/23)	62 (13/21)
Fertilization rate (%)	79 (19/24)	63 (15/24)	79 (19/24)	76 (16/21)
Cleavage rate (%)	80^{a} (263/327)	74^{b} (233/317)	77^{ab} (256/331)	76^{ab} (230/301)
Blastocyst formation (%)	31^{A} (101/327)	25^{B} (79/317)	27^{AB} (89/331)	24^{B} (72/301)
Blastocyst formation from cleaved zygotes (%)	38^{A} (101/263)	34^{AB} (79/233)	35^{AB} (89/256)	31^{B} (72/230)
Hatching rate (%)	39 (39/101)	28 (22/79)	36 (32/89)	32 (23/72)
Cell number (n)*	108 ± 25 (25)	110 ± 24 (27)	101 ± 21 (26)	110 ± 22 (26)
Apoptotic cell index (%)*	5.6 (6/108)	5.5 (6/110)	5.9 (6/101)	3.6 (4/110)

Data are presented as percentages or as means ± standard deviation. ^{ab}Data with a different superscript differ significantly ($P < 0.05$); ^{AB}Data tend to be different ($P < 0.1$). *Numeric variables between brackets represent the number of embryos stained (cell number) and the mean counts (mean number of apoptotic cells / mean cell number) of those embryos for each treatment group (apoptotic cell index).

Gene expression of *LDHA* was higher in lab-control and OBESE embryos, compared with NEGATIVE and POSITIVE embryos ($P < 0.05$, **Figure 1**). *DNMT3A* and *SLC27A1* expression was higher in lab-control, compared to POSITIVE embryos ($P < 0.05$). *TP53* and *ACCA* expression were higher in lab-control embryos, compared to POSITIVE, NEGATIVE and OBESE embryos ($P < 0.05$). Finally, *PPARGC1B* expression was higher in lab-control embryos, compared with NEGATIVE and OBESE embryos ($P < 0.05$). *PPARGC1B* expression was also higher in POSITIVE, compared to OBESE embryos ($P < 0.05$, **Figure 1**). mRNA transcript abundance of *GAPDH*, *MNSOD*, *IGF2R*, *GLUT1*, *GPX1* and *NRF2* was not affected by treatment.

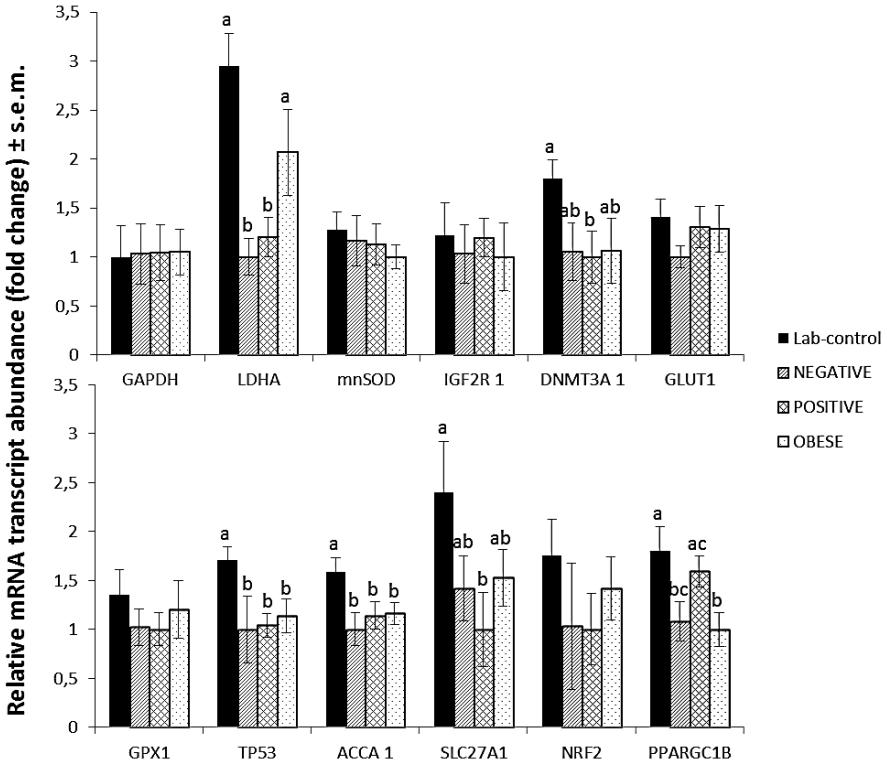


Figure 1: Gene expression analyses. Day 7.5 blastocyst transcript abundance for Lab-control, NEGATIVE, POSITIVE and OBESE treatment groups. Data are presented as relative fold change (± s.e.m.), with reference to the treatment with the lowest expression level. [ab]Data with a different superscript differ significantly ($P < 0.05$).

Discussion

For the first time, we used a bovine oocyte maturation model as a screening tool to specifically investigate the effect of human pre-ovulatory FF, from women with a differential IVF outcome or obesity, on *in vitro* bovine oocyte developmental competence and embryo quality. Support for the validity of the bovine model, which allowed the essential uncoupling of human intrinsic oocyte quality and FF, comes from the observation that embryo development was similar between the POSITIVE treatment and our Lab-control. Our results showed that nuclear maturation and fertilization potential were not affected by treatment. However, the data do suggest that oocyte developmental competence is impaired by the NEGATIVE and OBESE treatments, because (1) the NEGATIVE treatment reduced bovine cleavage rate ($P < 0.05$), (2) the NEGATIVE and OBESE treatments tended to reduce blastocyst formation ($P < 0.1$) and (3) the OBESE treatment tended to reduce blastocyst formation from cleaved zygotes ($P < 0.1$), compared with the lab-control. Most significant differences in bovine blastocyst mRNA transcript abundance were found between lab-control and embryos from the NEGATIVE, POSITIVE and OBESE FF groups: *LDHA, DNMT3A, TP53, ACCA, SLC27A1, PPARGC1B* and are as such caused by adding FF to the maturation medium. Interestingly, *LDHA* is responsible for the reversible oxidation of lactate to pyruvate with the production of reducing NADH and is overexpressed in OBESE embryos. *LDHA* expression is also increased in oocytes exposed to elevated NEFA concentrations (Van Hoeck et al., 2013a), and might act as a cytosolic reductant in the defense against oxidative stress. *PPARGC1B* regulates cellular differentiation, development and oxidative metabolism and is expressed to a higher degree in POSITIVE embryos, compared with OBESE embryos, suggesting that its function might be improved in POSITIVE embryos. Based on these results, it is possible that these pathways may be involved in the distinction between good and bad quality embryos during IVF procedures. In conclusion, although the described effect sizes were small, our results suggest that (1) changes in the composition of the FF may be responsible for decreased oocyte and embryo quality in this study and (2) the bovine model may be used as a screening tool to investigate the effect of human FF composition on IVF outcome.

References

Aardema H, Lolicato F, Van De Lest CH, Brouwers JF, Vaandrager AB. Van Tol HT, Roelen BA, Vos PL, Helms JB, Gadella BM. Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. *Biol Reprod, 2013;* **88,** 164.

Leroy JLMR, Rizos D, Sturmey R, Bossaert P, Gutierrez-Adan A, Van Hoeck V, Valckx S, Bols PEJ. Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: A focus on dairy cow fertility. *Reprod Fert Develop; 2012:* **24**, 1-12.

Menezo YJ, Herubel F. Mouse and bovine models for human ivf. *Reprod Biomed Online; 2002:* **4,** 170-5.

Valckx SDM, De Pauw I, De Neubourg D, Inion I, Berth M, Fransen E, Bols PEJ, Leroy JLMR. Bmi-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. *Hum Reprod;* 2012: **27,** 3531-3539.

Van Hoeck V, Leroy JLMR, Arias Alvarez M, Rizos D, Gutierrez-Adan A, Schnorbusch K, Bols PEJ, Leese HJ, Sturmey RG. Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: Mechanistic insights. *Reproduction*; 2013a: **145,** 33-44.

Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PEJ, Leroy JLMR. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *Plos One*; 2011: **6**, e23183.

Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ, Robker RL. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. *Fertil Steril*; 2012: **97,** 1438-43.

CHAPTER 5: FATTY ACID COMPOSITION OF THE FOLLICULAR FLUID OF NORMAL WEIGHT, OVERWEIGHT AND OBESE WOMEN UNDERGOING ASSISTED REPRODUCTIVE TREATMENT: A DESCRIPTIVE CROSS-SECTIONAL STUDY

Published in Reproductive Biology and Endocrinology. Valckx SDM, Arias-Alvarez M, De Pauw I, Fievez V, Vlaeminck B, Fransen E, Bols PEJ, Leroy JLMR (2014): Fatty acid composition of the follicular fluid of normal weight, overweight and obese women undergoing assisted reproductive treatment: a descriptive cross-sectional study. Reproductive Biology and Endocrinology 2014, 12:13

Abstract

Background: It has been well documented that the maturing oocyte is very vulnerable to changes in its micro-environment, the follicular fluid (FF). Recent research has focused on different components within this FF, like hormones, growth factors and metabolites, and how their concentrations are altered by diet and the metabolic health of the mother. It has been proposed that fatty acids (FAs) are potential factors that influence oocyte maturation and subsequent embryo development. However, a thorough study of the specific FF FA composition per lipid fraction and how this may be affected by BMI is currently lacking. Therefore, we investigated the BMI-related concentration of fatty acids (FAs) in the phospholipid (PL), cholesteryl-ester (CHE), triglyceride (TG) and non-esterified (NE) lipid fraction in the FF of women undergoing assisted reproductive treatment (ART).

Methods: In this descriptive cross-sectional study, the FF of normal weight ($18.5 \leq BMI < 25.0 kg/m^2$, n = 10), overweight ($25.0 \leq BMI < 30.0 kg/m^2$, n = 10) and obese ($BMI \geq 30.0 kg/m^2$, n = 10) women, undergoing ART, was sampled and analysed for 23 specific FAs in the PL, CHE, TG and NEFA fraction, using a gas chromatographic analysis method. Differences between BMI-groups were studied by means of univariate general linear models and post hoc Sheffé tests.

Results: Total FA concentrations in the PL and CHE fraction did not differ between BMI groups. Total TG concentrations tended to differ and total NEFA concentrations differed significantly between BMI groups. Interestingly, 42% and 34% of the total FAs was esterified in the PL and CHE fraction, respectively, while only 10% were present in both the TG and NEFA fraction. Only few individual FA concentrations differed in the PL, CHE and TG fraction between BMI groups, whereas abundant BMI-related differences were found in the NEFA fraction.

Conclusions: Our data show that differences in BMI are associated with alterations in the FA composition of the FF, an effect most pronounced in the NEFA fraction. These BMI-related variations could possibly affect granulosa cell viability, oocyte developmental competence and subsequent embryo quality possibly explaining differences in oocyte quality in obese patients described by others.

Introduction

Fatty acids (FAs) are important compounds in the micro-environment of the ovarian follicle. Besides their role as cellular energy source, they have important biological functions in cell membrane biogenesis (Sturmey *et al.*, 2009a, Renaville *et al.*, 2010) and signalling (McKeegan and Sturmey, 2012). In addition, they act as precursors for steroids and prostaglandins, which are essential for normal reproductive function (Mattos *et al.*, 2000). In the follicular fluid (FF), FAs are present in an esterified form [triglycerides (TG), cholesterol esters (CHE) and phospholipids (PL)] or as non-esterified FAs (NEFAs), mainly bound to albumin (Hughes *et al.*, 2011). Maternal diet can have a profound effect on the FA composition of the FF, which may subsequently affect the FA composition of the cumulus cells and the oocyte (Adamiak *et al.*, 2006, Wonnacott *et al.*, 2010, Zachut *et al.*, 2010). Numerous animal studies, mostly in cattle, sheep and rodents, have demonstrated the effect of diet-induced FA changes on ovarian physiology in general and on the quality of the oocyte more specifically (Petit *et al.*, 2002, Zeron *et al.*, 2002, Wonnacott *et al.*, 2010, Zachut *et al.*, 2010). Dietary induced FA changes or obesogenic diets during oocyte maturation or early embryo development affect oocyte and embryo quality (Igosheva *et al.*, 2010, Wu *et al.*, 2010) and these effects may even have consequences for the offspring's health (Jungheim *et al.*, 2010, Yi *et al.*, 2012). However, results of these studies are not always in agreement and difficult to compare as not only the quantity of the FA offered, but also the physiological status, the type of FA (ratios of omega-3, -6 or -9 FAs, number of double bonds, carbon chain length) and the duration of treatment may determine the final FA concentrations in the FF (McKeegan and Sturmey, 2012). Besides dietary effects, body condition and metabolic status may also impact on the profile of FAs in the FF (Leroy *et al.*, 2005). Metabolic disorders such as obesity and type II diabetes, but also a negative energy balance, are associated with upregulated lipolysis, leading to elevated NEFA concentrations in the serum (Karpe *et al.*, 2011), that are reflected in the FF of the dominant follicle (Leroy *et al.*, 2005, Valckx *et al.*, 2012) and could therefore directly affect oocyte quality and metabolism. It has been shown that TG and NEFA concentrations are increased in the FF of obese women subjected to ovum pick-up (Yang *et al.*, 2012), while such elevated NEFA and/or TG concentrations were negatively associated with human

cumulus-oocyte-complex morphology (Jungheim *et al.*, 2011a) and affected both murine oocyte maturation (Yang *et al.*, 2012) and bovine embryo quality (Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a). Other studies investigating the influence of serum or FF FAs on fertility in general and oocyte quality more specifically, are difficult to compare as experimental designs, number and treatment of patients included and the relation between specific FAs studied and fertility outcome parameters often differ (Jungheim *et al.*, 2011a, Shaaker *et al.*, 2012, Jungheim *et al.*, 2013c). Additional to the heterogeneity in study design, most research on FAs has focused on the total fat fraction, rather than investigating FA differences in different lipid fraction. This is important though, because the cellular response to FAs possibly depends on the lipid fraction the FA belongs to. For this reason, knowledge on the specific FA composition of each lipid fraction in human FF is essential. In this study, we hypothesized that the FF FA composition may be altered by the woman's metabolic body condition (as determined by her body mass index), being a well-documented risk factor for fertility failure (Koning *et al.*, 2010). Therefore, we aimed (1) to determine the quantity, the distribution and the relative abundance of FAs in different lipid fractions (PL, CHE, TG and NEFA) in the FF of the dominant follicle of normal weight, overweight and obese women, undergoing assisted reproductive treatment (ART) and (2) to define how this FA distribution is affected by BMI in these women. To our knowledge, this is the first time that a detailed description of FA concentrations and abundance in different lipid fractions in the FF of normal weight, overweight and obese women, undergoing ART is provided. This study further discusses potential implications of BMI-related FF FA changes on oocyte and embryo quality.

Material and methods

a) Patients and follicular fluid sampling

This descriptive cross-sectional study was approved by the ethical committees of the University of Antwerp (UA A09-04) and the ZNA Middelheim Hospital (EC 3352). Women seeking assisted reproductive services were recruited (voluntary informed consent) at the Centre for Reproductive Medicine of the ZNA Middelheim Hospital. Patients were treated with a long agonist protocol, followed by ovum pick-up, *in vitro* fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI) and embryo transfer on Day 3 of culture as described by Valckx *et al.* (2012). Height (m) and weight (kg) of the patients were used to calculate the body mass index (BMI) score (kg/m²). Ten patients were randomly selected for each BMI group out of a population of approximately 100 women from our previous study (Valckx *et al.*, 2012). The single criterion for this selection was $18.5 \leq BMI < 25.0$ kg/m² for the normal weight group, $25.0 \leq BMI < 30$ kg/m² for the overweight group and $BMI \geq 30.0$ kg/m² for the obese group, according to the standards of the World Health Organization (WHO, 2011). Male

and female subfertility etiologies were documented and further patient specific data included the age of the patient (years), IVF attempt number, ratio ICSI/IVF, dosage of gonadotropins used (IU), maximal serum estradiol values (pg/ml), the number of oocytes aspirated upon oocyte retrieval, the number of fertilized oocytes (presenting with 2 pronuclei, 2PN), the number of embryos produced, the number of top quality embryos, the number of embryos transferred and the number of live births. Besides, several ratios were calculated as surrogate markers for oocyte quality: percentage of 2PN (n. 2PN /n. oocytes), percentage of embryos (n. embryos /n. oocytes), percentage of top quality embryos (n. top quality embryos /n. of oocytes) and percentage of embryos and top quality embryos developing from fertilized oocytes (n. embryos /n. 2PN and n. top quality embryos /n. 2PN, respectively). A top quality embryo was defined as an embryo with: (i) four or five blastomeres on Day 2, and seven or more blastomeres on Day 3 (ii) 20% fragmentation or less on Day 3 and (iii) no multi-nucleated blastomeres ever as described by Van Royen *et al.* (1999).

For each patient, the FF of the largest and first punctured follicle was recovered during the oocyte retrieval procedure by means of a transvaginal follicular aspiration. Only patients with a blood free aspirate of a follicle ≥ 18 mm were considered for inclusion in the study. The FF samples were cooled immediately after aspiration and were transported on ice within 2h of collection. At the laboratory, FF samples were centrifuged (1500 g, 10 min) and the supernatant was stored at -80°C until all 30 samples were analysed for various FAs by means of gas chromatography.

b) Gas chromatographic analysis of follicular fluid

Lipids in the FF supernatant were extracted with methyl-tert-butyl-ether as described by Matyash *et al.* (2008). Lipid fractions were separated using solid phase extraction (SPE)-columns (Pinkart *et al.*, 1998, Burdge *et al.*, 2000). Total plasma lipid extracts were dissolved in chloroform (1.0ml) and applied to an aminopropyl silica column (pasteur pipette containing 100mg aminopropyl silica gel) under gravity. Cholesteryl-esters and TGs were eluted with chloroform (1.0ml and 0.5ml), combined, dried under N_2 and dissolved in 1.0ml hexane. Non-esterified FAs were eluted with diethyl ether/acetic acid (100:2; 1.0ml and 0.5ml) and PLs with 1ml methanol/chloroform (6:1) followed by 0.5ml 0.05M sodium acetate in methanol/chloroform (6:1). Cholesteryl-esters and TGs were further separated on a pre-packed 100mg aminopropyl column (Varian). The CHE and TG fractions were loaded in 1ml hexane and the CHE fraction was eluted with hexane (1.0ml and 0.5ml). Triglycerides were eluted with hexane/chloroform/ethyl acetate (100:5:5; 1.0ml and 0.5ml). Fatty acids in lipid extracts were methylated using a basic followed by an acid methylation step. Toluene (2ml) containing the internal standard (C13:0) and methanolic NaOH (2ml) was added and the mixture was incubated at 70°C (60 min) followed by 30 min at 50°C after addition of methanolic HCl (3ml), prepared by dissolving 10ml acetyl chloride in 50ml methanol. Fatty acid methyl esters (FAME) were

extracted with hexane. Fatty acids in the TG fraction were methylated as described by Chouinard *et al.* (1997) whereas NEFAs were methylated by an acid methylation step only. Fatty acids in PL and CHE were methylated using a basic followed by an acid methylation step.

Composition analyses of the FAs were conducted using a gas chromatograph (HP 7890A, Agilent Technologies, Diegem, Belgium) equipped with a 75-m SP-2560™ capillary column (i.d., 0.18mm, film thickness, 0.14µm; Supelco Analytical, Bellefonte, PA) and a flame ionization detector. The oven temperature program was 70°C before being raised to 175°C (50°C/min) for 13 min, after which it was raised to 215 °C (5°C/min) for 25 min. The inlet temperature was 250°C and the detector temperature 255°C. Various control materials were used: BR2/BR3, (Larodan Fine Chemicals, Malmö, Sweden), Supelco® 37 (Supelco Analytical, Bellefonte, PA), PUFA-3 (Matreya LLC, Pleasant Gap, PA) and GLC463 (NU-CHEK-PREP Inc., Elysian, MN, USA). Fatty acids for which no standards were commercially available were identified by order of elution according to Precht *et al.* (2001) and Kramer *et al.* (2008).

The FA analysis generated data on the concentrations of lauric acid (12:0), myristic acid (14:0), pentadecaenoic acid (15:0), palmitic acid (16:0), palmitoleic acid n-7 (16:1 n-7), palmitoleic acid n-9 (16:1 n-9), margaric acid (17:0), stearic acid (18:0), oleic acid (18:1 n-9), vaccenic acid n-11 (18:1 n-11), linoleic acid (18:2 n-6), arachidic acid (20:0), gamma-linolenic acid (18:3 n-6), alfa-linolenic acid (18:3 n-3), eicosenoic acid (20:1), eicosadienoic acid (20:2), di-homo-gamma-linolenic acid (20:3 n-6), arachidonic acid (20:4 n-6), eicosapentaenoic acid (20:5 n-3), docosatetraenoic acid (22:4 n-6), docosapentaenoic acid n-6 (22:5 n-6), docosapentaenoic acid n-3 (22:5 n-3), docosahexaenoic acid (22:6 n-3). For each FA measurement, both the absolute (µmol/l) and the relative concentration (mol%) was determined. Fatty acids were attributed to the PL, TG, CHE or NEFA fraction.

c) Statistical analyses

All statistical analyses were performed with PASW 18.0 (for Windows, Chicago, IL, USA) or R 2.13.1 . Several statistical hypothesis tests were carried out to study the relation between patient characteristics and reproductive outcome parameters, with the type of statistical test depending on the nature of the outcome parameter. Counted numbers were analysed using Quasipoisson regression (mean IVF attempt number, n. oocytes, n. 2PN, n. embryos, n. top quality embryos), with an F-test as described by Agresti (2002). Numeric variables were analysed using linear regression (age, gonadotropin dose, maximal estradiol values) and binary outcomes were analysed using logistic regression (live birth, percentage of 2PN, percentage of embryos and top quality embryos, calculated on the n. of oocytes and on the n. of 2PN). The association between the number of embryos transferred and BMI was tested by means of a Kruskal – Wallis test. Differences in the IVF/ICSI ratio

were studied by means of a Student's t test. In these tests, BMI was entered as a continuous variable. Univariate general linear models, with post hoc Sheffé tests, were used to study differences in FA concentrations within lipid fractions and between BMI groups. In these latter tests, BMI was entered as a categorical variable. The same linear model was used to study the follicular fluid FA composition between PCOS and non-PCOS patients. Statistical significance was set at $P < 0.05$. Fatty acid concentrations (absolute or relative) were log transformed in case their distribution was not normal. Data are presented as means ± standard deviation (SD).

Results

a) Patient characteristics

Patient characteristics are presented in **Table 1**. Our data showed that increasing BMI was significantly associated with a higher dosage of gonadotropins administered (IU, $P = 0.04$). Also, increasing BMI was associated with fewer 2PNs ($P < 0.01$) and lower 2PN percentages ($P = 0.03$). There was no association between BMI and age, mean IVF attempt number, IVF/ICSI ratio, the number of oocytes aspirated, the number of embryos, the percentage of embryos, the number of top quality embryos, the percentage of top quality embryos, the ratios 'n. embryos /n. 2PNs' and 'n. top quality embryos /n. 2PNs', the number of embryos transferred, the number of live births and maximal estradiol values. As presented in **Table 1**, PCOS patients were not excluded from the study. This choice was made based on the observation that there was no significant difference in follicular fluid FA concentrations in the different lipid fractions between PCOS and non-PCOS patients. Data from our previous study, from which the cohort in this study is a subset, also showed that primary infertility cause did not affect follicular fluid composition (Valckx *et al.*, 2012).

Table 1: Patient information according to BMI class. The statistical method used and the associated *P*-value are presented in the last two columns.

	18.5 ≤ BMI < 25.0 (n = 10)	**25.0 ≤ BMI < 30.0 (n = 10)**	**BMI ≥ 30.0 (n = 10)**	**Statistical method**	***P*-value**
Age (years)	31.5 ± 4.67	32.7 ± 4.3	35.0 ± 6.8	Linear regression	NS
Body Weight (kg)	61.6 ± 4.7	75.6 ± 11.8	95.5 ± 11.4	NA	NA
BMI (kg/m²)	21.5 ± 0.3	28.1 ± 1.5	34.2 ± 2.4	NA	
Mean IVF attempt number	1.3 ± 0.7	2.0 ± 1.3	2.2 ± 1.5	Quasipoisson regression	NS
ICSI/IVF	5/5	2/8	5/5	Student's t test	NS
Gonadotropins administered (IU)	1793 ± 554	2100 ± 1034	2645 ± 933	Linear regression	0.04
Infertility cause (n)				NA	
Idiopathic	8	7	7		
PCOS	1	1	2		
Endometriosis	0	2	0		
Tubal	1	0	1		
No. oocytes	9.1 ± 3.6	7.2 ± 2.9	6.7 ± 4.8	Quasipoisson regression	NS
No. 2PN	6.5 ± 2.2	4.5 ± 1.1	3.5 ± 2.1	Quasipoisson regression	< 0.01
No. 2PN/No. oocytes (%)	73.1 ± 12.0	67.3 ± 18.8	61.1 ± 24.9	Logistic regression	0.03
No. embryos	2.3 ±1.2	1.5 ± 0.5	1.8 ± 0.6	Quasipoisson regression	NS
No. embryos/No. oocytes (%)	30.2 ± 22.3	22.8 ± 9.1	42.0 ± 32.1	Logistic regression	NS
No. embryos/No. 2PN (%)	39.8 ± 24.9	35.6 ± 16.5	64.2 ± 28.2	Logistic regression	NS
No. top quality embryos	2.0 ± 1.5	0.8 ± 0.9	1.0 ± 1.1	Quasipoisson regression	NS
No. top quality embryos/No. oocytes (%)	26.9 ± 25.4	11.4 ± 15.2	19.4 ± 30.1	Logistic regression	NS
No. top quality embryos/No. 2PN (%)	34.5 ± 29.8	19.6 ± 24.8	30.2 ± 33.1	Logistic regression	NS
No. embryos transferred	1.1 ± 0.3	1.3 ± 0.5	1.5 ± 0.5	Quasipoisson regression	NS
No. live births	3	2	5	Logistic regression	NS
Maximal E_2 values (pg/ml)	1571 ± 445	1399 ± 769	1505 ± 928	Linear regression	NS

Data are presented as mean ± standard deviation. BMI: body mass index, IVF: *in vitro* fertilization, PCOS: poly cystic ovarian syndrome, 2PN: presence of 2 pronuclei, E_2: estradiol, NS: not significant ($P > 0.05$), NA: Not applicable.

b) Total FA concentrations in the FF per lipid fraction

The distribution of FAs in the different lipid fractions in FF from normal weight, overweight and obese women is presented in **Table 2**. In obese women, NEFA concentrations were elevated ($P < 0.05$) compared to normal weight and overweight women. The concentration of TG tended to be higher in overweight women, compared to normal weight women ($P = 0.1$). Total FA concentrations did not differ between BMI groups (**Table 2**). It is noteworthy that the individually measured FA concentrations, in all the lipid fractions, had a relatively high standard deviation. Forty-one up to 45% of the defined FAs in the FF could be attributed to the PL fraction, 32-36% to the CHE fraction, 8-13% to the TG fraction and 9-11% to the NEFA fraction and this distribution did not differ between BMI groups. Total concentrations, presented in **Table 2**, also included measures of unknown FAs (accounting for approximately 5% of the total FA concentration).

Table 2: Fatty acids in follicular fluid of normal weight, overweight and obese women. Total fatty acid concentrations (µmol/l) in different lipid fractions in the follicular fluid of normal weight (18.5 ≤ BMI < 25), overweight (25.0 ≤ BMI < 30) and obese (BMI ≥ 30.0) women.

Fat fraction	18.5 ≤ BMI < 25.0 (n = 10)	25.0 ≤ BMI < 30.0 (n = 10)	BMI ≥ 30.0 (n = 10)	*P*-value
Phospholipids (µmol/l)	1165.57 ± 180.55	1147.93 ± 247.63	1199.95 ± 296.46	NS
Cholesteryl esters (µmol/l)	920.04 ± 196.64	891.22 ± 117.35	1067.29 ± 269.40	NS
Triglycerides (µmol/l)	215.91 ± 57.89	355.39 ± 226.44	244.78 ± 74.65	< 0.1
NEFAs (µmol/l)	221.67 ± 23.00[a]	245.55 ± 35.98[a]	315.53 ± 82.68[b]	< 0.05
Total concentration (µmol/l)	2598.56 ± 422.68	2769.88 ± 477.07	2931.80 ± 684.58	NS

Data are presented as means ± standard deviation. *P*-values of the univariate general linear model analyses are presented in the last column. [ab]Data with a different superscript differ significantly ($P < 0.05$), NS: Not significant ($P > 0.05$), BMI: body mass index, NEFAs: non-esterified fatty acids.

c) BMI dependent distribution of FAs in different lipid classes

Total fat extract. The most abundant FAs in the FF were 16:0, 18:0, 18:1 n-9, 18:2 n-6 and 20:4 n-6. No significant differences could be found for neither absolute nor relative concentrations of the individual FAs between BMI groups.

*Phospholipid fraction (**Table 3A**).* The 4 most abundant FAs in the PL fraction of FF were 16:0, 18:0, 18:2 n-6 and 20:4 n-6, none of which were affected by BMI. On the contrary, the relative percentage of 18:1 n-9 was higher in normal weight women compared to overweight and obese women ($P < 0.01$) and the percentage of 20:3 n-6 was higher in overweight and obese women, compared to normal weight women ($P = 0.04$). There were no other BMI-related differences in FF FAs.

*Cholesteryl-ester fraction (**Table 3B**).* The most abundant FAs in this fraction were 16:0, 18:1 n-9, 18:2 n-6 and 20:4 n-6. Both the absolute and the relative concentration of 20:3 n-6 were significantly different between obese women and normal weight women (P = 0.04 and P = 0.02, respectively). There were no other BMI-related differences in the remaining FF FAs.

*Triglyceride fraction (**Table 3C**).* 16:0, 18:0, 18:1 n-9 and 18:2 n-6 were major FAs in the TG fraction. A significant elevation in the absolute concentration of 18:2 n-6 (P = 0.04) and 22:6 n-3 (P = 0.03) was found for overweight women, compared to normal weight women. There were no significant differences in the relative concentrations of the FAs.

*Non-esterified fatty acid fraction (**Table 3D**).* The most abundant FAs in the NEFA fraction were 16:0, 18:0, 18:1 n-9 and 18:2 n-6. Absolute concentrations of 14:0, 15:0, 16:1 n-7, 16:1 n-9, 17:0, 18:0, 18:1 n-9, 18:1 n-11, 18:2 n-6, 20:2, 20:3 n-6, 20:5 n-3, 22:5 n-6, 22:5 n-3 and 22:6 n-3 were higher in obese compared to normal weight women (P < 0.05). Additionally, absolute concentrations of 16:0, 18:3 n-6, 20:4 n-6 and 22:4 n-6 were higher in obese compared to normal weight and overweight women (P < 0.05). Furthermore, obese women had higher concentrations of 20:1 compared to overweight women (P < 0.05). Relative concentrations only showed a significant elevation in 20:4 n-6 for overweight and obese women, compared to normal weight women (P < 0.05) and an elevation in 20:3 n-6 for obese women, compared to normal weight women (P < 0.01).

Table 3: Fatty acid concentrations in different lipid fractions. Absolute (μM) and relative (mol%) fatty acid concentrations in the (A) phospholipid, (B) cholesteryl ester, (C) triglyceride and (D) non-esterified fat fraction in the follicular fluid of normal weight (18.5 ≤ BMI < 25.0), overweight (25.0 ≤ BMI < 30.0) and obese (BMI ≥ 30.0) women undergoing assisted reproductive services.

A	**Absolute concentrations (μM)**			**Relative concentrations (mol%)**		
Phopholipid	**Normal Weight**	**Overweight**	**Obese**	**Normal Weight**	**Overweight**	**Obese**
12:0	0,0 ± 0,0	0,2 ± 0,3	0,2 ± 0,6	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
14:0	4,1 ± 1,1	4,6 ± 1,8	4,1 ± 1,1	0,3 ± 0,1	0,4 ± 0,1	0,3 ± 0,1
15:0	2,7 ± 0,6	2,7 ± 0,7	2,4 ± 0,4	0,2 ± 0,0	0,2 ± 0,0	0,2 ± 0,0
16:0	359,4 ± 53,1	360,9 ± 92,0	383,5 ± 129,0	30,9 ± 1,1	31,3 ± 1,7	31,5 ± 2,6
16:1 n-7	1,7 ± 0,3	1,7 ± 0,4	1,6 ± 0,2	0,1 ± 0,0	0,2 ± 0,0	0,1 ± 0,0
16:1 n-9	5,8 ± 1,8	6,2 ± 2,8	5,5 ± 1,8	0,5 ± 0,1	0,5 ± 0,1	0,5 ± 0,1
17:0	4,3 ± 0,8	4,3 ± 0,8	4,2 ± 0,8	0,4 ± 0,0	0,4 ± 0,0	0,4 ± 0,1
18:0	160,0 ± 26,6	160,6 ± 24,2	164,2 ± 25,0	13,7 ± 1,0	14,2 ± 1,5	14,0 ± 2,1
18:1 n-9	104,2 ± 16,9	91,4 ± 26,4	95,1 ± 25,6	9,0 ± 0,7[a]	7,9 ± 0,8[b]	7,9 ± 0,7[b]
18:1 n-11	17,8 ± 2,9	16,9 ± 4,4	15,8 ± 4,0	1,5 ± 0,2	1,5 ± 0,2	1,3 ± 0,2
18:2 n-6	218,1 ± 39,7	191,1 ± 36,7	190,1 ± 26,1	18,7 ± 1,7	16,8 ± 2,2	16,3 ± 2,7
20:0	0,9 ± 0,1	0,8 ± 0,1	0,8 ± 0,1	0,1 ± 0,0	0,1 ± 0,0	0,1 ± 0,0
18:3 n-6	0,3 ± 0,1	0,4 ± 0,2	0,4 ± 0,1	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
18:3 n-3	2,7 ± 0,7	2,4 ± 0,8	2,1 ± 0,6	0,2 ± 0,1	0,2 ± 0,1	0,2 ± 0,1
20:1	2,3 ± 0,3	2,3 ± 0,5	2,2 ± 0,4	0,2 ± 0,0	0,2 ± 0,0	0,2 ± 0,0
20:2	4,9 ± 1,3	5,3 ± 1,2	5,0 ± 0,9	0,4 ± 0,1	0,5 ± 0,1	0,4 ± 0,1
20:3 n-6	40,1 ± 14,2	45,8 ± 8,5	48,7 ± 12,5	3,4 ± 0,7[a]	4,0 ± 0,6[b]	4,1 ± 0,6[b]
20:4 n-6	126,2 ± 19,4	132,2 ± 34,4	154,2 ± 63,4	10,9 ± 0,7	11,5 ± 1,6	12,5 ± 2,1
20:5 n-3	9,2 ± 3,0	12,3 ± 7,2	12,7 ± 5,7	0,8 ± 0,2	1,0 ± 0,5	1,0 ± 0,4
22:4 n-6	4,8 ± 0,7	5,1 ± 1,3	5,3 ± 2,0	0,4 ± 0,0	0,4 ± 0,1	0,4 ± 0,1
22:5 n-6	3,6 ± 1,0	3,8 ± 1,4	3,5 ± 1,4	0,3 ± 0,1	0,3 ± 0,1	0,3 ± 0,1
22:5 n-3	12,2 ± 1,6	12,6 ± 3,4	11,5 ± 3,4	1,1 ± 0,1	1,1 ± 0,1	1,0 ± 0,2
22:6 n-3	48,6 ± 11,0	54,7 ± 18,1	57,9 ± 19,7	4,2 ± 0,7	4,7 ± 0,9	4,8 ± 0,9
unknown	31,8 ± 9,6	29,7 ± 8,5	29,1 ± 7,7	-	-	-
total	1165,6 ± 180,6	1147,9 ± 247,6	1200,0 ± 296,5	-	-	-

B	Absolute concentrations (µM)			Relative concentrations (mol%)		
Cholesteryl ester	**Normal Weight**	**Overweight**	**Obese**	**Normal Weight**	**Overweight**	**Obese**
12:0	19,6 ± 14,7	23,1 ± 15,0	22,7 ± 14,4	2,1 ± 1,6	2,6 ± 2,1	1,8 ± 1,4
14:0	15,9 ± 7,9	17,7 ± 8,5	18,1 ± 8,0	1,7 ± 0,8	2,0 ± 1,7	0,9 ± 0,7
15:0	2,2 ± 0,7	2,0 ± 0,7	2,2 ± 0,6	0,2 ± 0,1	0,2 ± 0,2	0,0 ± 0,1
16:0	104,8 ± 21,8	103,9 ± 20,7	131,3 ± 45,3	11,4 ± 0,3	11,6 ± 12,1	1,1 ± 1,1
16:1 n-7	4,1 ± 1,1	4,0 ± 0,9	4,7 ± 1,8	0,4 ± 0,0	0,4 ± 0,4	0,1 ± 0,1
16:1 n-9	19,2 ± 7,9	21,1 ± 10,3	25,0 ± 16,5	2,0 ± 0,5	2,3 ± 2,2	1,0 ± 1,0
17:0	1,2 ± 0,2	1,3 ± 0,2	1,4 ± 0,5	0,1 ± 0,0	0,2 ± 0,1	0,0 ± 0,0
18:0	7,7 ± 1,5	8,1 ± 0,7	12,9 ± 13,7	0,8 ± 0,1	0,9 ± 1,1	0,1 ± 0,9
18:1 n-9	163,1 ± 34,4	147,6 ± 24,6	188,1 ± 66,4	17,8 ± 0,9	16,5 ± 17,3	1,4 ± 1,9
18:1 n-11	11,2 ± 2,0	10,3 ± 2,6	12,2 ± 4,2	1,2 ± 0,1	1,1 ± 1,1	0,2 ± 0,1
18:2 n-6	415,0 ± 102,5	386,7 ± 52,4	437,6 ± 65,9	45,0 ± 3,6	43,5 ± 42,0	4,0 ± 5,7
20:0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
18:3 n-6	3,7 ± 2,1	3,9 ± 1,3	5,1 ± 2,2	0,4 ± 0,1	0,4 ± 0,5	0,1 ± 0,2
18:3 n-3	4,2 ± 1,3	4,3 ± 1,2	5,5 ± 4,4	0,5 ± 0,1	0,5 ± 0,5	0,1 ± 0,3
20:1	0,0 ± 0,0	0,0 ± 0,0	0,2 ± 0,7	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
20:2	0,0 ± 0,0	0,0 ± 0,0	0,1 ± 0,4	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
20:3 n-6	7,3 ± 2,8^{a}	8,5 ± 1,6^{ab}	10,1 ± 2,3^{b}	0,8 ± 0,2^{a}	1,0 ± 1,0^{ab}	0,2 ± 0,1^{b}
20:4 n-6	65,8 ± 14,4	71,6 ± 18,6	94,7 ± 39,7	7,2 ± 0,8	8,0 ± 8,8	1,4 ± 2,1
20:5 n-3	6,5 ± 2,3	8,4 ± 4,9	10,4 ± 5,4	0,7 ± 0,2	0,9 ± 1,0	0,5 ± 0,4
22:4 n-6	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
22:5 n-6	0,1 ± 0,2	0,1 ± 0,2	0,2 ± 0,3	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
22:5 n-3	1,3 ± 0,3	1,2 ± 0,1	1,0 ± 0,3	0,1 ± 0,1	0,1 ± 0,1	0,0 ± 0,0
22:6 n-3	5,6 ± 1,6	6,6 ± 2,1	8,0 ± 2,8	0,6 ± 0,1	0,7 ± 0,8	0,2 ± 0,2
unknown	61,6 ± 8,0	60,7 ± 5,5	75,9 ± 37,6	-	-	-
total	920,0 ± 196,6	891,2 ± 117,4	1067,3 ± 269,4	-	-	-

C	Absolute concentrations (µM)			Relative concentrations (mol%)		
Triglyceride	**Normal Weight**	**Overweight**	**Obese**	**Normal Weight**	**Overweight**	**Obese**
12:0	2,0 ± 0,6	3,3 ± 3,1	2,4 ± 0,5	1,0 ± 0,3	0,9 ± 0,3	1,0 ± 0,2
14:0	5,6 ± 2,1	9,8 ± 8,1	6,8 ± 2,8	2,6 ± 0,7	2,7 ± 0,6	2,8 ± 0,6
15:0	1,1 ± 0,2	1,4 ± 0,6	1,1 ± 0,3	0,5 ± 0,1	0,4 ± 0,1	0,5 ± 0,1
16:0	51,4 ± 15,4	90,7 ± 70,1	60,8 ± 21,9	23,6 ± 1,2	24,4 ± 2,5	24,5 ± 2,4
16:1 n-7	2,2 ± 0,6	3,2 ± 1,7	2,3 ± 0,4	1,0 ± 0,1	1,0 ± 0,2	1,0 ± 0,3
16:1 n-9	6,5 ± 3,3	11,8 ± 11,6	7,1 ± 2,9	2,9 ± 0,8	3,1 ± 0,8	2,9 ± 0,5
17:0	0,8 ± 0,2	1,2 ± 0,7	0,9 ± 0,3	0,4 ± 0,0	0,4 ± 0,1	0,4 ± 0,1
18:0	11,2 ± 4,3	19,5 ± 14,7	15,2 ± 10,5	5,2 ± 1,1	5,5 ± 2,0	5,9 ± 2,1
18:1 n-9	76,7 ± 22,9	120,9 ± 74,0	83,4 ± 29,3	35,3 ± 1,9	34,1 ± 2,3	33,9 ± 1,9
18:1 n-11	4,2 ± 1,5	7,4 ± 5,0	4,7 ± 1,8	1,9 ± 0,2	2,1 ± 0,3	1,9 ± 0,2
18:2 n-6	27,2 ± 7,7[a]	46,8 ± 26,0[b]	30,5 ± 8,0[ab]	12,6 ± 1,4	13,6 ± 1,9	12,8 ± 2,5
20:0	0,3 ± 0,0	0,4 ± 0,2	0,3 ± 0,1	0,1 ± 0,0	0,1 ± 0,0	0,1 ± 0,0
18:3 n-6	0,4 ± 0,2	0,7 ± 0,5	0,5 ± 0,3	0,2 ± 0,0	0,2 ± 0,1	0,2 ± 0,1
18:3 n-3	2,4 ± 0,9	4,4 ± 3,3	3,0 ± 2,0	1,2 ± 0,3	1,2 ± 0,5	1,2 ± 0,6
20:1	0,7 ± 0,2	1,4 ± 1,0	0,8 ± 0,5	0,3 ± 0,1	0,4 ± 0,1	0,3 ± 0,1
20:2	0,7 ± 0,2	1,0 ± 0,5	0,7 ± 0,2	0,4 ± 0,1	0,3 ± 0,1	0,3 ± 0,1
20:3 n-6	0,4 ± 0,1	0,9 ± 0,8	0,5 ± 0,1	0,2 ± 0,0	0,2 ± 0,1	0,2 ± 0,1
20:4 n-6	2,1 ± 0,7	4,2 ± 3,5	3,5 ± 1,7	1,0 ± 0,2	1,3 ± 0,6	1,5 ± 0,7
20:5 n-3	0,6 ± 0,2	1,2 ± 0,9	0,8 ± 0,4	0,3 ± 0,1	0,3 ± 0,1	0,3 ± 0,1
22:4 n-6	0,5 ± 0,2	0,6 ± 0,5	0,4 ± 0,2	0,2 ± 0,1	0,2 ± 0,1	0,2 ± 0,1
22:5 n-6	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,1	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0
22:5 n-3	0,4 ± 0,1	0,8 ± 0,9	0,4 ± 0,1	0,2 ± 0,0	0,2 ± 0,1	0,2 ± 0,0
22:6 n-3	0,5 ± 0,2[a]	1,9 ± 2,9[b]	1,0 ± 0,4[ab]	0,2 ± 0,1	0,4 ± 0,3	0,4 ± 0,2
unknown	17,9 ± 3,6	21,8 ± 7,9	17,7 ± 4,1	-	-	-
total	215,9 ± 57,9	355,4 ± 226,4	244,8 ± 74,7	-	-	-

D	Absolute concentrations (µM)			Relative concentrations (mol%)		
NEFA	**Normal Weight**	**Overweight**	**Obese**	**Normal Weight**	**Overweight**	**Obese**
12:0	2,0 ± 0,6	2,6 ± 1,4	2,7 ± 0,9	0,9 ± 0,3	1,0 ± 0,6	0,9 ± 0,3
14:0	4,6 ± 0,6^{a}	5,1 ± 1,0^{ab}	6,4 ± 1,7^{b}	2,1 ± 0,2	2,1 ± 0,2	2,0 ± 0,2
15:0	0,5 ± 0,1^{a}	0,6 ± 0,1^{ab}	0,7 ± 0,2^{b}	0,2 ± 0,0	0,2 ± 0,0	0,2 ± 0,1
16:0	51,4 ± 6,4^{a}	55,7 ± 7,2^{a}	70,3 ± 17,1^{b}	23,2 ± 1,6	22,8 ± 1,6	22,5 ± 2,1
16:1 n-7	0,8 ± 0,1^{a}	1,0 ± 0,2^{ab}	1,4 ± 0,5^{b}	0,4 ± 0,0	0,4 ± 0,0	0,4 ± 0,1
16:1 n-9	6,3 ± 1,5^{a}	7,7 ± 1,7^{ab}	10,2 ± 3,8^{b}	2,8 ± 0,6	3,1 ± 0,4	3,2 ± 0,6
17:0	0,6 ± 0,1^{a}	0,7 ± 0,1^{ab}	0,8 ± 0,3^{b}	0,3 ± 0,0	0,3 ± 0,0	0,3 ± 0,1
18:0	19,7 ± 3,6^{a}	20,3 ± 2,9^{ab}	24,8 ± 5,6^{b}	8,9 ± 1,4	8,3 ± 0,9	8,1 ± 1,5
18:1 n-9	68,1 ± 8,3^{a}	76,9 ± 13,5^{ab}	102,5 ± 34,1^{b}	30,7 ± 1,8	31,3 ± 1,8	32,1 ± 3,3
18:1 n-11	5,8 ± 0,9^{a}	6,5 ± 1,6^{ab}	9,0 ± 3,0^{b}	2,6 ± 0,3	2,6 ± 0,4	2,8 ± 0,4
18:2 n-6	37,1 ± 5,3^{a}	40,0 ± 7,8^{ab}	49,7 ± 12,9^{b}	16,8 ± 2,1	16,3 ± 2,1	15,9 ± 1,7
20:0	0,4 ± 0,0	0,4 ± 0,0	0,5 ± 0,1	0,2 ± 0,0	0,2 ± 0,0	0,1 ± 0,0
18:3 n-6	0,4 ± 0,1^{a}	0,5 ± 0,1^{a}	0,7 ± 0,2^{b}	0,2 ± 0,0	0,2 ± 0,0	0,2 ± 0,1
18:3 n-3	1,2 ± 0,3	1,5 ± 0,5	2,1 ± 2,1	0,5 ± 0,1	0,6 ± 0,1	0,6 ± 0,4
20:1	1,7 ± 0,3^{ab}	1,7 ± 0,4^{a}	2,3 ± 0,7^{b}	0,8 ± 0,1	0,7 ± 0,1	0,7 ± 0,1
20:2	1,5 ± 0,4^{a}	1,6 ± 0,5^{ab}	2,1 ± 0,7^{b}	0,7 ± 0,2	0,6 ± 0,1	0,7 ± 0,2
20:3 n-6	0,5 ± 0,1^{a}	0,6 ± 0,1^{ab}	0,9 ± 0,3^{b}	0,2 ± 0,0^{a}	0,3 ± 0,0^{ab}	0,3 ± 0,1^{b}
20:4 n-6	1,2 ± 0,3^{a}	1,8 ± 0,4^{a}	2,3 ± 0,7^{b}	0,6 ± 0,1^{a}	0,7 ± 0,1^{b}	0,7 ± 0,1^{b}
20:5 n-3	0,2 ± 0,1^{a}	0,3 ± 0,1^{ab}	0,3 ± 0,1^{b}	0,1 ± 0,0	0,1 ± 0,0	0,1 ± 0,0
22:4 n-6	2,1 ± 0,6^{a}	2,0 ± 0,6^{a}	3,5 ± 1,2^{b}	0,9 ± 0,2	0,8 ± 0,2	1,1 ± 0,4
22:5 n-6	0,5 ± 0,1^{a}	0,5 ± 0,1^{ab}	0,7 ± 0,3^{b}	0,2 ± 0,1	0,2 ± 0,0	0,2 ± 0,1
22:5 n-3	0,6 ± 0,2^{a}	0,8 ± 0,3^{ab}	1,2 ± 0,4^{b}	0,3 ± 0,1	0,3 ± 0,1	0,4 ± 0,1
22:6 n-3	1,4 ± 0,5^{a}	1,9 ± 0,8^{ab}	2,7 ± 1,1^{b}	0,6 ± 0,2	0,8 ± 0,3	0,9 ± 0,2
unknown	13,1 ± 3,3	14,9 ± 3,8	17,8 ± 6,0	-	-	-
total	221,7 ± 23,0^{a}	245,6 ± 36,0^{a}	315,5 ± 82,7^{b}	-	-	-

Data are presented as means ± standard deviation. ^{ab}Data with a different superscript within absolute or relative concentrations, differ significantly ($P < 0.05$). BMI: Body Mass Index, NEFA: Non-esterified fatty acid.

Discussion

The aim of this study was to provide insight in the FA profile, specified per lipid fraction, in the FF of the pre-ovulatory follicle from normal weight, overweight and obese women, undergoing ART. To our knowledge, this is the first time that FAs in human FF lipid fractions are described in such detail. Our results showed that approximately 42% of the total FA concentration was esterified in the PL fraction and 34% in the CHE fraction, whereas only 10% were present in both the TG and NEFA fraction. Interestingly, only TG (trend) and NEFA concentrations were affected by BMI. Even though FA concentrations in the NEFA fraction were well below those of the other fractions, they showed the most BMI-related variability.

Total NEFA concentrations in the FF were elevated in obese women, which is confirmed by earlier work (Yang *et al.*, 2012). However, it is in contrast with our previous findings (Valckx *et al.*, 2012) and with the data of Robker *et al.* (2009), who could not show a BMI-related difference in FF total NEFA concentrations. The difference between these data and those of our previous study, could partially be explained by a large variation in NEFA concentrations, the use of a different (colorimetric versus gas chromatographic) analysis methods and the inequality of the number of patients within each BMI group in our previous study (Valckx *et al.*, 2012). Remarkably, our data also showed that even though absolute NEFA concentrations presented with a great BMI-related variability, relative concentrations only showed minor differences, suggesting that increasing BMI did not cause a shift in the relative abundance of the different FAs. Interestingly, Jungheim *et al.* (2011a) found that women with elevated levels of FF NEFAs displayed poorer cumulus-oocyte complex (COC) morphology. They differentiated between FF palmitic, stearic, oleic and linoleic acid in the NEFA fraction, but no correlations with BMI were found. Our data showed that 87% (20/23) of all FAs in the NEFA fraction, including palmitic, stearic, oleic and linoleic acid, correlated with BMI, which suggests that BMI causes changes in the FF NEFA concentrations that could directly affect COC function and quality. This is substantiated by the fact that we previously showed that bovine oocyte exposure, during the final stage of maturation, to elevated NEFA concentrations (oleic, palmitic and/or stearic acid) was detrimental for the oocyte's developmental capacity (Leroy *et al.*, 2005). It also altered gene expression patterns and energy/amino acid metabolism in blastocysts from oocytes exposed during the last 24h of maturation (Van Hoeck *et al.*, 2011). Aardema *et al.* (2011) showed that oocytes actively take up and metabolize NEFAs out of their environment (mitochondrial β-oxidation for the purpose of ATP production) and that this may influence lipid storage in lipid droplets within the oocyte, depending on the type and amount of FA offered. More specifically, oocytes exposed to palmitic and stearic acid presented with less intracellular fat storage and a hampered oocyte

developmental competence. This effect could be counteracted by the addition of oleic acid to the treatment (Aardema *et al.*, 2011). Such a protective effect of the mono-unsaturated FA oleic acid has also been proposed by Cnop *et al.* (2001) in pancreatic islet cells, where oleic acid was described to reduce palmitate induced lipotoxicity, possibly by promoting triglyceride formation (cytoprotective mechanism). Our recent research investigated the mechanisms behind this lipotoxicity and showed that the degree of mitochondrial FA β-oxidation has a strong impact on the development of NEFA exposed bovine oocytes and on the quality of the resulting embryos (Van Hoeck *et al.*, 2013a). Besides the oocyte, the somatic cells of the follicle might also be influenced by the composition of FAs in the FF. For example, palmitic acid has been shown to inhibit *in vitro* bovine granulosa and theca cell proliferation and to alter steroid production by inducing apoptosis (Vanholder *et al.*, 2005, Vanholder *et al.*, 2006). Arachidonic acid, on the other hand, protects human granulosa cells from palmitic and stearic acid-induced apoptosis (Mu *et al.*, 2001). The effects of poly-unsaturated FAs are less uniform as for example linoleic acid (18:2 n-6) hampers and linolenic acid (18:3 n-3) stimulates bovine nuclear oocyte maturation (Marei *et al.*, 2009, Marei *et al.*, 2010, Wonnacott *et al.*, 2010, Zachut *et al.*, 2010). Interestingly, linoleic acid was present in the follicular fluid as one of the most predominant fatty acids. Marei *et al.* (2010) showed that exposure of bovine cumulus-oocyte complexes to elevated linoleic acid concentrations during final maturation impaired oocyte maturation and decreased oocyte developmental competence. This may imply that elevated levels of linoleic acid could have harmful effects on fertility outcome in women. Under normal circumstances, linoleic acid concentrations are decreased in the follicular fluid of large follicles, compared to smaller follicles (Homa and Brown, 1992). This suggest that linoleic acid can play an important role in the regulation of oocyte maturation, with decreasing levels allowing progression of oocyte maturation.

In all the previously discussed *in vitro* studies, FAs are added to the medium as non-esterified FAs. It remains, however, unclear if the same FAs, but esterified to a different fat fraction might elicit a differential effect. For example, a FA derived from the hydrolysis of TGs, contained in for example very low-density lipoproteins, by lipoprotein lipase (Zhang *et al.*, 2010), could potentially exert a different effect at the level of the cell, compared to the same FA in the NEFA fraction.

Besides the total NEFA concentrations, also total TG concentrations tended to be increased in overweight women. Interestingly, when mouse oocytes were matured in medium supplemented with NEFA/TG rich human FF, a dramatic decrease in oocyte maturation to metaphase 2, an increased oocyte lipid content and an upregulation of genes related to endoplasmic reticulum stress could be observed (Yang *et al.*, 2012). This suggests a detrimental effect of elevated TG and/or NEFA concentrations on oocyte quality. It is however not clear whether this is caused by elevated TG or NEFA concentrations or both or to what particular compound in the FF this effect can be attributed.

Remarkably, 18:2 n-6 and 22:6 n-3 in the TG fraction were elevated in the FF of overweight, but not obese women, compared to normal weight women. A potential reason for this could be that in a first coping mechanism excess FAs, present in overweight individuals, are stored in lipid droplets (Klop *et al.*, 2013). However, in obese women, much of the circulating FAs originate from abdominal fat adipocytes (Klop *et al.*, 2013), which are rich in saturated and mono-unsaturated FAs (Garaulet *et al.*, 2006, Hostens *et al.*, 2012). High levels of the saturated palmitic and stearic acid have been shown to reduce lipid storage in maturing bovine oocytes (Aardema *et al.*, 2011). This is in agreement with our data, showing that obese women presented with elevated levels of many FA in the NEFA fraction, rather than in the TG fraction for potential storage in lipid droplets.

We have previously shown that a strong correlation exists between serum and FF metabolites, indicating that the serum composition influences the FF composition (Valckx *et al.*, 2012). However, these changes were not BMI-related, indicating that factors, other than serum composition have a large impact on FF composition. Furthermore, the potential impact of differential FAs in the FF on oocyte developmental competence depends on the actual presence and the ratios of FAs within the FF. Therefore, the primary focus of our study was to describe FF FAs, rather than to investigate their relation to the serum composition. We furthermore did not have any knowledge on diet or fasting before sampling, even though it has been well described that diet can change the FF FA composition and can thereby affect oocyte developmental competence and subsequent embryo quality (Zachut *et al.*, 2011). Another remark is that obese women required a higher dosage of gonadotropins to reach the same stimulatory effect on follicular development, compared to normal weight women. However, it has also been described that gonadotropin releasing hormone analogues can alter serum lipoprotein levels and can increase insulin resistance (Palomba *et al.*, 2004), potentially deteriorating the insulin resistant state in many obese women. Because of ethical restraints in proposing suboptimal gonadotropin concentration administration, we were unable to account for this potential confounding factor in our study.

In conclusion, this descriptive study reports on the FA concentrations in the PL, CHE, TG and NEFA fraction in the FF of normal weight, overweight and obese women, undergoing ART. Our study highlights that most FAs in the FF belong to the PL and CHE fat fraction, but that NEFAs presented with the greatest BMI-related variability, with most individual FA concentrations increased in obese women. These differences may affect oocyte quality and subsequent embryo development, possibly by acting directly on oocyte metabolism.

References

The R project for statistical computing [http://www.r-project.org/]

Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB and Gadella BM. Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biol Reprod* 2011; **85**: 62-69.

Adamiak SJ, Powell K, Rooke JA, Webb R and Sinclair KD. Body composition, dietary carbohydrates and fatty acids determine post-fertilisation development of bovine oocytes in vitro. *Reproduction* 2006; **131**: 247-258.

Agresti A. Categorical Data Analysis. 2nd edn, 2002. Wiley-Interscience, New York, USA.

Burdge GC, Wright P, Jones AE and Wootton SA. A method for separation of phosphatidylcholine, triacylglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction. *Brit J Nutr* 2000; **84**: 781-787.

Chouinard PY, Levesque J, Girard V and Brisson GJ. Dietary soybeans extruded at different temperatures: milk composition and in situ fatty acid reactions. *Journal of Dairy Science* 1997; **80**: 2913-2924.

Cnop M, Hannaert JC, Hoorens A, Eizirik DL and Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 2001; **50**: 1771-1777.

Garaulet M, Hernandez-Morante JJ, Lujan J, Tebar FJ and Zamora S. Relationship between fat cell size and number and fatty acid composition in adipose tissue from different fat depots in overweight/obese humans. *Int J Obes (Lond)* 2006; **30**: 899-905.

Homa ST and Brown CA. Changes in Linoleic-Acid during Follicular Development and Inhibition of Spontaneous Breakdown of Germinal Vesicles in Cumulus-Free Bovine Oocytes. *J Reprod Fertil* 1992; **94**: 153-160.

Hostens M, Fievez V, Leroy JLMR, Van Ranst J, Vlaeminck B and Opsomer G. The fatty acid profile of subcutaneous and abdominal fat in dairy cows with left displacement of the abomasum. *Journal of Dairy Science* 2012; **95**: 3756-3765.

Hughes J, Kwong WY, Li DF, Salter AM, Lea RG and Sinclair KD. Effects of omega-3 and-6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism. *Reproduction* 2011; **141**: 105-118.

Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR and McConnell J. Maternal Diet-Induced Obesity Alters Mitochondrial Activity and Redox Status in Mouse Oocytes and Zygotes. *Plos One* 2010; **5**: e10074.

Jungheim ES, Frolova AI, Jiang H and Riley JK. Relationship Between Serum Polyunsaturated Fatty Acids and Pregnancy in Women Undergoing In Vitro Fertilization. *J Clin Endocr Metab* 2013c; **98**: E1364-E1368.

Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS and Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. *Fertil Steril* 2011a; **95**: 1970-1974.

Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE and Moley KH. Diet-Induced Obesity Model: Abnormal Oocytes and Persistent Growth Abnormalities in the Offspring. *Endocrinology* 2010; **151**: 4039-4046.

Karpe F, Dickmann JR and Frayn KN. Fatty Acids, Obesity, and Insulin Resistance: Time for a Reevaluation. *Diabetes* 2011; **60**: 2441-2449.

Klop B, Elte JWF and Cabezas MC. Dyslipidemia in Obesity: Mechanisms and Potential Targets. *Nutrients* 2013; **5**: 1218-1240.

Koning AMH, Kuchenbecker WKH, Groen H, Hoek A, Land JA, Khan KS and Mol BWJ. Economic consequences of overweight and obesity in infertility: a framework for evaluating the costs and outcomes of fertility care. *Hum Reprod Update* 2010; **16**: 246-254.

Kramer JKG, Hernandez M, Cruz-Hernandez C, Kraft J and Dugan MER. Combining results of two GC separations partly achieves determination of all cis and trans 16 : 1, 18 : 1, 18 : 2 and 18 : 3 except CLA isomers of milk fat as demonstrated using ag-ion SPE fractionation. *Lipids* 2008; **43**: 259-273.

Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G and Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 2005; **130**: 485-495.

Marei WF, Wathes DC and Fouladi-Nashta AA. The Effect of Linolenic Acid on Bovine Oocyte Maturation and Development. *Biol Reprod* 2009; **81**: 1064-1072.

Marei WF, Wathes DC and Fouladi-Nashta AA. Impact of linoleic acid on bovine oocyte maturation and embryo development. *Reproduction* 2010; **139**: 979-988.

Mattos R, Staples CR and Thatcher WW. Effects of dietary fatty acids on reproduction in ruminants. *Reviews of Reproduction* 2000; **5**: 38-45.

Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A and Schwudke D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J Lipid Res* 2008; **49**: 1137-1146.

McKeegan PJ and Sturmey RG. The role of fatty acids in oocyte and early embryo development. *Reprod Fert Develop* 2012; **24**: 59-67.

Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K et al. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. *Endocrinology* 2001; **142**: 3590-3597.

Palomba S, Russo T, Orio F, Sammartino A, Sbano FM, Nappi C, Colao A, Mastrantonio P, Lombardi G and Zullo F. Lipid, glucose and homocysteine metabolism in women treated with a GnRH agonist with or without raloxifene. *Hum Reprod* 2004; **19**: 415-421.

Petit HV, Dewhurst RJ, Scollan ND, Proulx JG, Khalid M, Haresign W, Twagiramungu H and Mann GE. Milk production and composition, ovarian function, and prostaglandin secretion of dairy cows fed omega-3 fats. *Journal of Dairy Science* 2002; **85**: 889-899.

Pinkart HC, Devereux R and Chapman PJ. Rapid separation of microbial lipids using solid phase extraction columns. *Journal of microbiological methods* 1998; **34**: 9-15.

Precht D, Molkentin J, Destaillats F and Wolff RL. Comparative studies on individual isomeric 18 : 1 acids in cow, goat, and ewe milk fats by low-temperature high-resolution capillary gas-liquid chromatography. *Lipids* 2001; **36**: 827-832.

Renaville B, Bacciu N, Comin A, Motta M, Poli I, Vanini G and Prandi A. Plasma and Follicular Fluid Fatty Acid Profiles in Dairy Cows. *Reprod Domest Anim* 2010; **45**: 118-121.

Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M and Norman RJ. Obese Women Exhibit Differences in Ovarian Metabolites, Hormones, and Gene Expression Compared with Moderate-Weight Women. *J Clin Endocr Metab* 2009; **94**: 1533-1540.

Shaaker M, Rahimipour A, Nouri M, Khanaki K, Darabi M, Farzadi L, Shahnazi V and Mehdizadeh A. Fatty acid composition of human follicular fluid phospholipids and fertilization rate in assisted reproductive techniques. *Iran Biomed J* 2012; **16**: 162-168.

Sturmey RG, Reis A, Leese HJ and McEvoy TG. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod Domest Anim* 2009a; **44 Suppl 3**: 50-58.

Valckx SD, De Pauw I, De Neubourg D, Inion I, Berth M, Fransen E, Bols PE and Leroy JL. BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. *Hum Reprod* 2012; **27**: 3531-3539.

Van Hoeck V, Leroy JLMR, Arias-Alvarez M, Rizos D, Gutierrez-Adan A, Schnorbusch K, Bols PEJ, Leese HJ and Sturmey RG. Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: mechanistic insights. *Reproduction* 2013a; **145**: 33-44.

Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE and Leroy JL. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *Plos One* 2011; **6**: e23183.

Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, Eestermans W and Gerris J. Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod* 1999; **14**: 2345-2349.

Vanholder T, Leroy JL, Soom AV, Opsomer G, Maes D, Coryn M and de Kruif A. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci* 2005; **87**: 33-44.

Vanholder T, Leroy JL, Van Soom A, Maes D, Coryn A, Fiers T, de Kruif A and Opsomer G. Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci* 2006; **92**: 51-63.

WHO.Obesity and overweight. Fact sheet N° 311 (http://www.who.int/mediacentre/factsheets/fs311/en/index.html).

Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC and Sinclair KD. Dietary omega-3 and-6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. *Reproduction* 2010; **139**: 57-69.

Wu LLY, Dunning KR, Yang X, Russell DL, Lane M, Norman RJ and Robker RL. High-Fat Diet Causes Lipotoxicity Responses in Cumulus-Oocyte Complexes and Decreased Fertilization Rates. *Endocrinology* 2010; **151**: 5438-5445.

Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ and Robker RL. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. *Fertil Steril* 2012.

Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ and Robker RL. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. *Fertil Steril* 2012; **97**: 1438-1443.

Yi D, Zeng S and Guo Y. A diet rich in n-3 polyunsaturated fatty acids reduced prostaglandin biosynthesis, ovulation rate, and litter size in mice. *Theriogenology* 2012; **78**: 28-38.

Zachut M, Arieli A and Moallem U. Incorporation of dietary n-3 fatty acids into ovarian compartments in dairy cows and the effects on hormonal and behavioral patterns around estrus. *Reproduction* 2011; **141**: 833-840.

Zachut M, Dekel I, Lehrer H, Arieli A, Arav A, Livshitz L, Yakoby S and Moallem U. Effects of dietary fats differing in n-6:n-3 ratio fed to high-yielding dairy cows on fatty acid composition of ovarian compartments, follicular status, and oocyte quality. *Journal of Dairy Science* 2010; **93**: 529-545.

Zeron Y, Sklan D and Arav A. Effect of polyunsaturated fatty acid supplementation on biophysical parameters and chilling sensitivity of ewe oocytes. *Mol Reprod Dev* 2002; **61**: 271-278.

Zhang LY, Keung W, Samokhvalov V, Wang W and Lopaschuk GD. Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. *Bba-Mol Cell Biol L* 2010; **1801**: 1-22.

CHAPTER 6: ELEVATED NON-ESTERIFIED FATTY ACID CONCENTRATIONS DURING *IN VITRO* MURINE FOLLICLE GROWTH ALTER FOLLICULAR PHYSIOLOGY AND REDUCE OOCYTE DEVELOPMENTAL COMPETENCE

Published in Fertility and Sterility. Valckx SDM, Van Hoeck V, Arias-Alvarez M, Gutierrez-Adan A, Berth M, Cortvrindt R, Bols PEJ, Leroy JLMR (2014): Long-term exposure of murine secondary follicles to elevated NEFA concentrations impairs folliculogenesis, follicle quality and oocyte developmental competence *in vitro*. Fertility and Sterility. *In press (doi: 10.1016/j.fertnstert.2014.08.018).*

Abstract

Background: Lipolysis-linked elevated non-esterified fatty acid (NEFA) concentrations, typical in metabolic disorders like obesity and type II diabetes, have been shown to impair oocyte developmental competence and subsequent embryo quality and metabolism. However, the effect of long term elevated NEFA concentrations on the follicle as a whole, which is much more *in vivo* like, has never been studied. Therefore, we hypothesized that long term elevated NEFA concentrations affect murine follicular development, follicle quality and subsequent oocyte developmental competence *in vitro*.

Methods: Early secondary murine follicles were isolated out of the ovaries of 13-day old B6CBAF1 female mice and cultured individually. These follicles were exposed to elevated NEFA concentrations for 12 days. Treatments consisted of one or a mixture of NEFAs [stearic acid (SA), palmitic acid (PA), oleic acid (OA)] in physiological (BASAL) or pathological (HIGH SA/HIGH OA/HIGH NEFA) concentrations. Follicular development; follicle/oocyte diameters; progesterone, estradiol and inhibin B secretion in the medium and luteinized granulosa cell gene expression patterns were investigated. Furthermore, oocytes from NEFA exposed follicles, were fertilized *in vitro* and presumptive zygotes cultured until the blastocyst stage.

Results: Exposure to HIGH SA reduced follicle diameters and Day 12 antrum formation. Furthermore, elevated NEFA concentrations changed luteinized granulosa cell mRNA abundance of genes related to energy, fatty acid and steroid metabolism; apoptosis and oxidative stress. Also, HIGH NEFA and HIGH SA treatments increased progesterone synthesis, compared to HIGH OA follicles. Finally, oocyte developmental competence was substantially reduced in oocytes retrieved from HIGH OA, HIGH SA and HIGH NEFA exposed follicles, compared to BASAL treated follicles.

Conclusion: This study showed, for the first time, that lipolysis-linked, elevated NEFA concentrations can potentially impair fertility, by altering follicular physiology and reducing oocyte developmental competence.

Introduction

Metabolic disorders, such as obesity and type 2 diabetes, are characterized by an increased mobilization of body fats, which is associated with elevated serum non-esterified fatty acid (NEFA) concentrations (Boden and Shulman, 2002, Mooradian *et al.*, 2008). These lipolysis-linked elevated NEFA concentrations have been shown to be cytotoxic for several cell types, such as pancreatic β-cells and Leydig cells (Cnop *et al.*, 2001, Lu *et al.*, 2003), suggesting a potentially hazardous effect on somatic cell survival and function. High NEFA concentrations are also relevant in the pathogenesis of obesity and type 2 diabetes, by altering for example insulin signalling (Zhang *et al.*, 2010). Additionally, they have been proposed as an important link between maternal metabolic disorders and subfertility. In this regard, we previously showed that elevated serum NEFA concentrations were reflected in bovine (Leroy *et al.*, 2004) and human (Valckx *et al.*, 2012, Valckx *et al.*, 2014) follicular fluid from the pre-ovulatory follicle. Such elevated NEFA concentrations affect the proliferation and steroidogenesis of both granulosa and theca cells of the bovine ovarian follicle (Vanholder *et al.*, 2005, Vanholder *et al.*, 2006) and induce apoptosis in human granulosa cells (Mu *et al.*, 2001). In fact, elevated NEFA concentrations in human follicular fluid have been associated with inferior cumulus oocyte complex (COC) morphology (Jungheim *et al.*, 2011a). As others, we furthermore showed that elevated NEFA concentrations during the final maturation phase of bovine oocytes *in vitro* were detrimental for the oocyte's developmental competence and subsequent embryo quality (Jorritsma *et al.*, 2004, Leroy *et al.*, 2005, Aardema *et al.*, 2011, Van Hoeck *et al.*, 2011). Up until now, it has never been investigated if or to what extent folliculogenesis, follicle quality and the developmental competence of the enclosed oocyte are affected by a continuous exposure to elevated NEFA concentrations during follicle growth *in vitro*. Such a long term follicular NEFA exposure more closely resembles the *in vivo* situation in for example obese women, compared to the previously described short term exposure studies. Therefore, we hypothesized that, long term elevated NEFA concentrations alter follicular physiology ultimately leading to an impaired oocyte developmental competence, caused by either direct effects on the cumulus-oocyte complex (COC) or indirect effects through altered granulosa cell function, or both. Hence, the aim of this study was to investigate the effect of long term NEFA exposure on follicular growth, follicle quality and the developmental competence of the enclosed oocyte, by using a well-established murine follicle culture model (Cortvrindt *et al.*, 1996, Smitz and Cortvrindt, 2002).

Material and methods

a) Animals, follicle isolation and culture

Animals were housed and bred according to the national legislation and experiments were performed with ethical approval (Concession number LA12302070, EC-11-01). All products were purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise stated. For each experiment, 4 13-day old B6CBAF1 mice were sacrificed, after which the ovaries were dissected out and washed in L15 medium (Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (FBS, Greiner bio-one, Wemmel, Belgium) and 50IU/ml Penicillin G sodium salt. Follicles were mechanically isolated out of the ovaries and cultured individually in 75µl MEM (Life Technologies) supplemented with 5% FBS, 5µg/ml-5µg/ml-5ng/ml insulin-transferrin-selenium (ITS) and 0.75% bovine serum albumin (BSA) (MEM plus) in a 96-well plate. The same batch of FBS was used in every experiment (NEFA concentration of 110 µM, thus accounting for 5.5 µM in each medium sample originating from 5% FBS addition). Throughout the culture period, follicles were incubated at 37°C, 6% CO_2 and maximal humidity.

On Day 1, only early pre-antral follicles with a diameter between 100-130µm, with visible theca cells present over more than 1/3 of the circumference of the follicle and a good connection between the oocyte and the granulosa cells were selected for culture and transferred to 75µl MEM plus supplemented with 10mIU/ml follicle stimulating hormone (FSH, Merck-Serono, Overijse, Belgium) and 10mIU/ml luteinizing hormone (LH, Merck-Serono). On Days 4 and 8 of culture, 40% of the medium was renewed with fresh MEM plus supplemented with 10mIU/ml FSH. On Day 12 of culture, 40% of the medium was refreshed with MEM plus supplemented with 10mIU/ml FSH, 3.75IU/ml human Chorionic Gonadotrophin (hCG, Merck-Serono) and 25ng/ml epidermal growth factor (EGF) for ovulation induction and oocyte *in vitro* maturation. On Days 4, 8 and 12 of culture, follicular development was evaluated as previously described (2004), leading to a classification of follicles into follicular (F), follicular-diffuse (F/D), diffuse (D), diffuse-antral (D/A) and antral (A) stages. A follicle was considered to be of good quality (binary outcome measure) when it reached the F/D, D, D/A or A stage on Day 4; the D, D/A or A stage on Day 8 and the D/A or A stage on Day 12 of culture.

b) Follicle and oocyte diameters

Follicle diameters (µm) were assessed by measuring the distance between two sides of the follicle (excluding theca cells), straight through the centre of the oocyte with a ColorView camera (Olympus Soft Imaging Solutions GmbH, Germany) positioned on an inverted microscope in real time. Oocyte diameters included zona pellucida thickness.

c) Steroid and inhibin B concentrations in spent medium before and after the ovulatory stimulus

Culture medium samples were taken from all antral follicles (D/A and A) on Days 12 and 13 at the time of medium renewal and oocyte retrieval, respectively and pooled per plate. Day 12 medium samples were frozen directly at -80°C. Day 13 medium samples were frozen after pelleting of luteinized granulosa cells for gene expression analyses. Progesterone (P_4) and estradiol (E_2) measurements were conducted based on a competitive immunoassay format according to the manufacturer's instructions (Meso Scale Discovery, Rockville, Maryland, United States). Mean within and between assay variations were 3.9 and 13.8% for E_2 and 4.5 and 17.1% for P_4 measurements, respectively. Absolute P_4 rise (Day 13 P_4 – Day 12 P_4), E_2 reduction [(Day 13 E_2 – Day 12 E_2)*-1] and relative E_2 reduction (absolute E_2 reduction*100/Day 12 E_2) were calculated. Day 12 inhibin B concentrations were measured using the OBI MCA1312KZZ Inhibin B Enzyme Linked Immuno Sorbent Assay (DSL, United Kingdom), according to the manufacturer's instructions (mean within and between assay variations were 2.7 and 4.6%, respectively). Relative P_4 rise corrected for inhibin B concentrations, as a measure for granulosa cell number, was calculated by (Day 13 P_4*100/Day 12 P_4)/inhibin B concentrations.

d) Gene expression analyses

After the isolation of the COCs for fertilization, the remaining luteinized granulosa cells from those antral follicles were transferred to a sterile, RNase free 1.5ml microcentrifuge tube, by means of micropipette and a 200µl tip. A minimal contamination with theca cells from the monolayer formed below the follicle is to be assumed. Luteinized granulosa cells were pooled per plate, centrifuged (5min, 375g) and the pelleted granulosa cells snap frozen, by introduction of the eppendorf tube to liquid nitrogen. Samples were analysed by means of quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), as described by Van Hoeck *et al.* (2013b), with minor modifications. Results were normalized to the endogenous control histone *H2AFZ*. The choice of genes analysed in this study was based on the previously obtained knowledge of genes affected by short term NEFA exposure in bovine oocytes, cumulus cells and embryos (Van Hoeck *et al.*, 2013a). We furthermore opted to implement several genes specifically related to follicle quality. So, we implemented genes related to energy metabolism, protein metabolism, fatty acid metabolism, steroid metabolism, apoptosis, DNA damage and repair, oxidative stress and follicle quality. Details of the genes investigated, the primers used and the approximate size of the amplified fragments are presented in **Supplementary Table 1**.

e) Oocyte isolation, *in vitro* fertilization and embryo culture

For each replicate, two male B6CBAF1 mice of proven fertility were sacrificed, after which sperm was released from the epididymis in M16 medium supplemented with 3% BSA. After visual evaluation of

sperm motility and initial sperm count (Bürker counting chamber), the sperm suspension was diluted to a concentration of 10 000 motile spermatozoa/µl. Sperm capacitation continued for 2.5h in total.

On Day 13 of follicle culture, 20h after the Day 12 ovulatory stimulus, COCs from all antral (D/A and A) follicles were isolated with a small glass capillary and fertilized in groups of 10 in droplets of 30µl M16 medium with 3% BSA with mineral oil overlay by adding 10µl sperm suspension (10 000 motile spermatozoa/µl). After 3h of fertilization, presumptive zygotes were manually denuded with a small glass capillary in M16 medium with 300µg/ml hyaluronidase (422IU/ml), washed twice and cultured in groups of 10 in 75µl M16 medium in a 96-well plate. Plates were incubated at 37°C, 6% CO_2 and maximal humidity. Embryo cleavage was assessed on Day 14 (Day 1 p.i.), while embryo development was scored on Day 18 (Day 5 p.i.) of culture. Cleavage rate (n. 2-cell embryos/n. oocytes), blastocyst development (n. blastocysts/n. oocytes), blastocyst development from cleaved zygotes and hatching rate (n. hatching embryos/n. blastocysts) were calculated.

f) Experimental design

Follicles were exposed to one or a mixture of NEFAs in concentrations mimicking physiological and pathological serum NEFA concentrations, based on both bovine (Leroy *et al.*, 2005) and human (Valckx *et al.*, 2012, Valckx *et al.*, 2014) data. Three predominant NEFAs present in serum were used (Leroy *et al.*, 2005, Jungheim *et al.*, 2011a): stearic acid (SA), palmitic acid (PA) and oleic acid (OA) and dissolved in 100% ethanol to prepare the following NEFA stocks: 28mM/140mM/112mM SA, 21mM/105mM/210mM OA and 23mM/115mM/230mM PA for BASAL, MODERATE and HIGH NEFA treatment groups, respectively. These stock solutions were added in a volume of 25µl (112mM SA stock) or 10µl (other stock solutions) to 10ml MEM plus, to obtain the final concentrations described below. Media were placed in a sonication bath for 4h under continuous temperature control at 30-35°C, vortex mixed for 3 minutes and filtered (0.2µm). All medium samples were analysed for total NEFA concentrations using RX Daytona (Randox Laboratories). Preliminary experiments were set up to investigate the effect of 0.45% solvent (ethanol).

Experiment 1: Effect on follicular development. Selected follicles from 4 mice per replicate (4 replicates) were randomly assigned to experimental treatments from Day 1 until Day 13 of culture: 1) SOLVENT CONTROL (n = 153, 0.45% ethanol), 2) BASAL NEFA (n = 159, 28µM SA + 21µM OA + 23µM PA = 72µM NEFA), 3) MODERATE NEFA (n = 160, 140µM SA + 105µM OA + 115µM PA = 360µM NEFA) and 4) HIGH NEFA (n = 154, 280µM SA + 210µM OA + 230µM PA = 720µM NEFA). Follicular development was assessed as previously described on Days 4, 8 and 12 of culture.

Experiment 2: Effect on follicular development, steroidogenesis, granulosa cell gene expression patterns and oocyte developmental competence. Because of the importance of individual FAs and FA

ratios (Aardema *et al.*, 2011, Van Hoeck *et al.*, 2011), we opted to implement a HIGH SA and HIGH OA treatment group in experiment 2. Selected follicles from 4 mice per replicate (4 replicates) were randomly appointed to experimental treatments from Day 1 until Day 13 of culture: 1) BASAL NEFA (n = 154, see experiment 1), 2) HIGH SA (n = 160, 280µM SA), 3) HIGH OA (n = 159, 210µM OA) and 4) HIGH NEFA (n = 156, see experiment 1). Follicular development was assessed on Days 4, 8 and 12 of culture; follicle and oocyte diameters; P_4, E_2 and inhibin B concentrations in the culture medium and Day 13 luteinized granulosa cell gene expression patterns were investigated as previously described. Mature oocytes were fertilized and early embryo development was studied.

g) Statistical analyses

Following statistical analyses were performed with PASW statistics 18 (for Windows, Chicago, IL, USA). Differences in diameters (follicle/oocyte) and progesterone/estradiol/inhibin B concentrations between groups were investigated with a mixed model ANOVA with post hoc Sheffé tests, where treatment was entered as fixed factor and replicate as random factor. The interaction between replicate and treatment was first included in the model, only to be omitted from the final model when not significant. A binary logistic regression approach was used to study differences in follicular development (binary outcome: good or bad quality scoring on Days 4, 8 and 12 of culture as previously described), cleavage rate, blastocyst formation, blastocyst formation from cleaved zygotes and hatching rate. Similarly, the interaction between replicate and treatment was first included in the model, only to be omitted when not significant. Relative transcript abundance was analysed with SigmaStat software (Jandel Scientific, San Rafael, California), using a OneWay ANOVA with multiple pairwise comparisons and the Student-Newman-Keuls as post hoc method. Data are presented as means ± standard deviation or standard error of the means. Statistical significance was set at $P < 0.05$.

Results

a) Experiment 1

There was no significant difference in Day 4 follicular development. BASAL NEFA (77.4%) and MODERATE NEFA (76.9%) follicles showed less good quality scoring on Day 8 of culture, compared to SOLVENT CONTROL follicles (87.6%, $P = 0.02$). However, on Day 12 of culture, there was only a tendency for fewer follicles that reached the antral stage in HIGH NEFA (69.5%) follicles, compared to SOLVENT CONTROL follicles (79.1%, $P = 0.07$).

The preliminary experiments showed no significant difference between SOLVENT CONTROL (76.6%) and BASAL NEFA (73.5%) antrum formation, compared to our lab control without ethanol or NEFAs (79.7%). For this reason and to render the experimental design as biologically relevant as possible,

we implemented the BASAL NEFA treatment, which represents normal physiological NEFA concentrations, instead of the SOLVENT CONTROL treatment, as experimental control in experiment 2.

b) Experiment 2

Follicular development

Good quality scoring on Day 4 of culture was significantly lower for HIGH SA (85%) compared to HIGH NEFA follicles (93%, $P = 0.02$). On Day 8, follicular development was reduced in HIGH SA (81%) and HIGH OA (79%) follicles, compared to HIGH NEFA follicles (88%, $P = 0.05$ and $P = 0.03$, respectively). On Day 12 of culture, fewer HIGH SA follicles (68%) had reached the antral stage, compared to BASAL (79%) and HIGH NEFA follicles (83%, $P = 0.03$ and $P < 0.01$, respectively). Also, HIGH OA (72%) follicles displayed reduced antrum formation compared to HIGH NEFA follicles ($P = 0.04$). For all percentages of follicular development, the standard error of the proportion was 3-4%.

Follicle and oocyte diameters

Mean Day 4 oocyte diameters were lower in BASAL NEFA, compared to HIGH SA follicles (**Table 1**, $P = 0.035$). Furthermore, Day 8 follicle diameters were higher for HIGH OA follicles, compared to HIGH SA follicles (**Table 1**, $P < 0.01$). Day 12 follicle diameters were significantly lower for HIGH SA follicles, compared to BASAL NEFA, HIGH OA and HIGH NEFA follicles (**Table 1**, $P = 0.045$, $P = 0.010$ and $P = 0.027$, respectively), but oocyte diameters were similar among groups.

Table 1: Follicular and oocyte diameters (µm) on Days 4, 8 and 12 of culture for BASAL NEFA, HIGH SA, HIGH OA and HIGH NEFA treated follicles.

		BASAL NEFA	HIGH SA	HIGH OA	HIGH NEFA
Day 4	Follicle diameter (µm)	189 ± 68	194 ± 83	219 ± 93	210 ± 78
	Oocyte diameter (µm)	64 ± 6^{a}	66 ± 7^{b}	66 ± 7^{ab}	65 ± 6^{ab}
Day 8	Follicle diameter (µm)	541 ± 235^{ab}	472 ± 223^{a}	601 ± 262^{b}	549 ± 225^{ab}
	Oocyte diameter (µm)	71 ± 8	70 ± 7	74 ± 8	73 ± 9
Day 12	Follicle diameter (µm)	815 ± 241^{b}	726 ± 254^{a}	834 ± 274^{b}	820 ± 253^{b}
	Oocyte diameter (µm)	80 ± 8	79 ± 9	82 ± 9	80 ± 7

Data are presented as means ± standard deviation. ^{ab}Data with a different superscript in a row differ significantly between treatments ($P < 0.05$).

Steroidogenesis before and after the ovulatory stimulus

Day 12 and Day 13 E_2 concentrations, Day 12 P_4 concentrations and absolute E_2 reduction were not affected by treatment. Day 13 P_4 levels were higher in HIGH NEFA follicles, compared to HIGH SA follicles ($P = 0.002$, **Figure 1**). Also, P_4 rise was significantly higher in HIGH NEFA follicles, compared to both HIGH SA and HIGH OA follicles ($P = 0.012$ and $P = 0.017$, respectively, **Figure 1**). However, relative P_4 rise corrected for inhibin B concentrations was higher for HIGH SA and HIGH NEFA follicles,

compared to HIGH OA follicles (P = 0.045, P < 0.01, respectively). Furthermore, HIGH OA follicles had a higher relative E_2 reduction, compared to HIGH NEFA follicles (70.15 ± 8.91 vs. 62.33 ± 8.64%, P = 0.026).

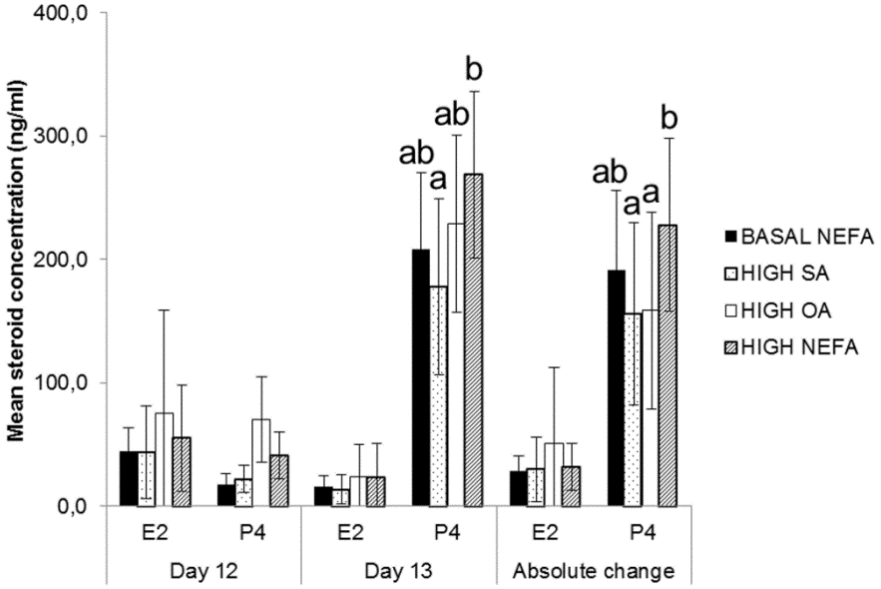


Figure 1: Follicle steroidogenesis. Estradiol (E2) and progesterone (P4) concentrations in the culture medium of Day 12 and Day 13 follicles cultured in BASAL NEFA, HIGH SA, HIGH OA and HIGH NEFA conditions. Absolute E_2 reduction [(E2 Day 13 – E2 Day 12)*-1] and P_4 rise (P4 Day 13 – P4 Day 12) between Day 12 and Day 13 are also shown. Data are presented as means ± standard deviation. [ab]Bars with a different superscript differ significantly between treatments (P < 0.05).

<u>*Day 13 granulosa cell gene expression*</u>

The mRNA transcript abundance (**Figure 2**) of *Tp53*, *Bax* and the ratio *Bax/Bcl2* was elevated in BASAL and HIGH NEFA, compared to HIGH OA luteinized granulosa cells. *Gadd45b* expression was higher in HIGH OA granulosa cells, compared to BASAL and HIGH NEFA. *Gapdh* expression was higher in BASAL and HIGH NEFA, compared to HIGH SA luteinized granulosa cells. *Slc2a1* expression was higher in HIGH NEFA, compared HIGH OA and HIGH SA granulosa cells. *Rgs2* expression was higher in HIGH NEFA, compared to HIGH OA and BASAL granulosa cells. *Acaca* was overexpressed in BASAL, compared to HIGH OA cells, while *Hsl* expression was higher in HIGH NEFA and HIGH OA, compared to BASAL luteinized granulosa cells. *Lhcgr* expression was higher in HIGH NEFA, compared to HIGH OA cells and *Cyp19a1* expression was elevated in HIGH SA, compared to HIGH NEFA granulosa cells. Furthermore, *Sod1* expression was higher in BASAL and HIGH NEFA, compared to HIGH OA. Expression of *Gpx1* and *Clu* was higher in HIGH SA, compared to BASAL granulosa cells.

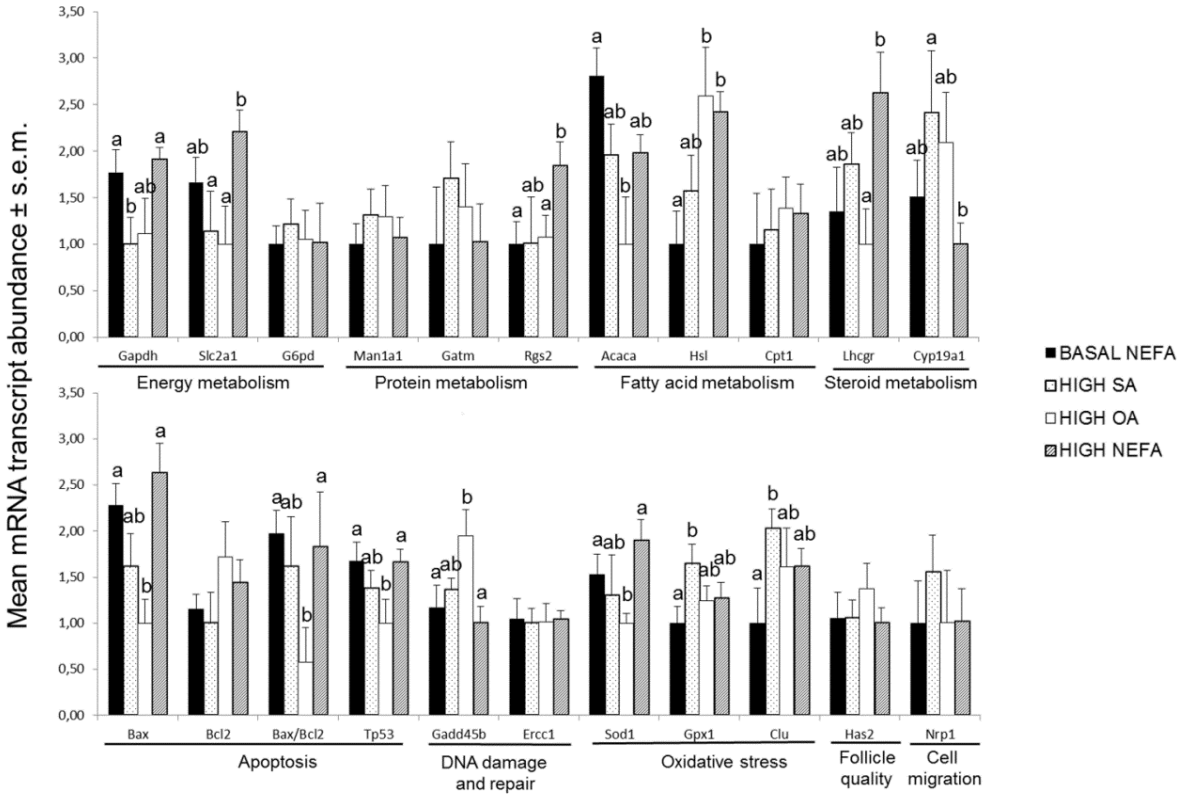


Figure 2: Granulosa cell gene expression patterns. Comparison of relative transcript abundance in murine Day 13 granulosa cells. Follicles were cultured for 12 days in BASAL NEFA, HIGH SA, HIGH OA or HIGH NEFA conditions (4 replicates) and granulosa cells were obtained on Day 13 of culture, 20h after a final maturation stimulus. [ab]Bars with different superscripts, differ significantly between treatments ($P < 0.05$). Data are presented as relative transcript abundance ± standard error of the means (s.e.m.).

Cleavage and embryo development

Data on cleavage and embryo development are presented in **Table 2**. Cleavage rate (Day 1 p.i.) was significantly reduced for HIGH NEFA oocytes, compared to BASAL NEFA oocytes ($P < 0.01$). Furthermore, blastocyst development was reduced for HIGH SA, HIGH OA and HIGH NEFA treatment, compared to BASAL NEFA ($P < 0.01$ for all 3 comparisons). Blastocyst formation from cleaved zygotes was reduced for HIGH SA embryos, compared to both BASAL and HIGH NEFA embryos ($P < 0.05$). Also, HIGH OA embryos had a reduced blastocyst formation from cleaved zygotes, compared to BASAL embryos. Hatching rate was reduced for HIGH OA embryos compared to BASAL NEFA, HIGH SA and HIGH NEFA embryos ($P < 0.05$).

Table 2: Details on cleavage rate (Day 1 p.i.) and embryo development (Day 5 p.i.) from oocytes originating from BASAL NEFA, HIGH SA, HIGH OA and HIGH NEFA treated follicles.

	BASAL NEFA n = 118	**HIGH SA n = 88**	**HIGH OA n = 97**	**HIGH NEFA n = 124**
Cleavage rate (%)	69.5[a]	59.1[ab]	56.7[ab]	52.8[b]
Blastocyst rate (%)	59.3[a]	30.7[b]	36.1[b]	42.3[b]
Blastocyst rate from cleaved zygotes (%)	85.4[a]	51.9[b]	63.6[bc]	80.0[ac]
Hatching rate from total n. of blastocyst (%)	40.0[a]	48.1[a]	14.3[b]	44.2[a]

[abc]Data with a different superscript in a row differ significantly between treatments ($P < 0.05$).

Discussion

Elevated NEFA concentrations, typical in metabolic disorders such as obesity and type II diabetes (Zhang *et al.*, 2010), have been proposed as a key factor linking maternal metabolic disorders and disappointing fertility results. In this study, we investigated how the biological unit of the growing follicle and the resulting oocyte quality is affected by a long term exposure to elevated NEFA concentrations. We showed that long term elevated NEFA concentrations only moderately affect follicular growth and antrum formation, with the most pronounced effect induced by the HIGH SA treatment. Elevated NEFA concentrations altered gene expression patterns in Day 13 luteinized granulosa cells, revealing that NEFA exposure mainly affected pathways involved in apoptosis, lipid metabolism, oxidative stress and steroidogenesis, which was also evidenced by P_4, E_2 and inhibin B analyses in spent medium. Most importantly, the oocytes originating from the NEFA exposed follicles displayed a significantly reduced developmental competence (blastocyst formation).

Follicular development and quality. Our results showed that the HIGH SA treatment caused the most pronounced effect on follicular development, by reducing Day 12 antrum formation and follicular diameters. Interestingly, the same SA concentration, but in a mixture of NEFAs (HIGH NEFA treatment) did not affect follicular growth. This is in agreement with the observation that mono- and poly-unsaturated FAs can prevent the pro-apoptotic effect of PA in pancreatic β-cells (Cnop *et al.*, 2001, Eitel *et al.*, 2002). Oleic acid also prevented the negative effect of PA and SA during *in vitro* bovine oocyte maturation on oocyte developmental competence (Aardema *et al.*, 2011), possibly through an increased shuttling of PA and SA into neutral lipid droplets. Treatment with HIGH OA also seemed to reduce apoptosis in Day 13 luteinized granulosa cells, as indicated by *Tp53* and *Bax* mRNA transcript abundance and the ratio *Bax/Bcl2*, which is ultimately responsible for the release of cytochrome c from the mitochondrion (Van Cruchten and Van den Broeck, 2002). The observation that *Gadd45b* expression was upregulated in HIGH OA granulosa cells substantiates this, since it is a cell cycle checkpoint regulator that is proposed to be involved in the prevention of apoptosis or DNA damage in granulosa cells of bovine dominant follicles (Mihm *et al.*, 2008).

Follicle metabolism. It is suggested that treatment with HIGH SA may reduce glucose uptake and consumption (glycolysis) by mural luteinized granulosa cells (decreased expression levels of *Slc2a1* and *Gapdh*), compared to BASAL and/or NEFA treated follicles. However, these data need to be functionally analysed. Furthermore, the increased mRNA abundance of *Sod1* in HIGH NEFA and *Gpx1* in HIGH SA exposed follicles, compared to HIGH OA and BASAL follicles, respectively, suggest increased exposure to oxidative stress. Elevated NEFA concentrations may indeed alter glucose

metabolism by inducing insulin resistance and increase fatty acid β-oxidation, leading to increased ROS production (Zhang *et al.*, 2010).

Follicle steroidogenic profile. Progesterone plays a key role in the acquisition of oocyte developmental competence (Fair and Lonergan, 2012). The P_4 data, corrected for the number of granulosa cells by taking inhibin B concentrations into account (Smitz and Cortvrindt, 1998, Lenie and Smitz, 2009), suggest that HIGH SA and HIGH NEFA follicles reacted stronger to the Day 12 ovulatory stimulus, compared to HIGH OA follicles. It was previously suggested that apoptotic bovine granulosa cells can maintain steroidogenesis as long as the steroidogenic organelles remain intact (Amsterdam *et al.*, 1997, Vanholder *et al.*, 2005) and that these organelles cluster during the process of apoptosis (Kerental *et al.*, 1995, Vanholder *et al.*, 2005), which could create the image of a higher steroidogenic capacity, while it in fact is a consequence of apoptosis. Furthermore, HIGH OA follicles showed a high Day 12 P_4 concentration, which could be indicative of pre-mature luteinization (rise in P_4 levels on the day of hCG administration, Elnashar, 2010), that could potentially lower the sensitivity of the granulosa cells to the subsequent ovulatory stimulus. This is substantiated by the decreased *Lhcgr* mRNA transcript abundance in HIGH OA follicles compared to HIGH NEFA follicles. To our knowledge, we show for the first time that altered NEFA conditions may affect the follicular steroid synthesis and the responsiveness to an ovulatory stimulus when studied in a whole follicle culture model. This may explain the higher incidence of cycle and ovulatory disorders, often seen in women suffering from metabolic disorders (Pasquali *et al.*, 2007).

Oocyte developmental competence. Elevated NEFAs during the final maturation phase of bovine oocytes have been shown to increase oocyte NEFA uptake and metabolism (Aardema *et al.*, 2011), which has been associated with hampered oocyte maturation, increased oxidative stress and impaired developmental competence of the oocyte as well as an altered physiology and metabolism of the resultant embryo (Leroy *et al.*, 2005, Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a, Van Hoeck *et al.*, 2013b). In agreement, we showed that oocyte developmental competence was substantially reduced in oocytes originating from NEFA exposed follicles. Interestingly, hatching rate was reduced for HIGH OA embryos. Alterations in the composition of the zona pellucida and altered embryonic ability to crack the zona pellucida, have been proposed as mechanisms that could explain differences in hatching rate between *in vivo* and *in vitro* derived embryos at different diameters (Holm *et al.*, 2002, Van Soom *et al.*, 2003). Additionally, numerous factors, like proteases, growth factors and transcription factors, may affect the process of blastocyst hatching (Seshagiri *et al.*, 2009). Interestingly, we showed that oxidative stress was reduced in HIGH OA luteinized granulosa cells (reduced *Sod1* expression, compared to BASAL follicles), which may have influenced the oxidative status of the resultant embryo. Since it has been shown that an oxyradical burst causes

hatching in mouse blastocysts (Thomas *et al.*, 1997), this might explain the reduced hatching rate in HIGH OA embryos. However, research investigating the exact mechanism by which elevated OA concentrations during follicular development, affect hatching rate, is mandatory to strengthen this hypothesis.

Because of the pathophysiological and biological relevance of exposing follicles as a functional unit, for a longer period of time, we particularly chose to use the murine follicle culture model, that has been proven suitable for *in vitro* testing of ovarian function (Cortvrindt and Smitz, 2002), and supports follicular growth, steroid production, antrum formation and the acquisition of oocyte developmental competence, much like the *in vivo* situation (Cortvrindt *et al.*, 1996, Smitz and Cortvrindt, 2002). But, even though we were able to implement a NEFA exposure for a longer period of time, the culture model used presents the limitation that it still does not cover the whole period of folliculo- and oogenesis. Future research should continue to focus on optimizing *in vitro* culture systems that allow the growth of follicles from the primordial stage up until the antral stage, with the development of a competent oocyte *in vitro*, in order to study how for example elevated NEFA concentrations may affect follicular growth from the primordial stage on.

In conclusion, we studied the effect of long term NEFA exposure on folliculogenesis, follicle quality, oocyte growth and subsequent oocyte developmental competence. Our data show for the first time that such a long term exposure to elevated NEFA concentrations, only moderately affects follicular growth and antrum formation, but substantially alters pre-ovulatory follicular steroid synthesis, the responsiveness to the ovulatory stimulus and luteinized granulosa cell gene expression patterns. This ultimately resulted in a severely impaired oocyte developmental competence. From a clinical point of view, our data help to understand the pathways by which lipolysis-linked elevated NEFA concentrations may contribute to subfertility in metabolically compromised women, by altering follicular physiology and oocyte developmental competence. Ongoing research focusses on the physiology and metabolism of the resultant and surviving pre-implantation embryos and the potential consequences for post natal health.

References

Aardema H, Vos PLAM, Lolicato F, Roelen BAJ, Knijn HM, Vaandrager AB, Helms JB and Gadella BM. Oleic Acid Prevents Detrimental Effects of Saturated Fatty Acids on Bovine Oocyte Developmental Competence. *Biol Reprod* 2011; **85**: 62-69.

Adriaens I, Cortvrindt R and Smitz J. Differential FSH exposure in preantral follicle culture has marked effects on folliculogenesis and oocyte developmental competence. *Hum Reprod* 2004; **19**: 398-408.

Amsterdam A, Dantes A, Selvaraj N and Aharoni D. Apoptosis in steroidogenic cells: Structure-function analysis. *Steroids* 1997; **62**: 207-211.

Boden G and Shulman GI. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. *European Journal of Clinical Investigation* 2002; **32**: 14-23.

Cnop M, Hannaert JC, Hoorens A, Eizirik DL and Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 2001; **50**: 1771-1777.

Cortvrindt R, Smitz J and Van Steirteghem AC. In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system. *Hum Reprod* 1996; **11**: 2656-2666.

Cortvrindt RG and Smitz JEJ. Follicle culture in reproductive toxicology: a tool for in-vitro testing of ovarian function? *Hum Reprod Update* 2002; **8**: 243-254.

Eitel K, Staiger H, Brendel MD, Brandhorst D, Bretzel RG, Haring HU and Kellerer M. Different role of saturated and unsaturated fatty acids in beta-cell apoptosis. *Biochem Bioph Res Co* 2002; **299**: 853-856.

Elnashar AM. Progesterone rise on the day of HCG administration (premature luteinization) in IVF: An overdue update. *Journal of Assisted Reproduction and Genetics* 2010; **27**: 149-155.

Fair T and Lonergan P. The Role of Progesterone in Oocyte Acquisition of Developmental Competence. *Reprod Domest Anim* 2012; **47**: 142-147.

Holm P, Booth PJ and Callesen H. Kinetics of early in vitro development of bovine in vivo- and in vitro-derived zygotes produced and/or cultured in chemically defined or serum-containing media. *Reproduction 2002; **123**: 553-565.*

Jorritsma R, Cesar ML, Hermans JT, Kruitwagen CLJJ, Vos PLAM and Kruip TAM. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. *Anim Reprod Sci* 2004; **81**: 225-235.

Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS and Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. *Fertil Steril* 2011a; **95**: 1970-1974.

Kerental I, Suh BS, Dantes A, Lindner S, Oren M and Amsterdam A. Involvement of P53 Expression in Camp-Mediated Apoptosis in Immortalized Granulosa-Cells. *Experimental cell research* 1995; **218**: 283-295.

Lenie S and Smitz J. Steroidogenesis-disrupting compounds can be effectively studied for major fertility-related endpoints using in vitro cultured mouse follicles. *Toxicology Letters* 2009; **185**: 143-152.

Leroy JLMR, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PEJ, Dewulf J and de Kruif A. Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. *Theriogenology* 2004; **62**: 1131-1143.

Leroy JLMR, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G and Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 2005; **130**: 485-495.

Lu ZH, Mu YM, Wang BA, Li XL, Lu JM, Li JY, Pan CY, Yanase T and Nawata H. Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. *Biochem Bioph Res Co* 2003; **303**: 1002-1007.

Mihm M, Baker PJ, Fleming LM, Monteiro AM and O'Shaughnessy PJ. Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. *Reproduction* 2008; **135**: 253-265.

Mooradian AD, Haas MJ, Wehmeier KR and Wong NCW. Obesity-related changes in high-density lipoprotein metabolism. *Obesity* 2008; **16**: 1152-1160.

Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K et al. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. *Endocrinology* 2001; **142**: 3590-3597.

Pasquali R, Patton L and Gambineri A. Obesity and infertility. *Curr Opin Endocrinol Diabetes Obes* 2007; **14**: 482-487.

Seshagiri PB, Sen Roy S, Sireesha G and Rao RP. Cellular and molecular regulation of mammalian blastocyst hatching. *J Reprod Immunol* 2009; **83**: 79-84.

Smitz J and Cortvrindt R. Inhibin A and B secretion in mouse preantral follicle culture. *Hum Reprod* 1998; **13**: 927-935.

Smitz JEJ and Cortvrindt RG. The earliest stages of folliculogenesis in vitro. Reproduction 2002; 123: 185-202.

Thomas M, Jain S, Kumar GP and Laloraya M. A programmed oxyradical burst causes hatching of mouse blastocysts. *J Cell Sci* 1997; **110** (Pt 14): 1597-1602.

Valckx SD, Arias-Alvarez M, De Pauw I, Fievez V, Vlaeminck B, Fransen E, Bols PE and Leroy JL. Fatty acid composition of the follicular fluid of normal weight, overweight and obese women undergoing assisted reproductive treatment: a descriptive cross-sectional study. *Reprod Biol Endocrinol* 2014; **12**: 13.

Valckx SD, De Pauw I, De Neubourg D, Inion I, Berth M, Fransen E, Bols PE and Leroy JL. BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. *Hum Reprod* 2012; **27**: 3531-3539.

Van Cruchten S and Van den Broeck W. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anatomia Histologia Embryologia* 2002; **31**: 214-223.

Van Hoeck V, Leroy JLMR, Arias-Alvarez M, Rizos D, Gutierrez-Adan A, Schnorbusch K, Bols PEJ, Leese HJ and Sturmey RG. Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: mechanistic insights. *Reproduction* 2013a; **145**: 33-44.

Van Hoeck V, Rizos D, Gutierrez-Adan A, Pintelon I, Jorssen E, Dufort I, Sirard MA, Verlaet A, Hermans N, Bols PEJ, Leroy JLMR. Interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations. *Reprod Fertil Dev* 2013b; **23**: Epub ahead of print

Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PEJ and Leroy JLMR. Elevated Non-Esterified Fatty Acid Concentrations during Bovine Oocyte Maturation Compromise Early Embryo Physiology. *Plos One* 2011; **6**.

Van Soom A, Mateusen B, Leroy J and De Kruif A. Assessment of mammalian embryo quality: what can we learn from embryo morphology? *Reprod Biomed Online* 2003; **7**: 664-670.

Vanholder T, Leroy JL, Soom AV, Opsomer G, Maes D, Coryn M and de Kruif A. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci* 2005; **87**: 33-44.

Vanholder T, Leroy JL, Van Soom A, Maes D, Coryn A, Fiers T, de Kruif A and Opsomer G. Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci* 2006; **92**: 51-63.

Zhang LY, Keung W, Samokhvalov V, Wang W and Lopaschuk GD. Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. *Bba-Mol Cell Biol L* 2010; **1801**: 1-22.

Supplementary Table 1: Details of primers used for qRT-PCR analyses.

Gene	Primer sequence (forward/reverse primer)	Fragment size (bp)	GenBank accession no.
Gapdh	ACCCAGAAGACTGTGGATGG/AYGCCTGCTTCACCACCTTC	247	BC102589
Slc2a1	CTGATCCTGGGTCGCTTCAT/ACGTACATGGGCACAAAACCA	68	NM_174602.2
G6pd	CGCTGGGACGGGGTGCCCTTC/ATCCGCCAGGCCTCCCGCAGTTCATCA	347	XM_583628.4
Acaca	AAACCAGCACTCCCGATTCAT/ GGCCAAACCATCCTGTAAGC	175	FN185963.1
Hsl	GGGAGCTCCAGTCGGAAGA/ GCTGTGTGCACCAAACTACG	287	NM_001039507.2
Cpt1a	CTGCCCGCCTGGGAAATGCTGTCAGTCTCTCCTCCCCGGGCTGG	328	NM_001034349.2
Lhcgr	GCCAACCCATTTCTGTACGC/CAGCCTGGGAAGGCTTACTT	178	NM_013582.2
Cyp19a1	TCCACACTGTTGTGGGTGAC/AGGGAAGTACTCGAGCCTGT	218	NM_007810.3
Bax	CTACTTTGCCAGCAAACTGGTCCCAAAGTAGGAGAGGA	158	NM_173894.1
Bcl2	GTCCCGCCTCTTCACCTTTCAG/GATTCTGGTGTTTCCCCGTTGG	147	NM_009741.4
Tp53	CTCAGTCCTCTGCCATACTAGGATCCAGGATAAGGTGAGC	364	NM_174201.2
Gadd45b	CTTCTGGTCGCACGGGAAGG/GCTCCACCGCGGCAGTCACC	277	AF441860.1
Ercc1	GTGCTGCTGGTTCAAGTGGA/GCAGTCAGCCAAGATGCACAT	80	NM_001127324.1
Sod1	GTGCAAGGCACCATCCACTTCG/ CACCATCGTGCGGCCAATGATG	309	NM_174615
Gpx1	GCAACCAGTTTGGGCATCACTCGCACTTTTCGAAGAGCATA	116	NM_174076.3
Has2	TCAGCGAAGTTATGGGCAGG/GATGAGGCAGGGTCAAGCAT	265	NM_008216.3
Man1a1	GTCACTGCAGAGTGAACGGA/AAGGATGAGCCTCGGTGTTG	183	NM_008548.4
Gatm	GTGGGCAGAGCTGAAAATGC/GGCACCACGATGGAAGTAGT	387	NM_025961.5
Rsg2	TCCTGTCACTTACCAACCGC/CACTGCGGAGAGGAACCATT	216	NM_009061.4
Clu	AGCCGTGCGGAATGAGATAG/TTCTTCCCGAGAGCAGCAAG	196	NM_013492.2
Nrp1	AGCATCCAATCAAGCCGACA/CCGAAGCTCAGGTGTGTCAT	313	NM_008737.2

CHAPTER 7: LONG TERM EXPOSURE OF MURINE EARLY PRE-ANTRAL FOLLICLES TO ELEVATED NEFA CONCENTRATIONS ALTERS THE QUALITY AND METABOLISM OF THE RESULTANT EMBRYOS

In preparation. Valckx SDM, McKeegan P, Simintiras C, Pintelon I, De Rijck D, Cortvrindt R, Bols PEJ, Leroy JLMR.

***Author contributions**: Valckx SDM and Leroy JLMR designed the experiments and wrote the manuscript, Valckx SDM performed all follicle and embryo cultures, visited the Centre for Cardiovascular Research in Hull to perform the medium analyses (HPLC) under the guidance of Simintiras C, performed the Nile Red staining and subsequent data analyses under guidance of Pintelon I and De Rijck D. McKeegan P performed the glucose analyses. Cortvrindt R and Bols PEJ critically revised the work.*

Abstract

Background: Lipolysis-linked elevated non-esterified fatty acid (NEFA) concentrations, typical in metabolic disorders like obesity and type II diabetes, have been shown to impair bovine oocyte developmental competence and subsequent embryo quality and metabolism after a short term NEFA exposure during final oocyte maturation. Furthermore, long term elevated NEFA concentrations modestly affect murine *in vitro* follicular growth to the antral stage, but they substantially alter follicular physiology, ultimately resulting in a decreased oocyte developmental competence. However, the effect of such a long term NEFA exposure during murine follicular growth on subsequent embryo metabolism, has never been studied. Furthermore, it is not known whether long term and short term elevated NEFA concentrations elicit a similar effect on oocyte developmental competence. Therefore, we hypothesized that murine oocyte developmental competence is more severely impaired by a long term NEFA exposure, compared to a short term NEFA exposure, only during final oocyte maturation. Furthermore, we hypothesized that long term elevated NEFA concentrations affect the resultant embryo's metabolism and thus quality.

Methods: Early secondary murine follicles were isolated out of the ovaries of 13-day old B6CBAF1 female mice and cultured individually. These follicles were exposed to elevated NEFA concentrations for 12 days or only during the final phase of oocyte maturation (Day 12-13). Treatments consisted of one or a mixture of NEFAs [stearic acid (SA), palmitic acid (PA), oleic acid (OA)] in physiological (BASAL) or pathological (HIGH SA/HIGH OA/HIGH NEFA) concentrations. Subsequent murine blastocyst formation, morula glucose consumption, amino acid turnover and total lipid content were studied.

Results: Embryos resulting from HIGH SA exposed follicles consumed little or no glucose. Furthermore, HIGH NEFA embryos tended to have a higher overall amino acid production. We could not find a significant difference in intracellular lipid content. Long term elevated NEFA concentrations more severely impaired oocyte developmental competence, compared to short term NEFA exposure.
Conclusion: This study showed that lipolysis-linked elevated NEFA concentrations during follicular growth can result in an altered embryo metabolism. Furthermore, we showed for the first time that long term elevated NEFA concentrations during follicular growth impair oocyte developmental competence more than short term elevated NEFA concentrations during final oocyte maturation.

Introduction

Elevated non-esterified fatty acid (NEFA) concentrations are a typical characteristic of metabolic disorders, such as obesity and type II diabetes, due to an upregulated lipolysis (Boden and Shulman, 2002, Mooradian *et al.*, 2008). Besides cytotoxic effects on several cell types *in vitro* (Cnop *et al.*, 2001, Lu *et al.*, 2003), elevated NEFA concentrations are involved in the pathogenesis of multiple metabolic disorders, for example by altering insulin signalling (Zhang *et al.*, 2010). These elevated NEFA concentrations have furthermore been proposed as a major link between maternal metabolic disorders and subfertility. This is substantiated by the observation that elevated serum NEFA concentrations are reflected in the follicular fluid (Leroy *et al.*, 2004, Valckx *et al.*, 2012). Interestingly, in women this was associated with inferior cumulus-oocyte complex (COC) morphology (Jungheim *et al.*, 2011a) and exposure of murine oocytes to triglyceride and NEFA rich human follicular fluid, negatively affected oocyte maturation (Yang *et al.*, 2012). Short term elevated NEFA concentrations, during the last 24h of bovine oocyte maturation, do alter oocyte developmental competence and may hamper subsequent embryo quality and metabolism (Jorritsma *et al.*, 2004, Leroy *et al.*, 2005, Aardema *et al.*, 2011, Van Hoeck *et al.*, 2011). The metabolism of these *in vitro* Day 7 bovine embryos may thus be pre-programmed by changes in the oocyte's micro-environment during final maturation. Several potential mechanisms of action were discussed, such as an increased rate of β-oxidation with a subsequent increased production of reactive oxygen species (ROS), ceramide formation leading to the induction of apoptosis related caspases and lipid biosynthesis in triglyceride, cholesteryl ester and phospholipid fat fractions (Van Hoeck *et al.*, 2013b, Van Hoeck *et al.*, submitted). An increased metabolic activity of the early pre-implantation embryo is possibly related to an inferior embryo quality, as the quiet embryo hypothesis states that pre-implantation embryo survival is best served by a relatively low level of metabolism (Leese, 2002). This also means that the metabolic profile of the embryo, as determined by for example carbohydrate and amino acid

turnover (Sturmey *et al.*, 2008a, Sturmey *et al.*, 2010, Gardner *et al.*, 2011), is a valuable tool to study embryo quality.

Because routine bovine models are currently limited to *in vitro* oocyte maturation and embryo culture, without preceding follicle culture, the previously described studies only investigated the effect of elevated NEFA concentrations during the restricted time period of final oocyte maturation (24h). Therefore, we recently used a murine follicle culture system and showed that long term elevated NEFA concentrations only moderately affect follicular growth, but we revealed important changes in murine follicular physiology, such as an altered steroid synthesis and changes in expression patterns of developmentally and metabolically important genes, that ultimately resulted in a drastically impaired oocyte developmental competence (Valckx *et al.*, 2014). These observations are particularly relevant in light of the recent of findings of Carvalho *et al.* (2014), who found no effect of body weight change during the first three weeks after calving on the number of ovulations, total number of embryos collected and percentage of oocytes that were fertilized (after a standard superstimulation treatment 10 weeks later). However, more loss of body condition post-partum was related to a reduced potential of fertilized oocytes to develop to transferable embryos and a higher proportion of degenerated embryos. So prolonged exposure of the growing follicle to the negative energy balance in the cow *in vivo* resulted in a practically normal follicular growth, but a reduced oocyte developmental competence. In agreement, our *in vitro* murine data showed that follicular development was not severely affected by continuous elevated NEFA concentrations, but oocyte developmental competence was impaired (Valckx *et al.*, 2014). However, the potential effect on subsequent embryo physiology and quality was not investigated so far. Therefore, in this study, we hypothesized that murine embryos originating from oocytes that were isolated out of long term NEFA exposed follicles, are metabolically impaired, resulting in reduced embryonic development and reduced embryo quality. Furthermore, it is not known what time period during follicular development is the most sensitive to a metabolic insult (critical window of susceptibility). It has for example been shown that final oocyte maturation is susceptible to elevated NEFA concentrations (Leroy *et al.*, 2005, Van Hoeck *et al.*, 2011), but how vulnerable are the steps preceding final oocyte maturation to a metabolic insult? Follicular differentiation, oocyte cytoplasmic maturation as well as the differentiation and function of granulosa and cumulus cells, are all established well before final oocyte maturation (Fair, 2003, Collado-Fernandez *et al.*, 2012) and could be sensitive to insult. Therefore, we also hypothesized that long term elevated NEFA concentrations throughout murine follicular growth *in vitro* more severely impair oocyte developmental competence, compared to a NEFA exposure only during the final phase of maturation. The specific aims of this study were (1) to determine the metabolic profile and intracellular fat content of murine embryos originating from

long term NEFA exposed follicles and (2) to investigate the difference between short and long term follicular NEFA exposure on murine oocyte developmental competence.

Material and methods

1. Follicle and embryo culture

Animals were housed and bred according to the national legislation and experiments were performed with ethical approval (Concession number LA12302070, EC-11-01). All products were purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise stated. For each experiment, 4 13-day old B6CBAF1 mice were sacrificed, after which the ovaries were dissected out and washed in L15 medium (Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (FBS, Greiner bio-one, Wemmel, Belgium) and 50IU/ml Penicillin G sodium salt. Follicles were mechanically isolated out of the ovaries and cultured individually in 75µl MEM (Life Technologies) supplemented with 5% FBS, 5µg/ml-5µg/ml-5ng/ml insulin-transferrin-selenium (ITS) and 0.75% bovine serum albumin (BSA) (MEM plus) in a 96-well plate. The same batch of FBS was used in every experiment (NEFA concentration of 110 µM, thus accounting for 5.5 µM in each medium sample originating from 5% FBS addition). Throughout the culture period, follicles were incubated at 37°C, 6% CO_2 and maximal humidity.

On Day 1, only early pre-antral follicles with a diameter between 100-130µm, with visible theca cells present over more than 1/3 of the circumference of the follicle and a good connection between the oocyte and the granulosa cells were selected for culture and transferred to 75µl MEM plus supplemented with 10mIU/ml follicle stimulating hormone (FSH, Merck-Serono, Overijse, Belgium) and 10mIU/ml luteinizing hormone (LH, Merck-Serono). On Days 4 and 8 of culture, 40% of the medium was renewed with fresh MEM plus supplemented with 10mIU/ml FSH. On Day 12 of culture, 40% of the medium was refreshed with MEM plus supplemented with 10mIU/ml FSH, 3.75IU/ml human Chorionic Gonadotrophin (hCG, Merck-Serono) and 25ng/ml epidermal growth factor (EGF) for ovulation induction and oocyte *in vitro* maturation.

On Day 13 of culture, two male B6CBAF1 mice of proven fertility were sacrificed by means of cervical dislocation, after which the epididymi and vas deferens were isolated in M2 medium supplemented with 50 U/ml Penicillin-50µg/ml Streptomycin (Life Technologies, Merelbeke, Belgium). The caudae epididymi and the first part of the vas deferens were isolated and the sperm was allowed to swim out for 15 minutes, after making diagonal slices on the tissue surface in M16 medium supplemented with 3% BSA. After visual evaluation of sperm motility and initial sperm count (Bürker counting

chamber), the sperm suspension was diluted to a total concentration of 10 000 motile spermatozoa/µl in M16 medium with 3% BSA. Sperm capacitation continued for 2.5h in total.

Exactly 20h after the Day 12 ovulatory stimulus, COCs from all antral follicles were isolated, fertilized and presumptive zygotes were cultured routinely, as previously described (Valckx *et al.*, 2014) with following modification: embryos were cultured at 37°C, 6% CO_2 and 5% O_2. Embryo cleavage was assessed on Day 14 (Day 1 p.i.), while embryo development was scored on Day 15, 16 and/or 18 of culture, depending on the experiment.

2. Metabolic profiling and intracellular fat content

NEFA exposure

Follicles were exposed to one or a mixture of NEFAs in concentrations mimicking physiological and pathological serum NEFA concentrations, based on both bovine (Leroy *et al.*, 2005) and human (Valckx *et al.*, 2012, Valckx *et al.*, 2014) data. Three predominant NEFAs present in serum were used (Leroy *et al.*, 2005): stearic acid (SA), palmitic acid (PA) and oleic acid (OA) and dissolved in 100% ethanol to prepare the following NEFA stocks: 28mM/112mM SA, 21mM/210mM OA and 23mM/230mM PA for BASAL, HIGH SA and HIGH NEFA treatment groups. These stock solutions were added in a volume of 25µl (112mM SA stock) or 10µl (other stock solutions) to 10ml MEM plus, to obtain the final concentrations described below. Media were placed in a sonication bath for 4h under continuous temperature control at 30-35°C, vortex mixed for 3 minutes and filtered (0.2µm). On Day 12 of culture, hCG and EGF were added after filtration. Media were allowed to equilibrate for at least 2h at 37°C, 5% CO_2 and maximal humidity, before use. All medium samples were analysed for total NEFA concentrations using RX Daytona (Randox Laboratories). Preliminary experiments showed no effect of adding 0.45% of the solvent ethanol to the culture medium on follicular dynamics (Valckx *et al.*, 2014).

Follicles were exposed to elevated NEFA concentrations from Day 1 until Day 13 of culture. Following treatments were implemented:

- **BASAL NEFA** (72µM NEFA mix = 23µM PA, 28µM SA and 21µM OA),
- **HIGH SA** (280µM SA) and
- **HIGH NEFA** (720µM NEFA mix = 230µM PA, 280µM SA and 210µM OA).

Embryonic glucose and amino acid turnover (4 replicates)

In each replicate, 40 follicles were cultured in the BASAL treatment group and 60 follicles in both the HIGH SA and HIGH NEFA groups, to ensure equal groups (number of 4- to 8-cell stage embryos) for the metabolic assay. These numbers are based on our previous experience (Valckx *et al.*, 2014),

concerning the percentage of antral follicles, fertilizable oocytes and subsequent embryos in each treatment group. On Day 2 p.i., 4- to 8-cell staged embryos were selected. These embryos were transferred in groups of 10 to 5µl droplets of home-made assay medium under oil. The home-made assay medium was prepared by adding all the compounds of commercially available M16 medium from Sigma, but with a lower concentration of 1mM glucose (Fiers, Kuurne, Belgium), to B60 water (Braun, Diegem, Belgium). Furthermore, a physiological amino acid mix (data from vascularly perfused human oviducts, Tay *et al.*, 1997) was added to the medium. Embryos were cultured in droplets of this medium for 24h, alongside empty medium control droplets, at 37°C, 6% CO_2 and 5% O_2. The exact time of incubation and embryo developmental stage at the beginning and the end of culture were recorded. The embryos were fixed in 4% paraformaldehyde for 24h and then stored in PBS at 4°C until further analyses (Nile Red staining, see next section). The petri-dishes with droplets under oil were sealed with Tesla tape and frozen at -80°C, until sample analyses. After thawing, 2µl of the micro-droplets was recuperated for the analysis of glucose in the spent medium. Glucose concentrations were determined by an ultrafluorometric assay as previously described (Leese and Barton, 1984, Gardner and Leese, 1986) by using a BMG Fluostar spectrophotometer. The remaining volume in the drops was then diluted under oil by adding sterile water, resulting in 25µl sample that could be recuperated from each droplet to perform amino acid analyses on. Samples were analysed by means of reverse phase HPLC as previously described (Sturmey *et al.*, 2010). Final amino acid concentrations were calculated by comparison with standard amino acid mixtures. All peak signals were normalized to the signal of internal standards.

Morula intracellular fat content

Fixed morulas (see previous section) were stained with Nile Red and DAPI to determine intracellular fat content and embryonic cell number, respectively. Morulas were washed twice in droplets of PBS, supplemented with 1mg/ml polyvinylpyrrolidinone (PBS-PVP) and then individually incubated in a drop of 5µg/ml DAPI for 5 minutes at room temperature. Embryos were washed again twice in PBS-PVP, after which they were incubated in a drop of 1 µg/ml Nile Red (Life Technologies) for 2 hours at room temperature. After that, embryos were mounted on a petri-dish with glass bottom in 5µl droplets of Nile Red solution with mineral oil overlay and analysed in a double blinded way by visualization with a confocal laser scanning microscope LSM510 Meta (Carl Zeiss Microimaging GmbH, Göttingen, Germany) equipped with an argon laser (excitation with 488 nm) and a 2- photon Ti-Sapphire laser (excitation with 780 nm) for imaging of Nile Red and DAPI staining, respectively (**Figure 1**). For each morula, a Z-stack of 20µm (1µm interval) was consistently taken a the lower mid equatorial level. Data image analyses were performed with Velocity 6.0.1 software. Individual

droplets were not analysed, as they were indistinguishable from each other. Total volume of Nile Red staining (μm^3) per cell was calculated.

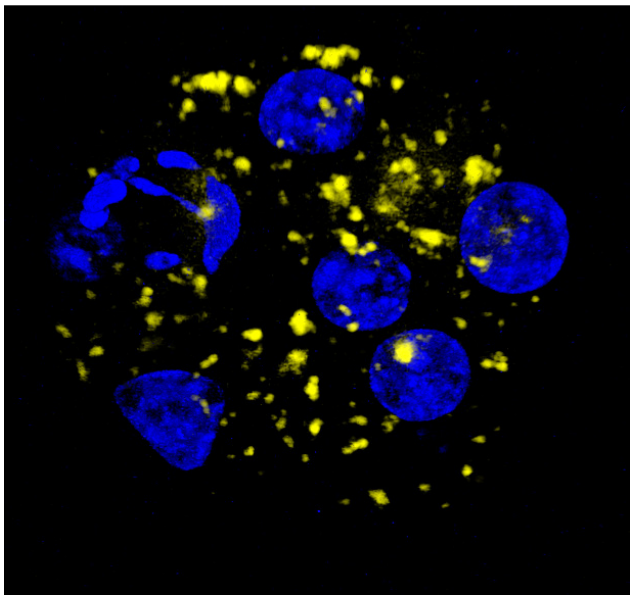


Figure 1: Image of a Nile Red (yellow) and DAPI (blue) stained murine morula.

3. Differential effect of short and long term NEFA exposure on oocyte developmental competence

The previously described experimental design was altered in order to be able to study differences between the effects of short term (during final oocyte maturation) and long term (throughout follicular growth from early secondary to antral follicle stages) NEFA exposure.

Collection of Day 12 conditioned medium (6 replicates)

Because the experimental design described below required the removal of 100% of the culture medium from Day 12 follicles, conditioned Day 12 culture medium was needed to compensate for secretory products accumulated in the spent medium. For this purpose, follicles were cultured in MEM plus, supplemented with LH and/or FSH, as previously described and 100% of the medium was collected on Day 12 of culture. Medium from all antral follicles was pooled per replicate and frozen directly, without further handling, at -80°C. To provide an adequate volume of conditioned medium for further processing (adding NEFAs, filtration) and use on Day 12 of culture, 6 replicates were needed (2 for each of the 3 replicates in which the NEFA exposure was performed).

Day 12 medium preparation

MEM plus, supplemented with NEFAs, FSH, hCG and EGF, was prepared as previously described. Additionally, 5ml defrosted conditioned medium, pooled from 2 replicates, was supplemented with BASAL, HIGH SA or HIGH NEFA mixtures. Together with the stimulation medium, the conditioned medium was sonicated for 4h under continuous temperature control and then filtered. The final composition of the Day 12 medium was obtained by adding 3ml of the conditioned medium to 2ml of the stimulation medium, for each treatment group.

Experimental design (3 replicates, Figure 2)

Follicles were exposed to elevated NEFA concentrations for 12 days or only during final maturation (last 20h of follicle culture). Five experimental groups were used (40 follicles per treatment per replicate):

1. **BASAL-BASAL**: experimental control (BASAL treatment from Day 1-13)
2. **BASAL-HIGH SA**: short term HIGH SA exposure (BASAL treatment from Day 1-12, HIGH SA treatment from Day 12-13)
3. **BASAL-HIGH NEFA**: short term HIGH NEFA exposure (BASAL treatment from Day 1-12, HIGH NEFA treatment from Day 12-13)
4. **HIGH SA-HIGH SA**: long term HIGH SA exposure (HIGH SA treatment from Day 1-13)
5. **HIGH NEFA-HIGH NEFA**: long term HIGH NEFA exposure (HIGH NEFA treatment from Day 1-13)

Follicles were cultured from Day 1 until Day 12 in BASAL, HIGH SA or HIGH NEFA medium as previously described. On Day 12 of culture, 100% of the culture medium was replaced by the mixture of conditioned Day 12 medium and stimulation medium with hCG and EGF, supplemented with BASAL, HIGH SA or HIGH NEFA mixtures. Final maturation continued for 20h, after which oocytes were aspirated from all antral follicles and routinely fertilized (Valckx *et al.*, 2014). After manual denudation of the presumptive zygotes, embryo culture occurred for 5 days at 37°C, 6% CO_2 and 5% O_2.

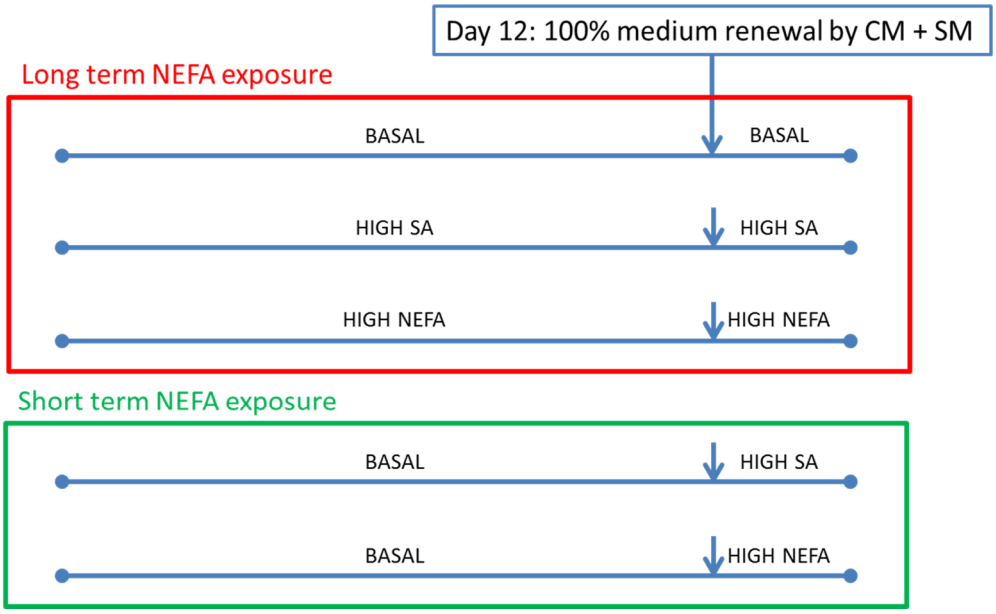


Figure 2: Experimental design to study differences between long term and short term NEFA exposure, during the longer period of folliculogenesis and only during final maturation, respectively. CM: conditioned medium, SM: stimulation medium.

4. Statistical analyses

Statistical analyses were performed with PASW statistics 18 (for Windows, Chicago, IL, USA). Differences in intracellular lipid content, glucose turnover and amino acid turnover between groups were investigated with a mixed model ANOVA with post hoc Sheffé tests. Treatment was entered as fixed factor and replicate as random factor. The interaction between replicate and treatment was first included in the model, only to be omitted from the final model when not significant. A T-test was used to study whether the observed differences in amino acid and glucose concentrations in the spent medium were different from zero. In the second experiment, a binary logistic regression approach was used to study differences in cleavage rate and blastocyst formation. Similarly, the interaction between replicate and treatment was first included in the model, only to be omitted when not significant. Statistical significance and statistical trends were set at $P < 0.05$ and $P < 0.1$, respectively.

Results

1. Metabolic profiling and intracellular fat content

Embryonic glucose and amino acid turnover

Glucose consumption was significantly higher in BASAL and HIGH NEFA embryos, compared to HIGH SA embryos (**Figure 3**), which did not consume any glucose ($P < 0.05$). Glucose consumption was significantly different from zero for BASAL ($P = 0.008$) and HIGH NEFA ($P = 0.004$) treatments, but not for the HIGH SA treatment. Amino acid analyses only showed a trend for an increased overall amino acid production in HIGH NEFA embryos ($P = 0.097$). There were no differences detected in the individual amino acids analysed (**Figure 4**). In the BASAL treatment group, aspartate, glutamate, glycine, arginine, tyrosine, tryptophan, methionine, isoleucine and leucine turnover differed from zero ($P < 0.05$). In the HIGH SA treatment group, aspartate, glycine, arginine and isoleucine turnover differed from zero ($P < 0.05$). Finally, in the HIGH NEFA treatment group, only arginine turnover differed significantly from zero ($P = 0.002$).

Morula intracellular fat content

Image analyses of Nile Red and DAPI stained embryos showed no significant differences in intracellular fat content per cell between BASAL (n = 15, 1412 ± 1067 µm³), HIGH SA (n = 37, 878 ± 606 µm³) and HIGH NEFA (n = 28, 1104 ± 1137 µm³) embryos.

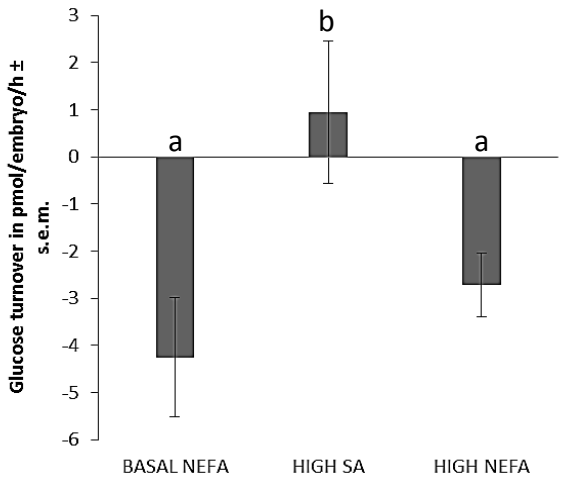


Figure 3: Mean glucose turnover in pmol per hour (± s.e.m.) for embryos originating from BASAL, HIGH SA and HIGH NEFA exposed follicles. [ab]Data with a different superscript differ significantly.

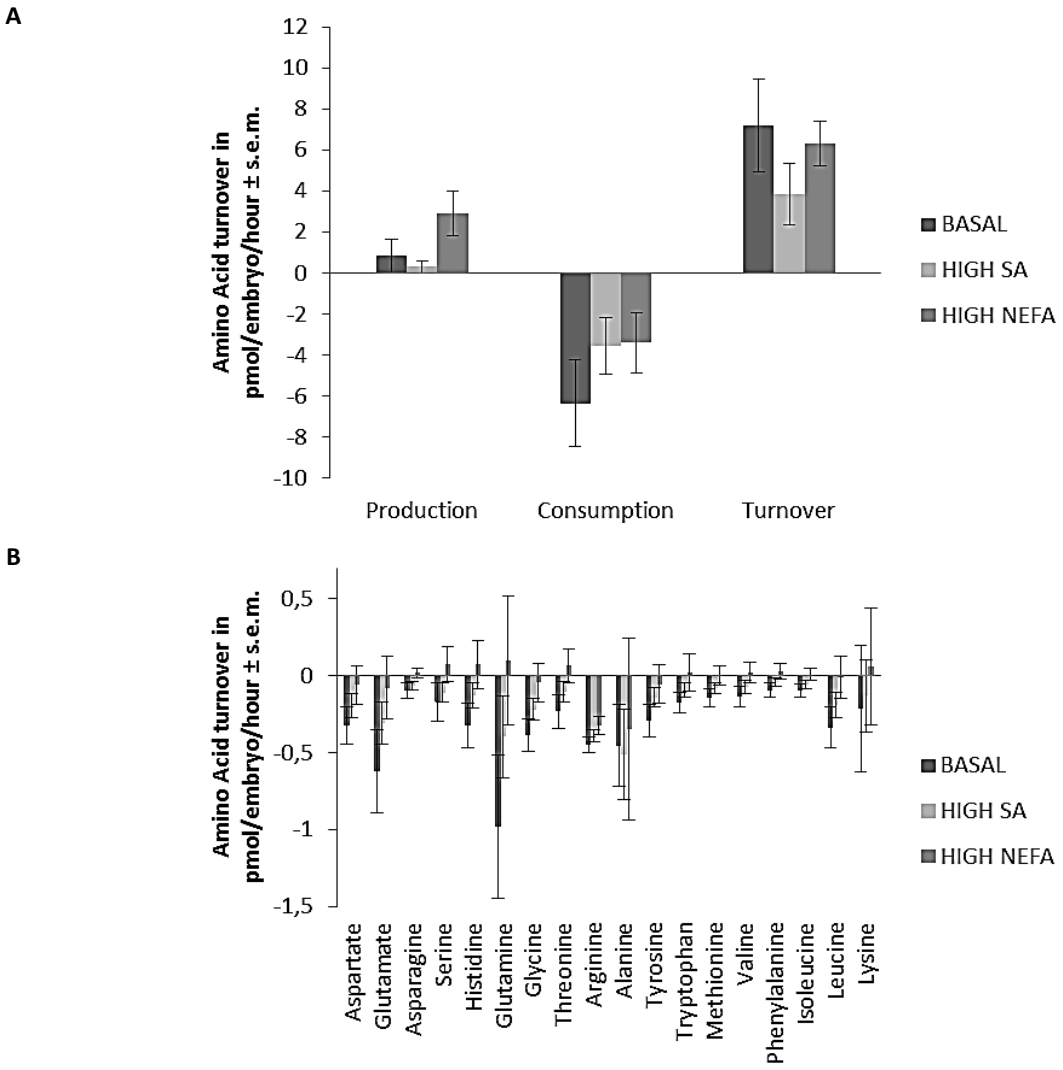


Figure 4: Mean amino acid turnover (sum of the absolute values for consumption and production) from embryos originating from BASAL, HIGH SA and HIGH NEFA exposed follicles (A). Amino acid turnover of individual amino acids (B). Data are presented as means (± s.e.m.).

2. Differential effect of short and long term NEFA exposure on oocyte developmental competence

Cleavage rate was reduced for BASAL-BASAL compared to BASAL-HIGH NEFA embryos (*P* = 0.045, **Figure 5**). The BASAL-HIGH NEFA treatment presented with a higher blastocyst percentage than BASAL-BASAL (*P* = 0.004), HIGH NEFA-HIGH NEFA (*P* = 0.037) and HIGH SA-HIGH SA (*P* = 0.001) treatments (**Figure 5**). The BASAL-HIGH SA treatment performed better than the HIGH SA-HIGH SA treatment (*P* = 0.049, **Figure 5**).

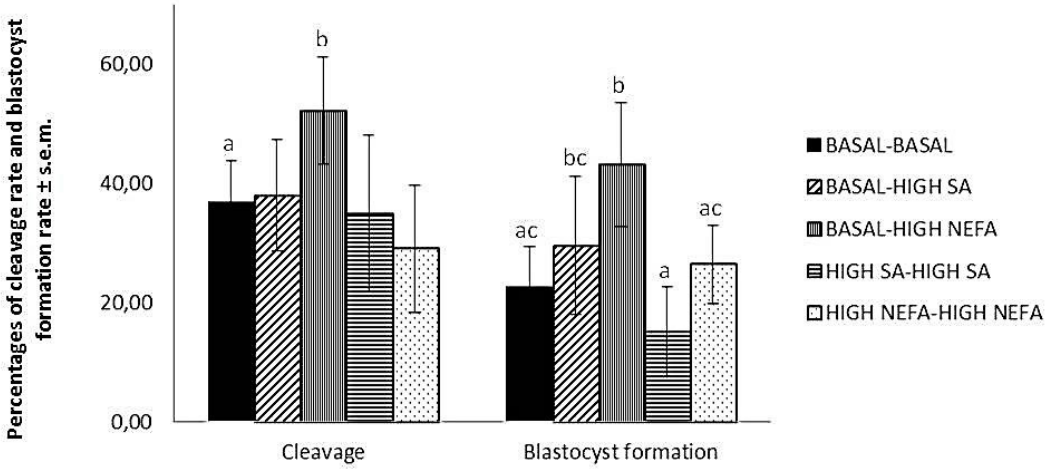


Figure 5: Representation of cleave rate and blastocyst formation rate (percentages) for embryos originating from BASAL-BASAL, BASAL-HIGH SA, BASAL-HIGH NEFA, HIGH SA-HIGH SA and HIGH NEFA-HIGH NEFA treatments. [abc]Data with a different superscript differ significantly.

Discussion

In this study, we hypothesized that follicular growth and oocyte maturation under continuous elevated NEFA concentrations hampers embryo quality and metabolism. We previously showed that such long term elevated NEFA concentrations affect follicular physiology in terms of steroid synthesis, granulosa cell gene expression patterns and a decreased oocyte developmental competence (Valckx *et al.*, 2014). This study investigated potential carry-over effects of such long term NEFA exposure during follicular growth and oocyte maturation, on subsequent embryo quality and metabolism. Our results showed that HIGH SA embryos consume little or no glucose in contrast to HIGH NEFA and BASAL embryos. Furthermore, HIGH NEFA embryos tend to produce more amino acids. The intracellular fat content was not affected by treatment. Interestingly, we showed for the first time that long term elevated NEFA concentrations throughout follicle culture elicit a more pronounced negative effect on oocyte developmental competence, compared to short term NEFA exposure, limited to the final phase of maturation.

It has previously been shown that *in vitro* produced bovine blastocysts have a higher **lipid content**, compared to *in vivo* derived embryos, which is associated with inferior embryo quality (Rizos *et al.*, 2002c, Barcelo-Fimbres and Seidel, 2011). Multiple studies in murine and bovine oocytes/embryos describe that exogenously provided fatty acids may be incorporated into neutral lipid droplets (Hillman and Flynn, 1980, Aardema *et al.*, 2011, Van Hoeck *et al.*, 2013b), which has been proposed as a protective mechanism for fatty acid induced lipotoxicity (Cnop *et al.*, 2001). Also, lipid droplets in maturing bovine oocytes are associated with the endoplasmic reticulum and mitochondria, suggesting that their distribution may be related to metabolic changes (Barcelo-Fimbres and Seidel, 2011). In our study, we assessed morula lipid content by staining the embryos with Nile Red. We used morula staged embryos because the lack of cell differentiation at that stage ensures a homogenous distribution of intracellular fat between cells and because the presence of a blastocoel could interfere with the visualization of the Nile Red/DAPI stain (Van Hoeck *et al.*, 2013b). We could not show a significant difference in intracellular fat content per cell, probably due to the high biological inter-oocyte variation within groups and the limited number of observations. However, the data of Van Hoeck *et al.* (2013b) showed that embryos, originating from oocytes exposed to HIGH SA concentrations during final maturation, present a lower volume of the lipid droplets in the blastomeres, compared to HIGH NEFA embryos, even though the size of the lipid droplets was similar among groups. Also, the number of lipid droplets per cell was lower in HIGH SA embryos, compared to HIGH NEFA and control embryos (Van Hoeck *et al.*, 2013b). These results are indicative for a lower

intracellular lipid storage of exogenously provided stearic acid and a higher use of stearic acid in other, potentially lipotoxic, pathways.

It has been well recognized that **glucose** metabolism during oocyte maturation, plays a crucial role in the acquisition of developmental competence (Sutton-McDowall *et al.*, 2010). Our results indicate that HIGH SA embryos do not consume glucose from the culture medium, compared to BASAL and HIGH NEFA embryos. The oocyte itself has low glycolytic rates and relies on glucose metabolites, such as pyruvate and lactate, provided by the surrounding cumulus cells (Sutton-McDowall *et al.*, 2010). However, as we previously showed that granulosa cells, originating from HIGH SA exposed follicles also presented a 'glucose-intolerant' gene expression profile (Valckx *et al*., 2014), we hypothesize that the follicular somatic cell physiology is responsible for pre-programming the oocyte's and subsequent embryo's metabolism. In other words, these results suggest that the environment in which the follicle and oocyte grow, not only alters follicular cell physiology, but also alters the oocyte's metabolism to a degree that it affects embryo metabolism.

As morphological assessment of embryo quality is often unreliable and maybe even unfit to predict developmental competence, alternative tests for embryo quality are being investigated. In this regard, **amino acid profiling** has been suggested as a valuable tool to study embryo quality *in vitro* (Sturmey *et al.*, 2008a). Amino acids are essential requirements for biosynthetic processes, mainly protein synthesis. It has been shown that an increased amino acid turnover, indicative of a higher metabolic activity, is associated with a higher degree of DNA damage in bovine, porcine and human embryos (Sturmey *et al.*, 2009b). This observation supports the Quiet embryo hypothesis, described by Leese *et al.* (2002), proposing that a quiet embryo metabolism is indicative of better embryo quality, when compared to embryos with a higher metabolic rate. The mechanism behind this is that viable embryos have less molecular and cellular damage than those which arrest and they have a reduced need to take up nutrients for repair mechanisms (Leese, 2002). In this study, we showed that embryos, originating from HIGH NEFA exposed follicles, present with a trend for an increased overall amino acid production, which corroborates with the quiet embryo hypothesis and with the findings of previous studies (Sturmey *et al.*, 2010, Van Hoeck *et al.*, 2011). We however could not find any differences in individual amino acid turnover. Even after correcting for embryos that did not develop until the morula stage after 24h, by removing the data from those droplets from the database, no significant differences could be found (non-parametric testing, data not shown). Furthermore, when correcting for the number of embryos that did develop to the morula stage in 24h, by multiplying the measured consumption/production with 'number of morulas/total number of embryos' in the drop, no significant differences were found (parametric testing). These results are somewhat unexpected, because previous research in bovine embryos, originating from NEFA

exposed oocytes, has shown plenty differences in both individual and overall amino acid turnover (Van Hoeck *et al.*, 2011). Nevertheless, overall murine embryo amino acid turnover in our study is in the same order of magnitude as those described in other studies (Lamb and Leese, 1994, Wale and Gardner, 2012), so the lack of statistical differences in individual amino acid turnover is probably due to a high biological variation within groups. Overall, the increased amino acid production in HIGH NEFA embryos does agree with Leese's quiet embryo hypothesis (Leese *et al.*, 2008), but more in depth research into the metabolic activity of embryos derived from NEFA exposed follicles, is needed to clarify these results.

Finally, we showed that oocyte developmental competence is impaired to a higher degree when follicles are exposed to NEFAs throughout the culture period, compared to only during final oocyte maturation. This suggests that not only final oocyte maturation, but also the prolonged follicular growth preceding final oocyte maturation, is pivotal for oocyte developmental competence, which is in agreement with the hypothesis of Britt, stating that follicles grown during the period of negative energy balance early post-partum in dairy cattle could be affected by the accompanying unfavourable metabolic changes and therefore contain a developmentally less competent oocyte (Britt, 1992). Subsequently, after a growing and maturation phase of several weeks, this inferior oocyte will be ovulated at the moment of the first insemination (Lucy, 2003). Our data expand on this hypothesis, in that the murine embryo, originating from a NEFA exposed follicle, might be of inferior quality (evidenced by an increased metabolic activity). From a clinical point of view, these data might suggest that chronic lipolytic conditions (such as obesity, type II diabetes), may have a more profound effect at the level of the oocyte, compared to acute lipolytic disorders (for example induced by acute weight loss or stress). However, a possible limitation in the interpretation of the differential effect of short versus long term NEFA exposure is that the control treatment (BASAL-BASAL) presented with unexpectedly low cleavage and blastocyst rates. We are currently unable to explain these differences with previous, preliminary trials. But, we do assume that the low results do not originate from the repeated freeze thaw of the medium, as for example steroids are stable for multiple freeze thaw cycles (Bauwman, 1982). More research is needed to confirm that no other compounds in the medium might have affected oocyte maturation.

In conclusion, the results described in this study show that oocyte developmental competence is impaired the most by a long term NEFA exposure throughout follicular growth. Furthermore, the resulting embryos have a deviating metabolism, which might be indicative of a lower embryo quality. These results may help elucidate the pathways that may partly explain infertility in women suffering from lipolytic disorders, such as obesity and type II diabetes.

References

Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB and Gadella BM. Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biol Reprod* 2011; **85**: 62-69.

Barcelo-Fimbres M and Seidel GE. Cross-validation of techniques for measuring lipid content of bovine oocytes and blastocysts. *Theriogenology* 2011; **75**: 434-444.

Bauman JE. Stability of radioimmunoassayable steroid and protein hormones after repeated freeze-thaw cycles. *Clin Chem* 1982; **28**: 2336-2337

Boden G and Shulman GI. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. *European Journal of Clinical Investigation* 2002; **32**: 14-23.

Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. *Proceedings of the Annual Convention of the American Association of Bovine Practitioners* 1992; **24**: 39-43.

Carvalho PD, Souza AH, Amundson MC, Hackbart KS, Fuenzalida MJ, Herlihy MM, Ayres H, Dresch AR, Vieira LM, Guenther JN et al. Relationships between fertility and postpartum changes in body condition and body weight in lactating dairy cows. *Journal of Dairy Science* 2014; **97**: 3666-3683.

Cnop M, Hannaert JC, Hoorens A, Eizirik DL and Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 2001; **50**: 1771-1777.

Collado-Fernandez E, Picton HM and Dumollard R. Metabolism throughout follicle and oocyte development in mammals. *The International journal of developmental biology* 2012; **56**: 799-808.

Fair T. Follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci* 2003; **78**: 203-216.

Gardner DK and Leese HJ. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. *Hum Reprod* 1986; **1**: 25-27.

Gardner DK, Wale PL, Collins R and Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod* 2011; **26**: 1981-1986.

Hillman N and Flynn TJ. The metabolism of exogenous fatty acids by preimplantation mouse embryos developing in vitro. *J Embryol Exp Morphol* 1980; **56**: 157-168.

Jorritsma R, Cesar ML, Hermans JT, Kruitwagen CLJJ, Vos PLAM and Kruip TAM. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. *Anim Reprod Sci* 2004; **81**: 225-235.

Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS and Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. *Fertil Steril* 2011a; **95**: 1970-1974.

Lamb VK and Leese HJ. Uptake of a mixture of amino acids by mouse blastocysts. *J Reprod Fertil* 1994; **102**: 169-175.

Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* 2002; **24**: 845-849.

Leese HJ and Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J Reprod Fertil* 1984; **72**: 9-13.

Leese HJ, Baumann CG, Brison DR, McEvoy TG and Sturmey RG. Metabolism of the viable mammalian embryo: quietness revisited. *Mol Hum Reprod* 2008; **14**: 667-672.

Leroy JLMR, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PEJ, Dewulf J and de Kruif A. Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. *Theriogenology* 2004; **62**: 1131-1143.

Leroy JLMR, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G and Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 2005; **130**: 485-495.

Lu ZH, Mu YM, Wang BA, Li XL, Lu JM, Li JY, Pan CY, Yanase T and Nawata H. Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. *Biochem Bioph Res Co* 2003; **303**: 1002-1007.

Lucy MC. Mechanisms linking nutrition and reproduction in postpartum cows. *Reprod Suppl* 2003; **61**: 415-427.

Mooradian AD, Haas MJ, Wehmeier KR and Wong NC. Obesity-related changes in high-density lipoprotein metabolism. *Obesity (Silver Spring)* 2008; **16**: 1152-1160.

Rizos D, Ward F, Duffy P, Boland MP and Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev* 2002c; **61**: 234-248.

Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ and Lonergan P. Amino Acid Metabolism of Bovine Blastocysts: A Biomarker of Sex and Viability. *Mol Reprod Dev* 2010; **77**: 285-296.

Sturmey RG, Brison DR and Leese HJ. Assessing embryo viability by measurement of amino acid turnover. *Reprod Biomed Online* 2008a; **17**: 486-496.

Sturmey RG, Hawkhead JA, Barker EA and Leese HJ. DNA damage and metabolic activity in the preimplantation embryo. *Hum Reprod* 2009b; **24**: 81-91.

Sutton-McDowall ML, Gilchrist RB and Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 2010; **139**: 685-695.

Tay JI, Rutherford AJ, Killick SR, Maguiness SD, Partridge RJ and Leese HJ. Human tubal fluid: production, nutrient composition and response to adrenergic agents. *Hum Reprod* 1997; **12**: 2451-2456.

Valckx S, Van Hoeck V, Arias-Alvarez M, Maillo V, Lopez-Cardona AP, Gutierrez-Adan A, Berth M, Cortvrindt R, Bols PEJ and Leroy JLMR. Elevated non-esterified fatty acid concentrations during in vitro murine follicle growth alter follicular physiology and reduce oocyte developmental competence. *Fertil Steril* 2014.

Valckx SD, Arias-Alvarez M, De Pauw I, Fievez V, Vlaeminck B, Fransen E, Bols PE and Leroy JL. Fatty acid composition of the follicular fluid of normal weight, overweight and obese women undergoing assisted reproductive treatment: a descriptive cross-sectional study. *Reprod Biol Endocrinol* 2014; **12**: 13.

Valckx SD, De Pauw I, De Neubourg D, Inion I, Berth M, Fransen E, Bols PE and Leroy JL. BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. *Hum Reprod* 2012; **27**: 3531-3539.

Van Hoeck V, De Bie J, Ramos-Ibeas P, Rizos D, Gutierrez-Adan A, dufort I, Sirard MA, Merckx E, Andries S, Bols PEJ et al. Elevated concentrations of non-esterified fatty acids during bovine embryo culture compromise pre-implantation embryo development and influence gene transcription. submitted.

Van Hoeck V, Rizos D, Gutierrez-Adan A, Pintelon I, Jorssen E, Dufort I, Sirard MA, Verlaet A, Hermans N, Bols PE et al. Interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations. *Reprod Fertil Dev* 2013b: [Epub ahead of print].

Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE and Leroy JL. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *Plos One* 2011; **6**: e23183.

Wale PL and Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod* 2012; **87**: 24, 21-28.

Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ and Robker RL. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. *Fertil Steril* 2012.

Zhang LY, Keung W, Samokhvalov V, Wang W and Lopaschuk GD. Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. *Bba-Mol Cell Biol L* 2010; **1801**: 1-22.

CHAPTER 8: GENERAL DISCUSSION

From embryonic differentiation to primordial germ cells, until the ovulation of a matured and developmentally competent oocyte, the female gamete is subjected to the micro-environment provided by the mother. It has become increasingly clear that the health of the oocyte at the time of ovulation is decisive for correct fertilization, early embryo development and normal fetal development. Even the slightest change within the oocyte may have severe repercussions for normal embryo development. Maternal metabolic disorders are associated with changes in circulating serum metabolites that may directly affect follicular growth or may be reflected in the follicular fluid (Leroy *et al.*, 2004, Robker *et al.*, 2009), potentially endangering the oocyte's acquisition of developmental competence. Knowing that the micro-environment of the oocyte is pivotal for and supports optimal oocyte growth, in the present thesis, the following **general hypothesis** was formulated:

> **Maternal metabolic disorders, like obesity, are associated with changes in the serum profile, that are reflected in the maternal micro-environment of the pre-ovulatory follicle. These changes alter folliculogenesis and oocyte growth, ultimately leading to a reduced oocyte developmental competence and subsequent embryo quality.**

So besides the notion that "*all things come from the egg"* (citation William Harvey), the egg originates from a micro-environment that may be equally important for successful reproduction.

A typical characteristic of metabolic disorders is **elevated serum NEFA concentrations** due to an increased lipolysis. These elevated NEFA concentrations have been implicated in both the origin and appearance of metabolic disorders (Zhang *et al.*, 2010). Furthermore, they have proven cytotoxic effects on several cell types *in vitro* (Cnop *et al.*, 2001, Lu *et al.*, 2003), including granulosa cells (human and bovine) and oocytes (bovine) (Mu *et al.*, 2001, Aardema *et al.*, 2011, Van Hoeck *et al.*, 2011). Up until now, studies have mainly focused on the effect of short term NEFA exposure at the level of the (bovine) oocyte. However, in metabolically compromised women, oocytes are exposed to elevated NEFA concentrations for a longer period of time, during their growth within the follicle. Therefore, we **specifically hypothesized that long term elevated NEFA concentrations during follicular growth affect follicular physiology, ultimately resulting in an incompetent oocyte and thus an embryo with an altered quality and metabolism.**

In this thesis, we tried to unravel several parts of the complex interactions between maternal metabolic disorders, the maternal micro-environment and the acquisition of oocyte developmental competence throughout folliculogenesis, as indicated by the following research questions (**Figure 1**):

- How are serum metabolic changes, related to maternal metabolic disorders, like obesity, reflected in the ovarian follicular fluid and how may this affect oocyte developmental competence and subsequent embryo quality? (Chapter 3 and 4)
- How are fatty acids distributed in the ovarian follicular fluid and how do they associate with lipolytic conditions, like obesity? (Chapter 5)
- How do prolonged elevated NEFA concentrations affect the growth and differentiation of the ovarian follicle as a whole and the maturing oocyte enclosed within? Furthermore, how are embryo development, quality and metabolism affected by adverse metabolic conditions during follicular development? (Chapter 6 and 7)

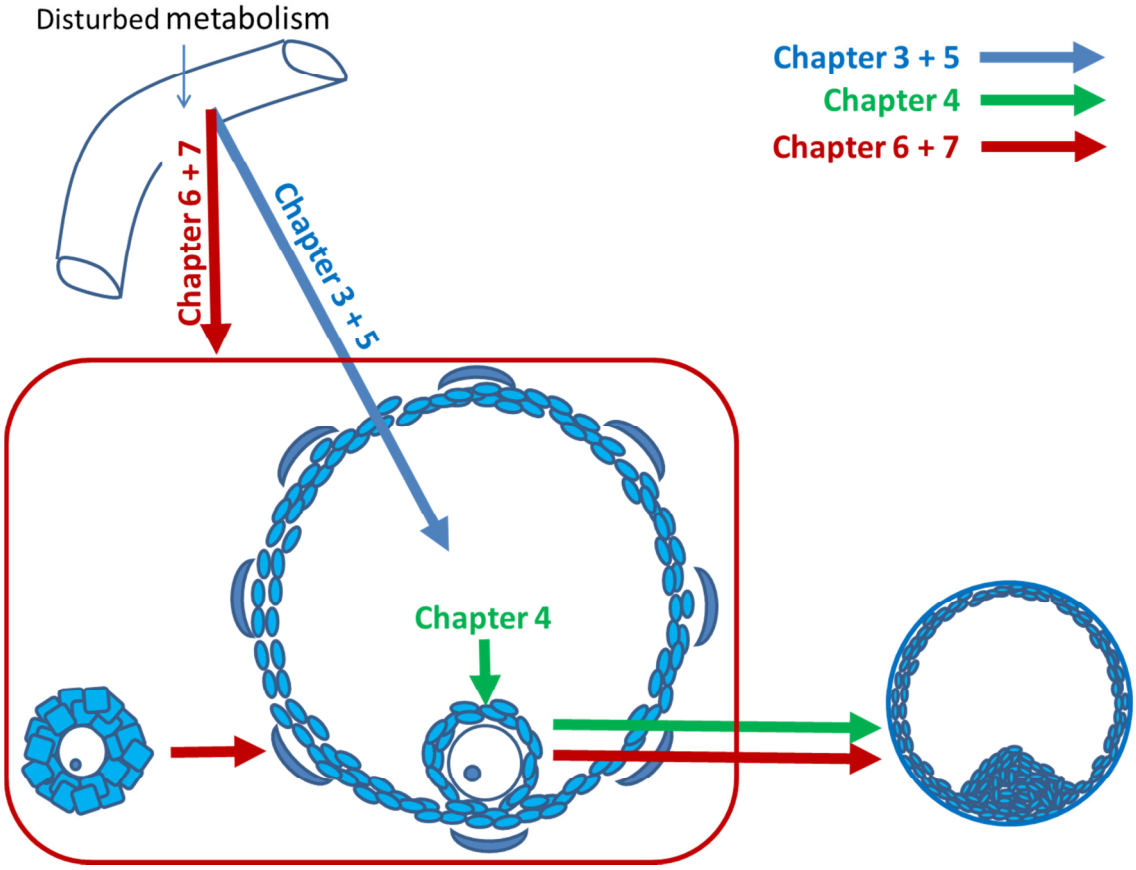


Figure 1: Schematic representation of the work described in this thesis. A disturbed maternal metabolism is reflected in the serum composition. Chapters 3 and 5 describe how serum metabolic changes are reflected in the follicular fluid and how this may be associated with oocyte developmental competence in women undergoing ART, Chapter 4 investigates the effect of the human follicular fluid composition on bovine oocyte developmental competence *in vitro* and Chapters 6 and 7 specifically study the effect of elevated NEFA concentrations during murine *in vitro* follicular culture on follicle growth, follicle quality, oocyte developmental competence and the quality of the resultant embryo.

1. The follicular fluid as a potential link between maternal metabolic disorders, oocyte developmental competence and embryo quality

a) Serum versus follicular fluid composition and the potential influence of BMI

In vivo research performed in cattle showed that the ovarian micro-environment may be altered by the metabolic state of the mother (Leroy *et al.*, 2004). However, how such changes may occur in women undergoing ART, needed to be clarified.

Women with metabolic disorders typically present an altered serum metabolic profile. In our first study (**Chapter 3**), we showed that the serum profile is partly reflected in the follicular fluid composition of women undergoing ART. The concentrations of most, but not all metabolites were lower in the follicular fluid, compared to the serum, an effect mainly attributed to the blood follicle barrier and the active metabolism of follicle cells (Gosden *et al.*, 1988, Sutton *et al.*, 2003, Hennet and Combelles, 2012). Serum concentrations of CRP, cholesterol, HDL cholesterol, triglycerides, ApoA1, lactate, insulin and IGF-1 were associated with BMI, while in the follicular fluid, only the concentrations of CRP, triglycerides, insulin and IGF-1 were associated with BMI. However, none of these BMI-related factors in the follicular fluid were associated with oocyte developmental competence. Only follicular fluid total protein and ApoA1 concentrations were negatively associated with oocyte developmental competence and morphological embryo quality.

The observation that only few follicular fluid compounds were associated with BMI, may be related to the **limited BMI spread** in the obese group of our study (from 30 to 39 kg/m²), because morbidly obese women were first asked to lose weight before starting fertility treatment. In contrast, in the study of Robker *et al.* (2009), maximum BMI values were around 50kg/m². Robker *et al.* (2009), like us, found that increasing BMI was correlated with elevated levels of triglycerides and CRP in the follicular fluid. They also found a correlation between BMI and insulin, glucose and lactate in the follicular fluid, whereas we found an additional association with IGF-1. They also showed that sex hormone binding globulin concentrations were decreased with increasing BMI, which contributed to an elevated intrafollicular free androgen index (Robker *et al.*, 2009).

Based on the above, the lipid and energy metabolism related composition of the follicular fluid may be changed by increasing BMI. This might ultimately offset the vulnerable balance within the follicular milieu. In this regard, it might not seem surprising that no clear association between one BMI-related compound in the follicular fluid and oocyte quality parameters was found, as there may be a great **interaction** between these compounds related to lipid and energy metabolism (ex. IGF-1, insulin, glucose, lactate, triglycerides, NEFAs), that may not act in a linear way (as assumed by the

statistical analyses). So complex, non-linear interactions between the compounds under study may interfere with the finding of a significant effect.

Notably, we did not have any knowledge on **dietary intake** before sampling. This could be a potential confounder, because dietary intake can influence the follicular fluid composition (reviewed by Leroy *et al.*, 2014), even though it remains to be investigated during what time frame dietary intake may affect follicular fluid composition. Importantly, we paid much attention to scrutinize the effect of the **female subfertility etiologies** on IVF outcome and follicular fluid composition, as that may have affected the results. We used linear discriminant analyses (LDA), combined with logistic regression to study this. The results showed that even though the LDA approach pointed to some interesting compounds that may be related to the subfertility etiology (endometriosis, tubal defects, etc.), logistic regression analyses could not always confirm this, probably due to the complex interaction between follicular fluid compounds. So we did not find strong statistical evidence that female subfertility etiologies affected the follicular fluid composition. It is furthermore debatable whether the women enrolled in this study are representative for all women of reproductive age. A first argument is that these women undergo an ART treatment for an identified or unidentified subfertility etiology, which makes it difficult to generalize the generated results without caution to the general population of women. In this regard, studying the follicular fluid of women from which only the partner has a known reason for subfertility, might partly resolve that issue. However, all women undergoing ART are subjected to a personalized **superstimulation treatment** to allow multiple oocytes to mature at the same time, that may affect the follicular fluid composition (Yding Andersen *et al.*, 1993). For example, intrafollicular concentrations of FSH and LH are affected by their circulating levels, which, in case of ART treatment, are determined by the amount of exogenously administered gonadotropins. Besides the observation that high concentrations of FSH, hCG and LH promote oocyte maturation and the fertilization potential, gonadotropins play an important role in regulating the secretion of several substances by granulosa cells (e.g. hyaluronic acid), in turn affecting oocyte development and maturation (reviewed by Revelli *et al.*, 2009). This confounder might partially be accounted for by only including women who have had the same type of superstimulation treatment. However, women react differently to these treatments, so this confounder is hard to correct for and remains a well acknowledged limitation of all studies relying on human follicular fluid from ART settings (including those described in Chapters 3-5). Another limitation of our study is that we were unable to investigate changes in the follicular fluid composition **over time**. Repeated sampling of follicular fluid in women, is both ethically and practically impossible. However, results of studies in high yielding dairy cows provide us with important information. It was found that typical postpartum biochemical changes in the serum are well reflected in the follicular fluid over time, even though the oocyte and granulosa cells seemed to

be protected from too low glucose and too high NEFA levels (Leroy *et al.*, 2004). **Other factors** that could not be accounted for in our study are the potential effects of male subfertility etiologies or intrinsic sperm quality, environmental pollution (Kamarianos *et al.*, 2003, Petro *et al.*, 2012), general health history, education and lifestyle (Kamarianos *et al.*, 2003), which could all affect the relation between obesity, the follicular fluid composition and oocyte developmental competence (or success rates of IVF treatment).

Concluding, for the first time in women, it was shown that the serum composition is partly reflected in the follicular fluid and that some compounds (total protein and ApoA1) in the follicular fluid may affect oocyte developmental competence. BMI was associated with lipid and energy metabolism related compounds in the serum and the follicular fluid, but these compounds were not directly associated with oocyte developmental competence *in vitro*.

b) Follicular fluid as a potential marker for oocyte quality

Many studies have focussed on factors in the follicular fluid and their relation to oocyte developmental competence (Revelli *et al.*, 2009). However, so far, no clear marker for oocyte quality has been found. This is mainly (1) because a multitude of factors is present within the follicular fluid and no clear sight on possible interactions between all of them exists and (2) because intrinsic oocyte quality may confound the results. This is why, in our second study (**Chapter 4**), we chose to change our strategy, from 'retrospectively' looking at oocyte developmental competence to trying to identify a causative link between the follicular fluid and oocyte developmental competence. We hypothesized that the pre-ovulatory follicular fluid composition may determine oocyte developmental competence after *in vitro* fertilization. To study this, we investigated the effect of the follicular fluid composition on oocyte developmental competence in an entirely different setting, in which intrinsic oocyte quality is uncoupled from the follicular fluid composition. Here, we added follicular fluid from women undergoing IVF to the *in vitro* maturation of bovine oocytes. The follicular fluid originated from women with (1) a high number of good quality embryos during IVF (a POSITIVE IVF outcome), (2) a low number of good quality embryos during IVF (a NEGATIVE IVF outcome) and (3) obesity (OBESE) during the *in vitro* maturation of bovine oocytes. We then compared bovine oocyte developmental competence and embryo quality between groups, including a routine lab-control. Importantly, we implemented very strict in- and exclusion criteria for the women enrolled in this study (see Chapter 4 for more details), to exclude known confounders (like age and male infertility) and included only women who reacted well to the gonadotropin treatment. We showed that adding follicular fluid from women with an overall bad IVF treatment outcome (NEGATIVE) or with obesity (OBESE) during bovine *in vitro* oocyte maturation impaired oocyte developmental

competence. So, even though we could not find a strong impact of BMI on the follicular fluid composition in our first study (Chapter 3), these results suggest that BMI does affect the follicular fluid composition to a degree that it may directly affect bovine oocyte developmental competence. Furthermore, bovine blastocyst gene expression analyses of developmentally important genes showed that *LDHA* and *PPARGC1B* mRNA abundance differed between treatment groups in which follicular fluid was added. This study thus provides us with some clues regarding potential pathways that may be affected by our treatment groups. However, more in depth pathway analyses, by means of both transcriptional and functional analyses, might be beneficial to get a more profound insight. Knowing which pathways are affected by follicular fluid from women with differential IVF outcome, can give us clues as to what to look for in the follicular fluid. **These results further substantiate that the follicular fluid composition may be partly responsible for the differentiation between good and bad quality embryos in an IVF setting.**

Other studies, in which intrinsic oocyte quality was uncoupled from the follicular fluid factor mainly include oocyte donation in women (see general introduction) and transplanting of oocytes/embryos into normal recipient animals. For example, Jungheim *et al.* (2011b) showed that murine embryos, exposed to elevated levels of palmitic acid *in vitro*, result in smaller birth weight with a catch-up growth, after transfer to a normal recipient mouse. In another study, an embryo transfer model was used to investigate the effects of exposure to either maternal obesity and/or weight loss before and for 1-week post-conception on the abundance of key molecules regulating hepatic fatty acid oxidation and lipid synthesis in the 4-month-old lamb. This study showed that such an exposure to adverse metabolic conditions changed lipid metabolism in the liver of the offspring (Nicholas *et al.*, 2014). In another study that investigated the potential impact of the follicular fluid composition on oocyte developmental competence, a single species model was used. Namely, bovine cumulus-oocyte complexes were exposed to bovine NEFA rich follicular fluid (aspirated in heifers after short term fasting). This resulted in massive intracellular lipid storage in the cumulus cells, but not in the oocyte, preventing oocyte developmental competence from being affected (Aardema *et al.*, 2013). The authors suggested that the cumulus cells may protect the oocyte from short term increased NEFA concentrations, an effect probably mediated by the high concentrations of mono-unsaturated fatty acid, oleic acid, in the follicular fluid. However, the observed effect cannot be attributed to elevated NEFAs alone, as fasting may cause a shift in numerous metabolites and hormones in the follicular fluid, that could influence cumulus cell function and final oocyte maturation. Of particular interest might be triglycerides (breakdown for energy provision), ROS (through an increased β-oxidation) and even steroid levels (released from stores in adipose tissue) (Michalakis *et al.*, 2013). The same holds true for a study of Yang *et al.* (2012), in which human follicular fluid from obese patients, rich in triglycerides and NEFAs, was added during murine oocyte maturation *in vitro*. What

other compounds in that follicular fluid may have contributed to the final effect? Because the results of the previously described studies are biased by selecting for specific compounds in the follicular fluid (like NEFAs or triglycerides), not accounting for other compounds in the follicular fluid, we specifically opted to create treatment groups based on the biological outcome (i.e. the developmental potential of the oocyte coming from that follicle), rather than a selection of compounds in the follicular fluid. We furthermore implemented an obese treatment group, because of the known increased risk for fertility failure in obese women and the rising evidence that oocyte and embryo quality may be impaired (Robker, 2008). An important remark on our study is the mismatch in endocrine timing, i.e. we exposed immature bovine COCs to post LH follicular fluid. Even though this may have confounded the results, by creating premature high progesterone concentrations, it was ethically impossible to collect human follicular fluid earlier. We also have no knowledge on interspecies effects. In this regard, it is important to mention that our lab-control showed similar results in term of oocyte developmental competence, compared to the POSITVE treatment.

Importantly, the potential role of the cumulus cells surrounding and protecting the oocyte should not be forgotten. Cumulus cells play a pivotal role in sustaining oocyte maturation and growth (Gilchrist *et al.*, 2004) and it has very recently been shown that bovine cumulus cells may even transfer large mRNA molecules to the oocyte (Macaulay *et al.*, 2014), contributing to the maternal mRNA stores that are used during embryonic growth before embryonic genome activation. So it is not surprising that markers for oocyte quality have been found by investigating cumulus cell gene expression patterns (Assidi *et al.*, 2008, Hamel *et al.*, 2008, Van Hoeck *et al.*, 2013a, Bunel *et al.*, In press). The substantial contribution and protective effect of cumulus cells to safeguard oocyte developmental competence, together with the limited effect size (due to the strict selection of samples) may explain the limited impact (statistical trends) of adding human follicular fluid during bovine oocyte maturation on oocyte developmental competence and embryo quality in our study (Chapter 4).

c) Emphasized: fat in the follicular fluid

Several researchers reported on the presence of fatty acids in the follicular fluid (Robker *et al.*, 2009, Jungheim *et al.*, 2011a) and have shown an important effect of specific fatty acids on reproductive function in general and on oocyte developmental competence more specifically (Fouladi-Nashta *et al.*, 2007). For example, supplementing dietary fat alters the physiology and steroidogenic capacity of the bovine pre-ovulatory follicle (Beam and Butler, 1997, Moallem *et al.*, 2007) and also alters the follicular fluid fatty acid profile in the cow (Adamiak *et al.*, 2005, Wonnacott *et al.*, 2010, Zachut *et al.*, 2010). At the level of the oocyte and embryo, a diet rich in n-3 polyunsaturated fatty acids reduced ovulation rate, disturbed mitochondrial activity and reduced litter size in mice (Wakefield *et*

al., 2008, Yi *et al.*, 2012), but improved embryo cleavage rate in the cow (Zachut *et al.*, 2010) and improved human embryo morphology (Hammiche *et al.*, 2011). Furthermore, *in vitro* bovine oocyte maturation in the presence of n-3 PUFA (linolenic acid) improved nuclear oocyte maturation, whereas the presence of n-6 PUFA (linoleic acid) reduced the resumption of meiosis (Marei *et al.*, 2009, Marei *et al.*, 2010). A list of important studies about the effects of dietary fat on oocyte and embryo quality, along with their main findings, can be found in a review by Leroy *et al.* (2014). **Importantly, the role of the follicular fluid composition in the previously described studies is not always clear**. **Furthermore, none of these studies has made the distinction between fatty acids in the different lipid fractions, as they mostly analysed fatty acids in the total lipid fraction.** This is important though, because a fatty acid esterified to one or another lipid fraction, may exert a different biological effect at the level of the cell. For example, Wonnacott *et al.* (2010) showed that PUFA enriched high density lipoproteins (HDLs) added to the culture medium reduced embryo development and altered embryo gene expression for scavenger receptor class B member 1, low-density lipoprotein receptor and stearoyl-CoA desaturase, even though no net fatty acid uptake could be observed. Subsequent research showed that net fatty acid uptake from the supplemented serum only occurred when the fatty acids were bound to albumin. This albumin-bound fatty acid uptake clearly affected embryo fatty acid profiles and increased cellular oxidative stress (Hughes *et al.*, 2011). Importantly, the fatty acid composition of the follicular fluid in (obese) women, specified for the different lipid fractions, had never been investigated.

Furthermore, a negative energy balance or acute dietary fasting in cows affects the follicular fluid fatty acid composition in the non-esterified fat fraction (Jorritsma *et al.*, 2003, Leroy *et al.*, 2005, Aardema *et al.*, 2013). Whether the same holds true for women suffering from metabolic disorders in the different lipid fractions, remained to be investigated. **This is why, in Chapter 5, we investigated the fatty acid composition of the different lipid fractions in the follicular fluid of women undergoing IVF treatment in relation to BMI**. In doing so, we created a state-of-the-art database on follicular fluid fatty acids. We showed that most of the fatty acids in the follicular fluid are esterified in the phospholipid and cholesteryl-ester fraction, whereas only a small part resided in the triglyceride and NEFA fraction. It were, however, only the triglyceride and NEFA fractions that were associated with BMI. Furthermore, the triglyceride and NEFA fractions showed the most BMI-related differences in individual fatty acids, suggesting that body weight impacts mostly on fatty acid storage (triglycerides) and fatty acid metabolism (NEFA). These results are partly in contrast with our first study, where we did not find an association between BMI and follicular fluid NEFA concentrations. This controversy may be attributed to the differences in experimental design. In our first study (Chapter 3), we allowed all women to participate in the study, resulting in a skewed distribution of women across BMI groups (60 women had a normal weight, 26 were overweight and 20 obese). In

our third study (Chapter 5), we randomly selected 10 women for each BMI group, resulting in equal groups and a different statistical throughput.

Approximately 35% of the follicular fluid fatty acids can be attributed to the **cholesteryl-ester fraction** (Chapter 5). Lipoprotein analyses of human follicular fluid revealed that HDL, but little or no LDL and very LDL are present (Simpson *et al.*, 1980, Jaspard *et al.*, 1997). In our first study (Chapter 6), we showed that follicular fluid and serum HDL cholesterol are positively correlated, indicating that changes in follicular fluid HDL cholesterol are related to serum changes. The observation that mostly HDL is present in the follicular fluid is probably the result of the selective permeability of the blood follicular fluid barrier (see General introduction). Normal HDL metabolism is pivotal for oocyte developmental competence and subsequent embryo development, since mice lacking the HDL receptor, scavenger receptor class B, member 1 (SRB1) are infertile due to failure of embryo development beyond the morula stage (Miettinen *et al.*, 2001). Increased HDL concentrations in human follicular fluid have been associated with low embryo fragmentation (Browne *et al.*, 2008, Browne *et al.*, 2009). On the contrary, we showed in Chapter 3 that **ApoA1** (lipoprotein mainly present in HDL particles) concentrations in the follicular fluid were negatively associated with the chance for an embryo to develop into a top quality embryo. This was substantiated by the findings of Wallace *et al.* (2012), who showed that HDL lipoprotein levels were elevated in the follicular fluid from follicles whose oocyte failed to cleave in an IVF setting. Furthermore, it has been shown that HDLs, isolated from the serum of ewes fed an n-6 PUFA rich diet, during ovine embryo culture *in vitro* decreased blastocyst formation, without any net uptake of fatty acids (Wonnacott *et al.*, 2010). As previously indicated, only albumin bound fatty acids are readily taken up by embryos (Hughes *et al.*, 2011). These contrasting result are probably caused by the multiple functions HDL particles may mediate, like increasing steroidogenesis by providing cholesterol or by scavenging cholesterol like it does in the blood circulation.

The **phospholipid** fractions account for 45% percent of the total follicular fluid fatty acids (Chapter 5). In the cow, Renaville *et al.* (2010) found that the follicular fluid phospholipid fraction of inactive follicles had lower concentrations of stearic acid and higher concentrations of oleic acid, arachidonic acid and docosahexaenoic acid, compared to active follicles and serum. It remains to be investigated whether these fatty acid ratios and the way they change during follicle development, are reflected in the oocyte's phospholipid fraction.

In dairy cows, **triglyceride** concentrations are lower in the follicular fluid compared to serum, but a significant correlation is present (Leroy *et al.*, 2004). We were able to confirm these results in women undergoing IVF treatment, even though the correlation between serum and follicular fluid

concentrations did not attain statistical significance (trend, Chapter 3). Follicular fluid triglyceride concentrations also tended to be higher in overweight women, compared to normal weight and obese women (Chapter 5). This is an interesting finding, that may be party explained by the triglyceride storage capacity of fat and other tissues. When a women develops overweight, she stores more intracellular triglycerides in fat droplets. However, when the capacity of the cells to store triglycerides is exceeded, lipids may be metabolised in other pathways and no excess triglycerides may be found in the follicular fluid (as this is an extracellular environment in which metabolites may accumulate).

Furthermore, **NEFA** concentrations are 60% lower in the follicular fluid than in serum, but are significantly correlated to serum concentrations in the cow (Leroy *et al.*, 2004). In women, follicular fluid NEFA concentrations are 50% lower than serum concentrations and a correlation between serum and follicular fluid concentrations exists (Chapter 3). Importantly, increased BMI was associated with increased follicular fluid NEFA concentrations (Chapter 5). Additionally, the most abundant fatty acids in the NEFA fraction of human follicular fluid were palmitic (16:0), stearic (18:0), oleic (18:1 n-9) and linoleic acid (18:2 n-6) (Chapter 5). This finding confirms similar observations made in dairy cattle (Leroy *et al.*, 2005). Also, our results confirm the data of Jungheim *et al.* (2011a), who found that oleic, linoleic and palmitic acid were the most prevalent in human follicular fluid and showed a weak but significant correlation with serum levels. However, this study only analysed 7 specific fatty acids in the NEFA fraction (Jungheim *et al.*, 2011a), while our study investigated a total of 23 fatty acids in each lipid fraction, thus expanding our knowledge on fatty acids in the follicular fluid.

Interestingly, the fatty acid distribution in the follicular fluid described in Chapter 5 seems to be similar to those within oocytes, as it has been shown in multiple species that the main fatty acids present within the oocyte are palmitic, stearic and oleic acids (McEvoy *et al.*, 2000). For example, lipid content analysis of fertilization-failed human oocytes showed that major fatty acids were stearic (38% of total fatty acids), palmitic (33%), oleic (10%), myristic (4%) and linoleic (4%) acids. Overall, saturated fatty acids represented 79%, mono-unsaturated fatty acid 14%, n-6 poly-unsaturated fatty acids 5% and n-3 poly-unsaturated fatty acids 1% of the intracellular lipid content of human oocytes (Matorras *et al.*, 1998). These findings are consistent in most species, which means that saturated fatty acids generally account for the vast majority of intracellular fatty acid stores (Dunning *et al.*, 2014). Interestingly, human embryos that developed beyond the 4-cell stage contained more oleic, linoleic and arachidonic acid, than embryos that did not develop past the 4-cell stage (fresh embryos, unsuited for clinical use, Haggarty *et al.*, 2006). Furthermore, Adamiak *et al.* (2005) and Rooke *et al.* (2006) found that a change in follicular fluid fatty acid concentrations was also reflected in the fatty

acid content and profile of bovine cumulus-oocyte complexes. So, BMI-related changes in the fatty acid composition of the follicular fluid may be reflected in the oocyte, which in turn may affect metabolic processes during subsequent embryonic growth. However, Fouladi-Nashta *et al.* (2009) could not find that fat feeding caused a difference in major fatty acids within bovine granulosa cells, so it is also possible that the ovary can protect oocytes, at least to some extent, against fluctuations in n-3 and n-6 fatty acids.

In the studies, described in Chapters 3 and 5, we analysed the composition of the pre-ovulatory follicular fluid. Because this is the micro-environment for final oocyte maturation, the results are very valuable in trying to elucidate of the effect of metabolic disorders during final maturation on oocyte developmental competence. However, these results do not allow us to assume anything about the environment in which the **pre-antral and early antral follicle grows**. In women, such research is ethically difficult, if not impossible to conduct, but *in vitro* animal models, like the one used in Chapters 6 and 7, may provide an alternative to study the influence of the maternal micro-environment on folliculogenesis and oocyte growth.

2. The effect of long term elevated NEFA concentrations during follicular growth

The effects of elevated NEFA concentrations on ovarian somatic cells have already been well-studied, including effects on bovine cumulus cells (Leroy *et al.*, 2005, Van Hoeck *et al.*, 2013a), granulosa cells (Jorritsma *et al.*, 2004, Vanholder *et al.*, 2005) and theca cells (Vanholder *et al.*, 2006). Additionally, the effects of elevated NEFA concentrations during final bovine oocyte maturation on oocyte developmental competence and embryo quality were assessed (Leroy *et al.*, 2005, Aardema *et al.*, 2011, Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a, Van Hoeck *et al.*, 2013b). Importantly, Britt (1992) hypothesized that bovine follicles grown during the period of negative energy balance early post-partum could be affected by the unfavourable metabolic changes (follicle development, activation and cyclicity). These follicles may therefore contain a developmentally incompetent or inferior oocyte. If such a follicle survives to a point where it reaches ovulation, its inferior oocyte may be ovulated and give rise to a low quality embryo. While efforts are being undertaken to establish a bovine follicle culture system (Jorssen *et al.*, 2014), the previously described studies were unable to investigate the effect of long term elevated NEFA concentrations, because of current (bovine) model-specific limitations in the exposure timeframe. Therefore, we relied on a different animal model, i.e. a mouse model, to study the effect of **long term elevated NEFA concentrations**, as present in individuals suffering from lipolytic disorders, on the **follicle as a whole** (**Chapters 6**). This model comprises the individual culture of murine early secondary pre-antral follicles until the antral stage *in vitro*, with the subsequent isolation and fertilization of the oocyte, followed by embryo culture. The murine follicle culture has been validated as a functional follicular unit, which much resembles the *in vivo* situation (Cortvrindt and Smitz, 1998, Cortvrindt and Smitz, 2001, Cortvrindt and Smitz, 2002). By using this model, both direct effects at the level of the COC and indirect effects, mediated through an altered granulosa cell and theca cell function, culminate in a final effect at the level of the oocyte. In this regard, the murine follicle culture model, is more appropriate to study the effects of elevated NEFAs on follicular development, with potential carry-over effects at the level of the oocyte, as described by Britt (1992), compared to previous bovine studies (Leroy *et al.*, 2005, Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a, Van Hoeck *et al.*, 2013b).

a) Effects on follicular growth and quality

We learned that exposure to high stearic acid (HIGH SA) concentrations reduced murine follicular diameters and development until the antral stage, while exposure to basal, physiological NEFA concentrations (BASAL NEFA), high oleic acid concentrations (HIGH OA) or a mixture of high stearic acid, oleic acid and palmitic acid (HIGH NEFA), did not affect **follicular development**. This observation

may be explained by the different physiological actions of saturated and unsaturated fatty acids. Saturated fatty acids, like palmitic and stearic acid, are almost universally toxic to cells in culture, while unsaturated fatty acids, like oleic acid, are often non-toxic or even cytoprotective (Nolan and Larter, 2009). The opposing effects of saturated and unsaturated fatty acids have been described in several cell types such as pancreatic β-cells (Cnop *et al.*, 2001, El-Assaad *et al.*, 2003), endothelial cells (Staiger *et al.*, 2006), cardiac myocytes (Miller *et al.*, 2005), breast cancer cell lines (Hardy *et al.*, 2003), hepatocytes (Ricchi *et al.*, 2009), oocytes (Aardema *et al.*, 2011) and now also whole follicles (Chapter 6). Besides the fact that HIGH OA exposure did not affect follicular development, combining high oleic acid with high stearic acid and high palmitic acid concentrations, reduced the negative effect caused by high stearic acid alone. This effect may be due to increased lipid storage, as storing saturated fatty acids in lipid droplets seems to be an initial cellular defence against lipotoxicity, that may be stimulated by unsaturated fatty acids (Cnop *et al.*, 2001, Listenberger *et al.*, 2003, Aardema *et al.*, 2011).

Our granulosa cell gene expression data furthermore suggest that **triglyceride formation** is decreased in HIGH OA follicles (decreased *Acaca* expression), compared to BASAL follicles and that less triglycerides are metabolized to glycerol and free fatty acids in BASAL follicles (lower *Hsl* expression), compared to HIGH NEFA and HIGH OA follicles. These results were somewhat unexpected, as it has been shown before that exposing oocytes to a mixture of NEFAs, including oleic, stearic and/or palmitic acid, induced increased lipid storage in neutral triglycerides in bovine oocytes and morulas (Aardema *et al.*, 2011, Van Hoeck *et al.*, 2013b). Furthermore, exposing bovine COCs to follicular fluid rich in NEFAs resulted in massive intracellular lipid storage in the cumulus cells (Aardema *et al.*, 2013). One question remains: How 'safe' are these lipid droplets? The ability to store NEFAs in lipid droplets has been proposed as a cytoprotective mechanism, by leading fatty acids away from other potential lipotoxic pathways (Cnop *et al.*, 2001, Li *et al.*, 2010). However, excessive accumulation of lipids in specialized cell types, such as adipocytes, macrophages and hepatocytes, may cause ER stress, which may contribute to the development of ER stress associated metabolic diseases like obesity, diabetes and fatty liver disease (Zhang and Zhang, 2012). In our study, it may be possible that we do not see signs of increased intracellular lipid storage, because the granulosa cells surpassed their capacity to store lipid droplets after the long term NEFA exposure, allowing fatty acids to be used in other fatty acid related pathways, like increasing β-oxidation and ceramide formation (Zhang and Zhang, 2012). However, more research is needed to confirm this hypothesis.

Treatment with HIGH SA also seemed to reduce **glucose uptake and consumption** by mural granulosa cells (decreased expression levels of *Slc2a1* and *Gapdh*), compared to BASAL and/or NEFA

treated follicles. However, these gene expression data shall need to be functionally analysed, because mRNA molecules are intermediate products in the synthesis of functional proteins and thus do not contain any information regarding post-translational modification or protein-protein interactions (Wolf *et al.*, 2003). Because stearic acid, as a saturated fatty acid, is not easily incorporated in triglycerides (Nolan and Larter, 2009), stearic acid is prone to be metabolized by means of β-oxidation and oxidative phosphorylation. Increased β-oxidation leads to the increased production of acetyl CoA, NADH, FADH2 and citrate, which inhibit pyruvate dehydrogenase (conversion of pyruvate to acetyl CoA) and phosphofructokinase (phosphorylation of fructose-6-phosphate to fructose-1,6-biphosphate during the glycolytic pathway for the conversion of glucose to 2 pyruvate and 1 NADH molecule) respectively (Zhang *et al.*, 2010). Furthermore, increased β-oxidation leads to the increased accumulation of β-oxidation by-products, that can interfere with insulin-signalling (Zhang *et al.*, 2010). Overall, these results on transcript abundance may be indicative for a reduced oxidation of glucose derived molecules for energy provision in the presence of elevated NEFAs (**Figure 2**). However, the gene product of *Slc2a1* (GLUT1) is responsible for facilitated glucose uptake by the cell and that of *Gapdh* serves as one of the enzymes to convert glucose to pyruvate. So, *strictu sensu*, these steps occur before glucose-derived pyruvate is chosen as a substrate for either oxidative phosphorylation or conversion to lactate. Importantly, these 'glucose intolerant' characteristics are also pre-programmed in the oocyte, as HIGH SA morula stage embryos consumed little or no glucose (see section c). The question remains whether these embryos cannot (impaired glucose metabolism) or will not (use other substrates) use glucose as a substrate.

Importantly, glucose is not only essential for proper granulosa cell function, it is also essential for COC function and oocyte maturation. The oocyte relies on its surrounding cumulus cells for the provision of glucose metabolites (bi-directional communication). During oocyte maturation, a large proportion of total glucose is metabolised via the glycolytic pathway to provide substrates such as pyruvate for energy production. However, glucose is also the substrate for many cellular functions during oocyte maturation, including regulation of nuclear maturation and REDOX state (glutathione provision) via the PPP and for the synthesis of substrates of extracellular matrices (cumulus expansion) and O-linked glycosylation (cell signalling) via the HBP (Sutton-McDowall *et al.*, 2010). So, the suggested decreased glucose uptake in HIGH SA granulosa cells, may have extensive consequences for oocyte developmental competence (**Figure 3**).

Furthermore, the NEFA induced increase in β-oxidation (oxidative metabolism) results in the accumulation of one undesired by-product, namely ROS leading to an increased **oxidative stress** (**Figure 2**), as also evidenced by the increased expression levels of *Sod1* in HIGH NEFA and *Gpx1* in HIGH SA exposed granulosa cells, compared to HIGH OA and BASAL granulosa cells, respectively.

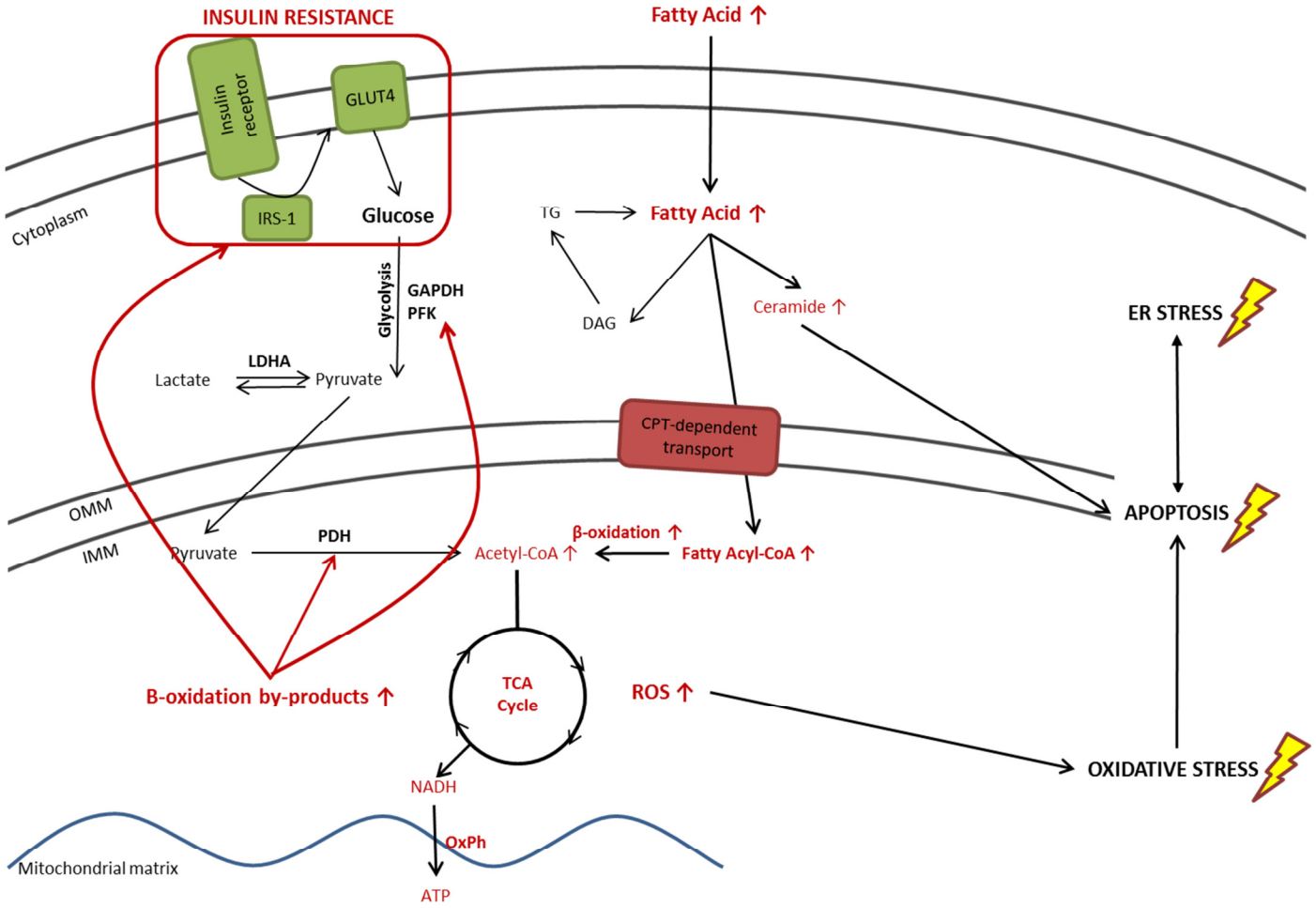


Figure 2: Proposed intracellular mechanism of action by which elevated NEFA concentrations may affect cell function and survival.
GLUT4: glucose transporter type 4, IRS-1: Insulin receptor substrate 1, TG: triglyceride, DAG: diacylglycerol, GAPDH : glyceraldehyde-3-phosphate dehydrogenase, PFK : phosphofructokinase, LDHA : lactate dehydrogenase A, PDH: pyruvate dehydrogenase, CPT: carnitine palmitoyl transferase 1, TCA: tricarboxyacid, ROS: reactive oxygen species, OxPh: oxidative phosphorylation, OMM: outer mitochondrial membrane, IMM: inner mitochondrial membrane.

If functionally translated, these gene products may serve to decrease oxidative stress to protect the oocyte. If bovine cumulus cells, however, fail to protect the oocyte from oxidative stress, the oocyte itself actively increases its defence against oxidative stress (increasing expression of *GPX1* and *LDHA*), as a compensatory mechanism (Van Hoeck *et al.*, 2013a).

In a very recent review by Turner and Robker (2015), evidence is provided that oocytes from obese or insulin-resistant mice exhibit abnormalities in mitochondrial parameters, like morphology and membrane potential. They describe findings that support a link between mitochondrial dysfunction and insulin resistance and propose that abnormalities in mitochondrial metabolism in oocytes may predispose the development of obesity and insulin resistance and thus contribute to the inter-generational programming of metabolic disease.

Like oxidative stress (Miller *et al.*, 2005), also **endoplasmic reticulum (ER) stress** may be involved in the NEFA induced cellular apoptosis by impairing mitochondrial function (Laybutt *et al.*, 2007). In this regard, mouse COCs exposed to lipid-rich follicular fluid had increased expression of ER stress genes in association with impaired nuclear maturation (Yang *et al.*, 2012). Co-treatment with an ER stress inhibitor during murine oocyte maturation reversed impaired cumulus expansion, altered oocyte mitochondrial activity and poor embryo development, induced by high levels of palmitic acid (Wu *et al.*, 2012), suggesting that the detrimental effects of the high lipid environment were mediated through a classic ER stress pathway. Disruption of the ER homeostasis results in the accumulation of unfolded or misfolded proteins. To cope with this stress, cells activate the unfolded protein response (UPR), a signal transduction systems that links the ER lumen with the nucleus and the cytoplasm (Ozcan *et al.*, 2004). When prolonged or exaggerated, this UPR may trigger β-cell apoptosis (Cnop *et al.*, 2007) and may interfere with the insulin-signalling cascade, potentially contributing to the development of insulin resistance (Ozcan *et al.*, 2004). In liver cells, palmitate and stearate induced ER stress and apoptosis, while unsaturated fatty acids did not (Wei et al., 2006). Interestingly, the study of Yoon *et al.* (2014) showed that *in vitro* bovine early embryo development was dependent on the orchestration between ROS and ER stress. ER stress increased in the presence of high ROS, but both were improved by reducing O_2 tension (reducing ROS) and by treatment with either an antioxidant or ER stress inhibitor. Studies in several cell types also demonstrated that palmitic acid can be converted to **diacylglycerol** and **ceramide**, instead of triglyceride accumulation, potentially contributing to the acquisition of insulin resistance (Zhang *et al.*, 2010) and apoptosis (Maedler *et al.*, 2001, Lu *et al.*, 2003), respectively (**Figure 2**). Interestingly, liver cells exposed to saturated fatty acids displayed increased ceramide concentrations, even though the inhibition of *de novo* ceramide synthesis did not prevent saturated fatty acid-induced ER stress and apoptosis (Wei *et al.*, 2006). These data suggest that saturated fatty acids disrupt ER homeostasis and induce apoptosis in liver cells via mechanisms that do not involve ceramide accumulation. Exposure to HIGH OA seemed to

reduce **apoptosis** in Day 13 granulosa cells (Chapter 6), since the expression of *Tp53*, *Bax* and the ratio *Bax/Bcl2* was significantly lower in HIGH OA granulosa cells, compared to BASAL and HIGH NEFA cultured follicles. The observation that *Gadd45b* expression was upregulated in HIGH OA granulosa cells substantiates this, since it is a cell cycle checkpoint regulator that is proposed to be involved in the prevention of apoptosis or DNA damage in granulosa cells of bovine dominant follicles (Mihm *et al.*, 2008). Again, these data were not functionally analysed, so assumptions must be made with caution.

As these results indicate that follicular growth itself was only moderately affected by NEFA treatment, we assume that follicle cells adapted to the elevated NEFA concentrations. Whether the embryos resulting from the exposed follicles are also able to survive after or adapt to the follicular NEFA exposure, will be discussed below.

b) Effects on follicle steroidogenesis

We showed that murine follicular steroidogenesis is affected by prolonged elevated NEFA concentrations (both functional and gene expression data, **Figure 3**). Even though it has been described that both bovine granulosa (Vanholder *et al.*, 2005) and theca cell (Vanholder *et al.*, 2006) steroid production is affected by elevated NEFA concentrations, this is the first time that the (murine) follicle is evaluated as a whole. The presented results are thus a reflection of the response of the follicle as a functional unit to these elevated NEFA concentrations. A detailed discussion on the potential mechanisms involved, is provided in Chapter 6. Overall, these data are in agreement with the hypothesis of Britt, stating that the health and steroidogenic capacity of a (bovine) follicle are determined by the specific biochemical environment during the long period of follicular growth prior to ovulation (Britt, 1992). Furthermore, after ovulation, these follicles may be less capable of producing adequate amounts of estrogens and progesterone to sustain early embryonic growth (Britt, 1992). Even more so, if follicular growth and maturation in these conditions results in ovulation, bovine oocyte quality may be impaired (see General introduction). Our results substantiate this, since the murine oocytes resulting from follicles that do present an altered steroidogenic profile, have an impaired developmental competence (see next section). Importantly, for a follicle to produce steroids in an adequate way, a functional communication between granulosa and theca cells is necessary (Boron and Boulpaep, 2005). Even though we have no data substantiating this, a miscommunication between theca and granulosa cells may be involved in the altered steroid profile of NEFA exposed follicles.

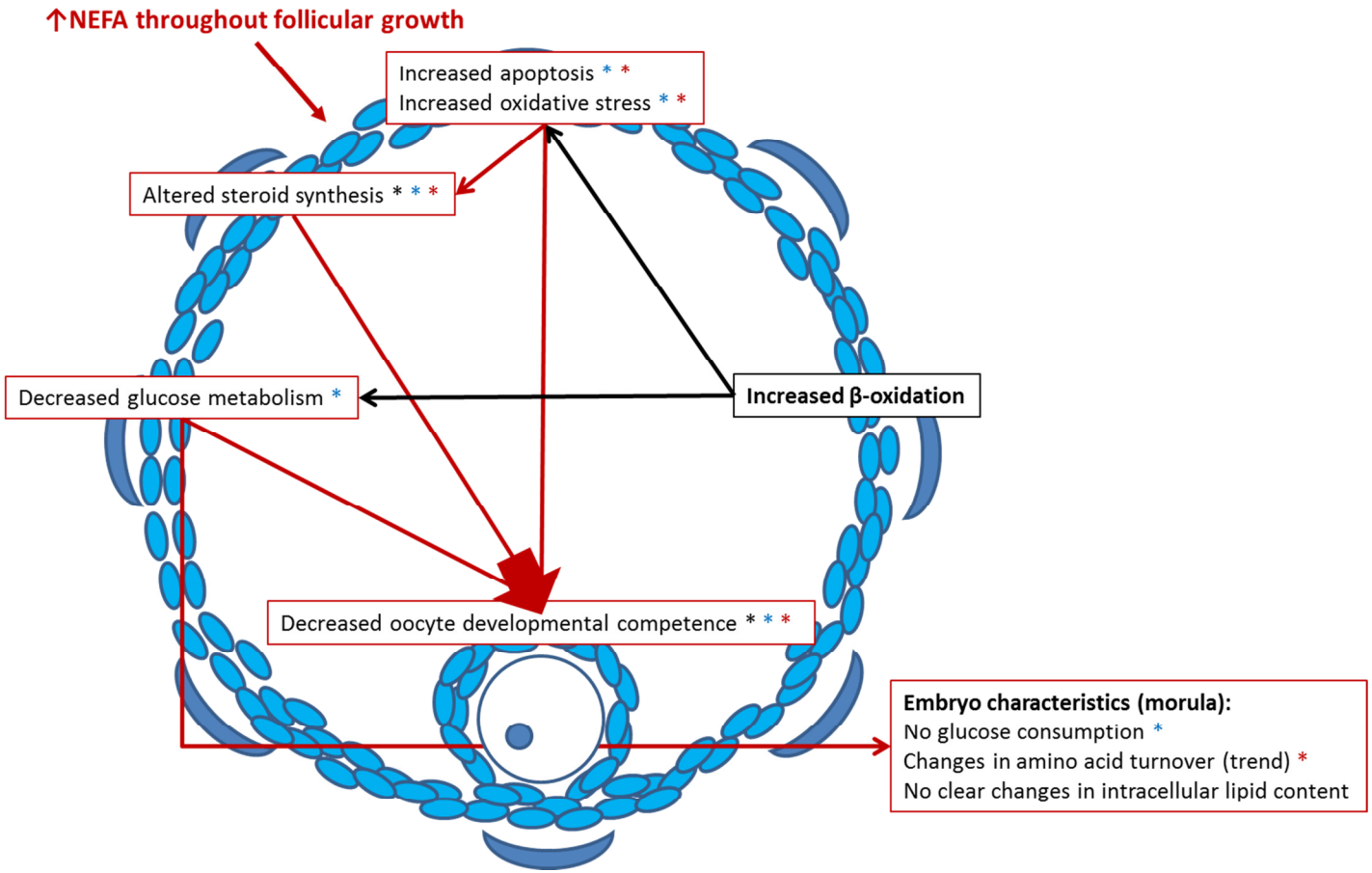


Figure 3: Schematic representation of proposed mechanisms of actions involved in the negative effects elicited by high NEFA concentrations during murine *in vitro* follicle culture. Specific results, described in this thesis are specified per treatment: black stars for HIGH OA treatment, blue stars for the HIGH SA treatment and red stars for the HIGH NEFA treatment. Long term elevated NEFA concentrations throughout follicular growth increase β-oxidation in granulosa cells, which increases the levels and ROS and oxidative stress and also decreases glucose metabolism. Increased oxidative stress and apoptosis in granulosa cells may directly contribute to a decreased oocyte developmental competence, or they may affect follicle steroid synthesis. An altered steroid synthesis may directly affect oocyte maturation and developmental competence. Furthermore, a decreased glucose metabolism in the granulosa cells may affect oocyte developmental competence and have consequences for glucose metabolism in the resultant embryos. Embryos may furthermore display an altered amino acid metabolism.

c) Effects on oocyte developmental competence and embryo quality

As stated before, embryos have an impressive capacity to adapt to their environment (Leese *et al.*, 2008). However, it remained to be investigated how the resultant embryos are affected by NEFA exposure during the whole period of follicular oocyte growth and maturation *in vitro*. Exposing murine follicles to elevated NEFA concentrations throughout follicular growth resulted in severely impaired oocyte developmental competence (**Figure 3**). So, even though follicular development was not or only moderately affected, embryo development was greatly reduced by HIGH SA, HIGH OA and HIGH NEFA treatments. It thus appears that unsaturated fatty acids, like oleic acid, can also be detrimental at high doses. In agreement, short term exposure of maturing bovine oocytes to high doses of oleic acid delayed progression to MII and reduced subsequent fertilization, cleavage and embryo development (Jorritsma *et al.*, 2004). Similarly, linoleic and linolenic acid, the latter only at supra-physiological concentrations, reduced cumulus expansion and impaired bovine oocyte maturation (Marei *et al.*, 2009, Marei *et al.*, 2010). Linoleic acid has a similar inhibitory effect on murine embryos (Nonogaki *et al.*, 1994). We also showed that murine cleaved zygotes originating from BASAL and HIGH NEFA oocytes sustained early embryo development better after the first cleavage division and thus after embryonic genome activation, compared to HIGH SA and/or HIGH OA embryos, confirming the importance of a well-balanced ratio between saturated and unsaturated fatty acids.

When looking at the murine embryos that were successfully fertilized and reached the 4- to 8-cell stage (**Chapter 7**), we learned that embryos originating from HIGH SA exposed follicles, consumed little or no **glucose**. In agreement, bovine blastocysts presented with a reduced oxygen, pyruvate and glucose consumption, an upregulated lactate production and a higher amino acid metabolism in response to elevated NEFA concentrations during final oocyte maturation (Van Hoeck *et al.*, 2011). Glucose, within the embryo is used aerobically to produce large amounts of ATP, but also anaerobically, with the production of lactate and few ATP. This previously explained Warburg effect, typical for both early embryos and cancer cells, may result in an increased production of glycolytic intermediates, like glucose-6-phosphate, that are important for the supply of the PPP (Vander Heiden *et al.*, 2009, Smith and Sturmey, 2013). The decreased oxygen, pyruvate and glucose consumption of bovine embryos points to a decreased oxidative metabolism and inhibition of the Warburg effect. On the contrary, the increased lactate production may be indicative for an increased turnover of glucose to lactate for the supply of the PPP. This together with a higher amino acid metabolism suggest that the embryos originating from *in vitro* NEFA exposed oocytes modulate their metabolism as an adaptive survival mechanism (Leese *et al.*, 2008).

As previously described (General introduction), increased NEFA concentrations may interfere with the insulin signalling cascade, leading to a decreased cellular glucose uptake (pathological mechanism) (Zhang *et al.*, 2010). However, a very recent study in mice proposed that glucose metabolism may be adapted to meet the energetic needs of the COC (physiological mechanism). More specifically, it was shown that inhibition of β-oxidation during murine COC maturation increased glucose consumption and stimulation of β-oxidation decreased glucose consumption and lipid storage (Paczkowski *et al.*, 2014). In another study from the same authors, etomoxir treatment (potent inhibitor of β-oxidation) at concentrations that did not inhibit nuclear maturation in bovine and murine COCs, increased glucose consumption (Paczkowski *et al.*, 2013). Also, glucose metabolism in pig embryos is increased when the embryos are denied the opportunity to metabolize lipids by means of methyl palmoxirate (Sturmey and Leese, 2008). So it seems that both COCs and embryos from a variety of species align glucose and fatty acid oxidation, in order to provide optimal energy substrates. Overall, our results strengthen the idea that the HIGH SA treatment during follicular growth causes an upregulation of β-oxidation in murine morulas, with a resulting decreased glucose consumption (via pathological or physiological mechanisms, Chapter 7). Strikingly, the same 'glucose intolerant' feature was proposed in granulosa cells originating from NEFA exposed follicles (Chapter 6), which suggests that the follicle's somatic cells may predispose some kind of metabolic profiling in the embryo.

By using a Nile Red staining, it has been shown that porcine oocytes have 2.4 times more **intracellular lipid** than bovine oocytes, which in turn have 2.8 fold more than mouse oocytes (Genicot *et al.*, 2005). We used this previously validated staining (Genicot *et al.*, 2005, Leroy *et al.*, 2005) to investigate the intracellular neutral lipid content in murine morulas, originating from NEFA exposed *in vitro* cultured follicles. We were, however, unable to detect any statistical significant differences in intracellular fat volume, which is probably due to a higher biological inter-oocyte variation within groups and a limited number of observations. Nevertheless, increased lipid accumulation has been associated with suboptimal mitochondrial function and a deviation in the relative abundance of developmentally important gene transcripts from stress responsive genes (Abe *et al.*, 2002, Rizos *et al.*, 2003, Leroy *et al.*, 2008). In bovine blastocysts originating from NEFA exposed oocytes, transcriptome analyses revealed an upregulation of genes related to lipid synthesis (Van Hoeck *et al.*, 2013b), which might be seen as a first coping mechanisms to shuttle fatty acids away from lipotoxic pathways.

Importantly, in most studies where β-oxidation is blocked, oocyte developmental competence is impaired (Dunning *et al.*, 2010, Dunning *et al.*, 2014). However, when β-oxidation of bovine oocytes exposed to elevated NEFA concentrations was blocked by means of β-mercapto-acetate, oocyte

developmental competence was rescued (Van Hoeck *et al.*, 2013a). This shows that it is the increased β-oxidation that is responsible for the developmental failure seen in embryos from oocytes exposed to elevated NEFA concentrations during final oocyte maturation. However, it is important to note that hormone induced oocyte maturation in mice requires a protein kinase A dependent increase in fatty acid oxidation (Valsangkar and Downs, 2013). It thus appears that physiological and pathological fatty acid oxidation mechanisms lie closely together and may even intertwine in case of high NEFA conditions.

Although many studies have focused on the effect of supplementing culture medium with amino acids on embryo development (Gardner and Lane, 1996, Lane and Gardner, 1998), much research has also investigated **amino acid turnover** as a potential marker for embryo quality and developmental potential (Houghton *et al.*, 2002, Sturmey *et al.*, 2008a, Sturmey *et al.*, 2010). Amino acid profiling has even been successfully used as a non-invasive marker for DNA damage in porcine and bovine blastocysts, as well as Day 2-3 human embryos (Sturmey *et al.*, 2009b). In this regard, amino acid analyses of the spent medium of murine morulas, originating from NEFA exposed follicles (Chapter 7) showed that embryos originating from HIGH NEFA exposed follicles had a higher overall amino acid production (trend). We however could not find any differences in individual amino acid turnover. These results are somewhat unexpected, because previous research in bovine embryos, originating from NEFA exposed oocytes, has shown plenty differences in both individual and overall amino acid turnover (Van Hoeck *et al.*, 2011). Nevertheless, overall embryo amino acid turnover in our study is in the same order of magnitude as those described in other studies (Lamb and Leese, 1994, Wale and Gardner, 2012), so the lack of statistical differences in individual amino acid turnover, is again probably attributable to a high biological inter-oocyte variation after an extended *in vitro* culture period. Overall, the increased amino acid production in HIGH NEFA embryos does agree with Leese's quiet embryo hypothesis (Leese *et al.*, 2008), but more in depth research into the metabolic activity of embryos derived from NEFA exposed follicles, is needed to clarify these results.

Overall, the data described in Chapters 6 and 7 show that follicular physiology is altered by follicle culture under high NEFA conditions. This ultimately results in a decreased oocyte development competence and an altered embryo metabolism.

d) Short versus long term NEFA exposure

Previous research has shown that short term elevated NEFA concentrations, during bovine *in vitro* maturation, are detrimental to oocyte and embryo quality (Leroy *et al.*, 2005, Aardema *et al.*, 2011, Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a, Van Hoeck *et al.*, 2013b). In this thesis, we showed

that long term elevated NEFA concentrations were also detrimental for murine oocyte developmental competence and embryo quality (Chapters 6 and 7). However, it remained unclear what specific time frame in follicular development is the most sensitive to a metabolic insult. In other words, it was questioned whether a long term exposure of whole follicles *in vitro* provokes a similar effect on oocyte developmental competence and embryo quality, compared to a short term exposure, only during the final phase of oocyte maturation. Therefore, we studied the difference between long term NEFA exposure throughout follicular growth and short term NEFA exposure, only during the final maturation phase. To be able to investigate this, it was essential to remove 100% of the follicle culture medium on Day 12 of culture. This is a challenging procedure, as is leaves the follicles without any nutrients for a limited period of time. Furthermore, it was essential to use conditioned medium, as the follicles have an *in vivo* like steroidogenic capacity at that stage (Day 12 of culture), which influences follicle growth and the responsiveness to the ovulatory stimulus.

We showed, for the first time, that long term elevated NEFA concentrations had a more pronounced negative effect on oocyte developmental competence, compared to a short term NEFA exposure. This was evidenced by the observation that both short term HIGH NEFA and HIGH SA treatment presented a better blastocyst formation than long term HIGH NEFA and HIGH SA treatments, an effect specific for the type of NEFA added. Even though preliminary experiments showed a good embryo development after the control treatment (basal, physiological NEFA concentrations throughout culture), this control in the actual experiments presented surprisingly low blastocyst rates. Caution is thus warranted when interpreting these results, because the removal of 100% of the medium on Day 12 of culture may possibly affect oocyte developmental competence. However, **these preliminary data do suggest that the maternal micro-environment throughout follicular growth and not only during final oocyte maturation is essential for optimal oocyte quality. Furthermore, long term exposure of follicles to potential toxicants may result in a more detrimental outcome than short term exposure, limited to the final phase of maturation.** These *in vitro* data, generated by using a murine follicle culture model, may be relevant for women suffering from lipolysis-linked metabolic disorders, like obesity, trying to get pregnant, as they suggest that adverse metabolic health throughout follicular growth has a more severe effect on oocyte developmental competence, than only during final oocyte maturation, which poses for example obese women at a higher risk of ovulating a low quality oocyte. These results are also relevant for other animal settings, like the cow in negative energy balance. Furthermore, our observations also imply that acute elevated NEFA concentrations, for example induced by acute stress at the time of ovulation and conception, induce a less severe effect on oocyte developmental competence, compared to when these stressful conditions are present for a longer period of time.

3. Increasing β-oxidation: friend or foe?

Many studies report on the beneficial effect of increasing β-oxidation, for example by means of supplementing L-carnitine, on oocyte developmental competence and embryo quality (Dunning *et al.*, 2010, Dunning *et al.*, 2011). This effect is proposed to be mediated by a reduction in the amount of intracellular lipid available for damage by oxidative stress. Indeed, supplementing porcine oocyte maturation with L-carnitine improved oocyte maturation, reduced intracellular ROS levels and increased the concentration of glutathione in porcine oocytes (Wu *et al.*, 2011). In contrast, increasing β-oxidation by an excess of fatty acids, as proposed in our work and that of others, is related to increased lipid storage, increased oxidative stress and reduced oocyte and embryo quality (Leroy *et al.*, 2005, Van Hoeck *et al.*, 2013b). In both cases, increased β-oxidation leads to the increased production of ROS. However, when using L-carnitine, this does not seem to affect developmental competence, while excess of fatty acids does have a detrimental effect. There may be two reasons for this. First, β-oxidation in case of high NEFA exposure does not decrease intracellular lipid storage, but is upregulated to cope with the excess of exogenously provided fatty acids. Second, the action of L-carnitine is 2-fold: it increases β-oxidation and it functions as an anti-oxidant. So, it is possible that the positive effects of L-carnitine may be mediated mainly by its anti-oxidant function, neutralizing any excess ROS produced.

4. Potential implications for post-natal health

The observation that embryo metabolism may be affected by NEFA exposure during follicular growth is of pivotal importance, because it illustrates that the metabolism of the embryo is influenced by the environment in which the oocyte developed for an extended period of time. Recent studies from our laboratory have indicated that elevated NEFA concentrations during maturation may affect epigenetic mechanisms in the resultant embryo (Van Hoeck *et al.*, 2011, Van Hoeck *et al.*, 2013a, Desmet *et al.*, 2014), which may have consequences for further development. Substantiating this, a study performed by Jungheim *et al.* (2011b), showed that preimplantation exposure of mouse embryos to palmitic acid results in fetal growth restriction followed by catch-up growth in the offspring. Interestingly, in humans it has been shown that decreased fetal size is associated with β-cell hyperfunction in early life and failure with age (Chakravarthy *et al.*, 2008), suggesting an intra-uterine signal for adult onset type II diabetes. Overall, these findings corroborate with the 'developmental origin of health and disease' (DOHaD) principle, often called the Barker hypothesis, stating that adverse influences early in development, and particularly during intrauterine life, can result in permanent changes in physiology and metabolism, which result in increased disease risk in adulthood (Barker and Osmond, 1986, Barker *et al.*, 1989, de Boo and Harding, 2006).

5. Potential confounders in studying the relation between maternal metabolism, oocyte developmental competence and subsequent embryo quality

a) Contribution of sperm

The contribution of sperm to the embryo consists mainly of genetic material, but also oocyte activation factor, centrosomes (not in mice) and little mRNA which are crucial for further development (Kumar *et al.*, 2013). Male obesity may affect sperm concentration (Jensen *et al.*, 2004, Koloszar *et al.*, 2005), motility (Braga *et al.*, 2012), morphology (MacDonald *et al.*, 2010) and the degree of DNA damage (Chavarro *et al.*, 2010). Importantly, several studies in mice report a negative effect of sperm with DNA damage on embryo quality and subsequent offspring's health (Fernandez-Gonzalez *et al.*, 2008, Ramos-Ibeas *et al.*, 2014). Even more so, several recent studies have pointed out that male obesity may directly affect embryo physiology. McPherson *et al*. (2014) propose two main theories for the origin of male obesity-induced paternal programming in the embryo: 1) accumulation of sperm DNA damage resulting in *de novo* mutations in the embryo and 2) changes in sperm epigenetic marks (microRNA, methylation or acetylation) altering the access, transcription and translation of paternally derived genes during early embryogenesis. "You are what your dad ate" by Ferguson-Smith and Patti (2011), describes in a short preview that the dietary or metabolic history of males affects the metabolism of the offspring. These phenotypes are likely mediated by sperm, potentially via epigenetic marks in germ cells (Ferguson-Smith and Patti, 2011). In Chapters 3, 4 and 5, we only have limited data on sperm quality (morphology, motility and sperm count in the ejaculate), but we do not have any knowledge about intrinsic sperm quality. Furthermore, in Chapters 6 and 7, we (only) evaluated sperm concentration and motility as quality parameters, even though we did pool the spermatozoa of two males of proven fertility in the described experiments. This emphasizes that intrinsic sperm quality can be a potential confounder in our studies.

b) Sex of the embryo

The sex of the embryo is a potential confounder. Male and female preimplantation mammalian embryos differ not only in their chromosomal constitution, but in their proteome and subsequent metabolome as well. In this regard, it has been shown that male and female embryos develop until the blastocyst stage at different rates, have a different capacity to respond to the maternal environment (Erickson, 1997) and respond to ROS differently (Perez-Crespo et al., 2005). Most importantly, sex-specific differences in glucose and amino acid metabolism (bovine and murine) (Gardner *et al.*, 2010, Sturmey *et al.*, 2010) as well as sex specific epigenetic changes in bovine

blastocysts (Bermejo-Alvarez *et al.*, 2008) have been described. Many other studies have looked at sex specific differences in embryo phenotype, genotype and subsequent offspring's health (for reviews, see Bermejo-Alvarez *et al.*, 2011, Bermejo-Alvarez *et al.*, 2012, Laguna-Barraza *et al.*, 2013). Especially because elevated NEFA concentrations have been associated with for example the generation of ROS, glucose metabolism and *de novo* DNA methylation, the sex of the embryos may be a confounder in our *in vitro* studies.

c) Isolating NEFAs from the maternal micro-environment

It is also important to mention that we specifically chose to study the effect of elevated NEFA concentrations and not the effect of fatty acids in other lipid fractions. The main advantage is that we are able to study the isolated effect of elevated NEFAs. However, *in vivo*, elevated NEFA concentrations are present in parallel with fatty acids in different lipid fractions. For example, dyslipidemia in obesity is associated with changes in triglycerides, cholesterol and free fatty acids (Klop *et al.*, 2013), with a continuous interplay between them (ex. NEFAs can be captured in other lipid fractions). It is difficult to find a good balance between mimicking the *in vivo* situation as closely as possible and trying to define etiologic mechanisms and pathways. The *in vitro* data described in this thesis must be evaluated next to a body of literature comprising both *in vivo* and *in vitro* trials that describe complementary results, to attain their maximal value.

d) The choice of NEFAs

In our experiments, we exposed the follicles to palmitic, stearic and/or oleic acid in concentrations that mimic basal physiological or pathological serum NEFA concentrations. These fatty acids and their concentrations were based on previous bovine data from Leroy *et al*. (2005). Because these bovine data might not be completely relevant to the situation in women, in Chapter 3, we showed that serum NEFA concentrations in obese women are in the same order of magnitude as the HIGH NEFA concentrations used in our *in vitro* studies. Preliminary studies in mice also showed serum NEFA concentrations in the same order of magnitude. In Chapter 3, we also showed a significant correlation between serum and follicular fluid NEFA concentrations. Furthermore, Chapter 5 strengthens the choice of individual NEFAs, as it was shown that palmitic, stearic and oleic acid are predominant NEFAs in human follicular fluid. It is, however, important to mention that the concentrations used in our *in vitro* studies, are based on serum and not follicular fluid concentrations, because *in vivo*, follicles as a biological unit are exposed to serum concentrations.

e) The use of serum in *in vitro* culture systems

The use of serum in *in vitro* culture systems has been a matter of debate for years and avoiding the use of serum is often aimed for. We adopted an already existing and validated *in vitro* murine culture model that contains serum in the culture medium. Even though much progress has been made, a routine serum free murine follicle culture system that allows the high throughput needed for these experiments, is currently lacking. However, serum may contain variable amounts of fatty acids in different lipid fractions. Therefore, we used the same batch of serum for every experiment. We also biochemically analysed our serum, which showed that our FBS, added in a concentration of 5%, contains 110 µM of NEFAs, thus only accounting for 5.5 µM in our final culture medium (5% FBS). In addition to that, we analysed our medium samples for total NEFA content, to ensure that the follicles were exposed to the intended NEFA concentrations.

f) Experimental controls

In our *in vitro* research, we used the BASAL treatment as a physiological control in the experiments. This BASAL treatment consists of a mixture of palmitic, stearic and oleic acid in normal physiological NEFA concentrations. By performing preliminary research, we showed that the BASAL treatment, the SOLVENT CONTROL and the routine CONTROL provided similar results concerning follicular and embryonic development. However, one criticism that may be posed is that we did not use the SOLVENT CONTROL or CONTROL to investigate all end points evaluated (such as gene expression, steroid synthesis etc.). We do believe that the BASAL treatment as a control is more valuable from a physiological point of view, because it more closely resembles the *in vivo* situation. We furthermore never implemented an *in vivo* control. *In vivo* maturation followed by *in vitro* fertilization and embryo culture only serves as an internal control for the murine IVF system. Instead, a threshold of 70% good follicular growth and 50% blastocyst development in the control group, always needed to be attained for the experiment to be considered valid.

6. Biological relevance for women trying to get pregnant

It is now generally accepted that metabolic disorders, like obesity and type II diabetes are risk factors for subfertility. More specifically, there is a growing amount of evidence that points to the potential role of an impaired oocyte developmental competence and embryo quality (Robker, 2008). The results described in this thesis contribute to the understanding of how maternal metabolic disorders may impair fertility. Overall, it is accepted that the serum composition is reflected in the ovarian follicular fluid (Chapters 3 and 5). Differences in the composition of the follicular fluid can impact on oocyte developmental competence and subsequent embryo quality (short term exposure, Chapter 4). Particularly long term elevated NEFA concentrations, as a result of increased lipolysis, can have profound negative effects. Our results suggest that follicular physiology may be altered, even though no cycle irregularities are to be expected (minor effect on follicular growth in Chapter 6). However, if an oocyte is ovulated, oocyte developmental competence may be severely impaired and subsequent embryo metabolism and quality may be altered (Chapters 6 and 7). Our results become particularly relevant in light of very recent observations in superovulated dairy cows postpartum. Here, no effect of body weight change during the first three weeks after calving was seen on the number of ovulations, total number of embryos collected and percentage of oocytes that were fertilized (after a standard superstimulation treatment 10 weeks later). However, more loss of body condition was related to a reduced potential of fertilized oocytes to develop to transferable stages and a higher proportion of degenerated embryos (Carvalho *et al.*, 2014). These bovine *in vivo* results corroborate with our *in vitro* findings on murine follicular development, cleavage and blastocyst formation (Chapter 6), suggesting that elevated NEFA *in vivo* may contribute partly to subfertility.

Reducing body weight is often encouraged in obese women and in some fertility centres even mandatory to start fertility treatment. Many researchers also share the common believe that ART treatment should have restrictions based on bodyweight, even though conflicting studies prevent such ‘measure’ to be officially taken (reviewed by Koning *et al.*, 2012). It has been suggested that even modest weight loss and lifestyle improvement may improve anovulatory fertility (Clark *et al.*, 1995, Norman *et al.*, 2004). However, in light of our observations, assessing the risks and benefits of weight loss before infertility treatment is necessary, because weight loss itself may influence the oocyte’s developmental capacity by inducing metabolic changes in the serum (increased lipolysis, elevated NEFA) and thus the ovarian micro-environment.

7. Ethics in fertility treatment and research

Since 1978, it has been possible for couples that cannot conceive naturally, to rely on techniques of *in vitro* fertilization. Many of these women subsequently get pregnant and a seemingly healthy baby is born. However, upcoming research has been focussing on the health of **children born from IVF or ICSI**. For example, IVF-conceived children have lower birth weights and higher peripheral fat, blood pressure and fasting glucose concentrations than spontaneously conceived children (Ceelen *et al.*, 2008). In contrast, no difference in growth, development and cognitive function was found between ART children and controls (Wagenaar *et al.*, 2008). Imprinting disorders have been documented in ART children, but not to a worrisome degree (reviewed by Fauser *et al.*, 2014). Furthermore, no increased risk for cancer has been documented in children born from ART, compared to naturally conceived children (Sundh *et al.*, 2014). Overall, a direct link between assisted reproduction and health-related outcomes in ART children could not be established so far (Fauser *et al.*, 2014). However, as most children born from ART are only now coming to adolescence, highly powered epidemiologic research is only recently emerging. Importantly, because most ART children have only now reached reproductive age, the question remains whether the infertility suffered by their parents, may persist into the children. In this regard, it might be argued that our technology has exceeded our humanity. We do not know what consequences IVF techniques have for long term health and reproductive function, even though there are many data from animal models. Studies using animal models have indeed provided us with information on the effect of maternal health around conception and early embryonic growth on the offspring's health and help us understand the potential mechanisms involved. Another ethical question poses when obese women, that cannot conceive naturally, sign up for fertility treatment. In most cases, obesity is preventable (World Health Organization, 2014), which means that in those cases where infertility is caused by obesity, the fertility problem may be cured by losing weight. Again, animal models and *in vitro* research have provided much information, where human material was scarce or unavailable and has warned us on the potential negative impact of lipolysis-linked metabolic disorders, like obesity, on oocyte and embryo quality.

8. Conclusions

In this thesis, we used several animal models to investigate the complex interaction between maternal metabolic disorders and fertility. We showed that the serum composition of women undergoing ART was reflected in the follicular fluid and that BMI was associated with both the serum and follicular fluid composition (Chapter 3). Adding human follicular fluid during bovine *in vitro* oocyte maturation, was able to change bovine oocyte developmental competence and subsequent embryo quality, indicating that the follicular fluid composition might be associated with embryo quality during ART (Chapter 4). Within the follicular fluid, fatty acids are mainly attributed to the phospholipid and cholesteryl-ester fraction, while only the triglyceride and NEFA fraction were associated with BMI (Chapter 5). The most predominant NEFAs in the serum and follicular fluid were used in the *in vitro* experiments described in this thesis. Long term elevated NEFA concentrations during follicular growth, affected follicular physiology, oocyte developmental competence and subsequent embryo metabolism (Chapters 6 and 7). From a clinical point of view, these data help elucidate mechanisms by which an altered maternal micro-environment, due to adverse metabolic health, associated with upregulated lipolysis, may lead to compromised follicular function, oocyte developmental failure and an altered embryo quality.

9. References

Aardema H, Lolicato F, van de Lest CH, Brouwers JF, Vaandrager AB, van Tol HT, Roelen BA, Vos PL, Helms JB and Gadella BM. Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. *Biol Reprod* 2013; **88**: 164.

Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB and Gadella BM. Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biol Reprod* 2011; **85**: 62-69.

Abe H, Yamashita S, Satoh T and Hoshi H. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Mol Reprod Dev* 2002; **61**: 57-66.

Adamiak S, Ewen M, Rooke J, Webb R and Sinclair KD. Diet and fatty acid composition of bovine plasma, granulosa cells, and cumulus-oocyte complexes. *Reprod Fert Develop* 2005; **17**: 200-201.

Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S and Sirard MA. Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro. *Biol Reprod* 2008; **79**: 209-222.

Barker DJ and Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1986; **1**: 1077-1081.

Barker DJ, Osmond C, Golding J, Kuh D and Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* 1989; **298**: 564-567.

Beam SW and Butler WR. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. *Biol Reprod* 1997; **56**: 133-142.

Bermejo-Alvarez P, Ramos-Ibeas P and Gutierrez-Adan A. Solving the "X'' in Embryos and Stem Cells. *Stem Cells Dev* 2012; **21**: 1215-1224.

Bermejo-Alvarez P, Rizos D, Lonergan P and Gutierrez-Adan A. Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease. *Reproduction* 2011; **141**: 563-570.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P and Gutierrez-Adan A. Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiol Genomics* 2008; **32**: 264-272.

Boron W and Boulpaep E. Medical Physiology, 2005. Elsevier Saunders, USA.

Braga DP, Halpern G, Figueira Rde C, Setti AS, Iaconelli A, Jr. and Borges E, Jr. Food intake and social habits in male patients and its relationship to intracytoplasmic sperm injection outcomes. *Fertil Steril* 2012; **97**: 53-59.

Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. *Proceedings of the Annual Convention of the American Association of Bovine Practitioners* 1992; **24**: 39-43.

Browne RW, Bloom MS, Shelly WB, Ocque AJ, Huddleston HG and Fujimoto VY. Follicular fluid high density lipoprotein-associated micronutrient levels are associated with embryo fragmentation during IVF. *J Assist Reprod Genet* 2009; **26**: 557-560.

Browne RW, Shelly WB, Bloom MS, Ocque AJ, Sandler JR, Huddleston HG and Fujimoto VY. Distributions of high-density lipoprotein particle components in human follicular fluid and sera and their associations with embryo morphology parameters during IVF. *Hum Reprod* 2008; **23**: 1884-1894.

Bunel A, Jorssen EPA, Merckx E, Leroy JLMR, Bols PEJ and Sirard MA. Individual bovine in vitro embryo production and cumulus cell transcriptomic analysis to distinguish cumulus-oocyte-complexes with high or low developmental potential. *Theriogenology* In press.

Carvalho PD, Souza AH, Amundson MC, Hackbart KS, Fuenzalida MJ, Herlihy MM, Ayres H, Dresch AR, Vieira LM, Guenther JN et al. Relationships between fertility and postpartum changes in body condition and body weight in lactating dairy cows. *Journal of Dairy Science* 2014; **97**: 3666-3683.

Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE and Delemarre-van de Waal HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin Endocrinol Metab* 2008; **93**: 1682-1688.

Chakravarthy MV, Zhu Y, Wice MB, Coleman T, Pappan KL, Marshall CA, McDaniel ML and Semenkovich CF. Decreased fetal size is associated with beta-cell hyperfunction in early life and failure with age. *Diabetes* 2008; **57**: 2698-2707.

Chavarro JE, Toth TL, Wright DL, Meeker JD and Hauser R. Body mass index in relation to semen quality, sperm DNA integrity, and serum reproductive hormone levels among men attending an infertility clinic. *Fertil Steril* 2010; **93**: 2222-2231.

Clark AM, Ledger W, Galletly C, Tomlinson L, Blaney F, Wang X and Norman RJ. Weight-Loss Results in Significant Improvement in Pregnancy and Ovulation Rates in Anovulatory Obese Women. *Hum Reprod* 1995; **10**: 2705-2712.

Cnop M, Hannaert JC, Hoorens A, Eizirik DL and Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 2001; **50**: 1771-1777.

Cnop M, Ladriere L, Hekerman P, Ortis F, Cardozo AK, Dogusan Z, Flamez D, Boyce M, Yuan J and Eizirik DL. Selective inhibition of eukaryotic translation initiation factor 2 alpha dephosphorylation potentiates fatty acid-induced endoplasmic reticulum stress and causes pancreatic beta-cell dysfunction and apoptosis. *J Biol Chem* 2007; **282**: 3989-3997.

Cortvrindt R and Smitz J. Early preantral mouse follicle in vitro maturation: Oocyte growth, meiotic maturation and granulosa-cell proliferation. *Theriogenology* 1998; **49**: 845-859.

Cortvrindt R and Smitz J. In vitro follicle growth: achievements in mammalian species. *Reprod Domest Anim* 2001; **36**: 3-9.

Cortvrindt RG and Smitz JEJ. Follicle culture in reproductive toxicology: a tool for in-vitro testing of ovarian function? *Hum Reprod Update* 2002; **8**: 243-254.

de Boo HA and Harding JE. The developmental origins of adult disease (Barker) hypothesis. *Aust N Z J Obstet Gynaecol* 2006; **46**: 4-14.

Desmet K, Van Hoeck V, Merckx E, Sirard MA, Bols PEJ and Leroy JLMR. Elevated non-esterified fatty acid concentrations during bovine embryo culture influences DNA methylation in blastocysts. . *EPICONCEPT Workshop 2014, Epigenomic Toolbox: from Methods to Models 07-09 May 2014, Las Palmas, Spain* 2014.

Dunning KR, Akison LK, Russell DL, Norman RJ and Robker RL. Increased beta-oxidation and improved oocyte developmental competence in response to l-carnitine during ovarian in vitro follicle development in mice. *Biol Reprod* 2011; **85**: 548-555.

Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ and Robker RL. Beta-Oxidation Is Essential for Mouse Oocyte Developmental Competence and Early Embryo Development. *Biol Reprod* 2010; **83**: 909-918.

Dunning KR, Russell DL and Robker RL. Lipids and oocyte developmental competence: the role of fatty acids and beta-oxidation. *Reproduction* 2014; **148**: R15-27.

El-Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L and Prentki M. Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* 2003; **144**: 4154-4163.

Erickson RP. Does sex determination start at conception? *Bioessays* 1997; **19**: 1027-1032.

Fauser BC, Devroey P, Diedrich K, Balaban B, Bonduelle M, Delemarre-van de Waal HA, Estella C, Ezcurra D, Geraedts JP, Howles CM et al. Health outcomes of children born after IVF/ICSI: a review of current expert opinion and literature. *Reprod Biomed Online* 2014; **28**: 162-182.

Ferguson-Smith AC, Patti ME. You are what your dad ate. *Cell Metabolism* 2011; **13**: 115-117.

Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, Hourcade JD, de Fonseca FR et al. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biol Reprod* 2008; **78**: 761-772.

Fouladi-Nashta AA, Gutierrez CG, Gong JG, Garnsworthy PC and Webb R. Impact of dietary fatty acids on oocyte quality and development in lactating dairy cows. *Biol Reprod* 2007; **77**: 9-17.

Fouladi-Nashta AA, Wonnacott KE, Gutierrez CG, Gong JG, Sinclair KD, Garnsworthy PC and Webb R. Oocyte quality in lactating dairy cows fed on high levels of n-3 and n-6 fatty acids. *Reproduction* 2009; **138**: 771-781.

Gardner DK and Lane M. Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. *Hum Reprod* 1996; **11**: 2703-2712.

Gardner DK, Larman MG and Thouas GA. Sex-related physiology of the preimplantation embryo. *Mol Hum Reprod* 2010; **16**: 539-547.

Genicot G, Leroy JLMR, Van Soom A and Donnay I. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. *Theriogenology* 2005; **63**: 1181-1194.

Gilchrist RB, Ritter LJ and Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. *Anim Reprod Sci* 2004; **82-83**: 431-446.

Gosden RG, Hunter RHF, Telfer E, Torrance C and Brown N. Physiological Factors Underlying the Formation of Ovarian Follicular-Fluid. *J Reprod Fertil* 1988; **82**: 813-825.

Haggarty P, Wood M, Ferguson E, Hoad G, Srikantharajah A, Milne E, Hamilton M and Bhattacharya S. Fatty acid metabolism in human preimplantation embryos. *Hum Reprod* 2006; **21**: 766-773.
Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A and Sirard MA. Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Hum Reprod* 2008; **23**: 1118-1127.
Hammiche F, Vujkovic M, Wijburg W, de Vries JHM, Macklon NS, Laven JSE and Steegers-Theunissen RPM. Increased preconception omega-3 polyunsaturated fatty acid intake improves embryo morphology. *Fertil Steril* 2011; **95**: 1820-1823.
Hardy S, El-Assaad W, Przybytkowski E, Joly E, Prentki M and Langelier Y. Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. *J Biol Chem* 2003; **278**: 31861-31870.
Hennet ML and Combelles CM. The antral follicle: a microenvironment for oocyte differentiation. *The International journal of developmental biology* 2012; **56**: 819-831.
Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ and Leese HJ. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod* 2002; **17**: 999-1005.
Hughes J, Kwong WY, Li D, Salter AM, Lea RG and Sinclair KD. Effects of omega-3 and -6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism. *Reproduction* 2011; **141**: 105-118.
Jaspard B, Fournier N, Vieitez G, Atger V, Barbaras R, Vieu C, Manent J, Chap H, Perret B and Collet X. Structural and functional comparison of HDL from homologous human plasma and follicular fluid. A model for extravascular fluid. *Arterioscler Thromb Vasc Biol* 1997; **17**: 1605-1613.
Jensen TK, Andersson AM, Jorgensen N, Andersen AG, Carlsen E, Petersen JH and Skakkebaek NE. Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fertil Steril* 2004; **82**: 863-870.
Jorritsma R, Cesar ML, Hermans JT, Kruitwagen CLJJ, Vos PLAM and Kruip TAM. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. *Anim Reprod Sci* 2004; **81**: 225-235.
Jorritsma R, de Groot MW, Vos PLAM, Kruip TAM, Wensing T and Noordhuizen JPTM. Acute fasting in heifers as a model for assessing the relationship between plasma and follicular fluid NEFA concentrations. *Theriogenology* 2003; **60**: 151-161.
Jorssen EPA, Langbeen A, Fransen E, Martinez EL, Leroy JLMR and Bols PEJ. Monitoring preantral follicle survival and growth in bovine ovarian biopsies by repeated use of neutral red and cultured in vitro under low and high oxygen tension. *Theriogenology* 2014; **82**: 387-395.
Jungheim ES, Louden ED, Chi MMY, Frolova AI, Riley JK and Moley KH. Preimplantation Exposure of Mouse Embryos to Palmitic Acid Results in Fetal Growth Restriction Followed by Catch-Up Growth in the Offspring. *Biol Reprod* 2011b; **85**: 678-683.
Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS and Moley KH. Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during in vitro fertilization. *Fertil Steril* 2011a; **95**: 1970-1974.
Kamarianos A, Karamanlis X, Goulas P, Theodosiadou E and Smokovitis A. The presence of environmental pollutants in the follicular fluid of farm animals (cattle, sheep, goats, and pigs). *Reprod Toxicol* 2003; **17**: 185-190.
Klop B, Elte JWF and Cabezas MC. Dyslipidemia in Obesity: Mechanisms and Potential Targets. *Nutrients* 2013; **5**: 1218-1240.
Koloszar S, Fejes I, Zavaczki Z, Daru J, Szollosi J and Pal A. Effect of body weight on sperm concentration in normozoospermic males. *Archives of andrology* 2005; **51**: 299-304.
Koning AMH, Mutsaerts MAQ, Kuchenbecker WKH, Broekmans FJ, Land JA, Mol BW and Hoek A. Complications and outcome of assisted reproduction technologies in overweight and obese women (vol 27, pg 457, 2012). *Hum Reprod* 2012; **27**: 2570-2570.
Kumar M, Kumar K, Jain S, Hassan T and Dada R. Novel insights into the genetic and epigenetic paternal contribution to the human embryo. *Clinics* 2013; **68 Suppl 1**: 5-14.
Laguna-Barraza R, Bermejo-Alvarez P, Ramos-Ibeas P, de Frutos C, Lopez-Cardona AP, Calle A, Fernandez-Gonzalez R, Pericuesta E, Ramirez MA and Gutierrez-Adan A. Sex-specific embryonic origin of postnatal phenotypic variability. *Reprod Fert Develop* 2013; **25**: 38-47.
Lamb VK and Leese HJ. Uptake of a mixture of amino acids by mouse blastocysts. *J Reprod Fertil* 1994; **102**: 169-175.

Lane M and Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998; **13**: 991-997.
Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV and Biden TJ. Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia* 2007; **50**: 752-763.
Leese HJ, Baumann CG, Brison DR, McEvoy TG and Sturmey RG. Metabolism of the viable mammalian embryo: quietness revisited. *Mol Hum Reprod* 2008; **14**: 667-672.
Leroy J, Sturmey R, Van Hoeck V, De Bie J, McKeegan P and Bols P. Dietary fat supplementation and the consequences for oocyte and embryo quality: hype or significant benefit for dairy cow reproduction? *Reprod Domest Anim* 2014; **49**: 353-361.
Leroy JL, Van Soom A, Opsomer G, Goovaerts IG and Bols PE. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part II. Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows. *Reprod Domest Anim* 2008; **43**: 623-632.
Leroy JLMR, Genicot G, Donnay I and Van Soom A. Evaluation of the lipid content in bovine oocytes and embryos with nile red: A practical approach. *Reprod Domest Anim* 2005; **40**: 76-78.
Leroy JLMR, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PEJ, Dewulf J and de Kruif A. Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. *Theriogenology* 2004; **62**: 1131-1143.
Leroy JLMR, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G and Van Soom A. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction* 2005; **130**: 485-495.
Li LO, Klett EL and Coleman RA. Acyl-CoA synthesis, lipid metabolism and lipotoxicity. *Biochim Biophys Acta* 2010; **1801**: 246-251.
Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Jr., Ory DS and Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci U S A* 2003; **100**: 3077-3082.
Lu ZH, Mu YM, Wang BA, Li XL, Lu JM, Li JY, Pan CY, Yanase T and Nawata H. Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. *Biochem Bioph Res Co* 2003; **303**: 1002-1007.
Macaulay AD, Gilbert I, Caballero J, Barreto R, Fournier E, Tossou P, Sirard MA, Clarke HJ, Khandjian EW, Richard FJ et al. The Gametic Synapse; RNA Transfer to the Bovine Oocyte. *Biol Reprod* 2014.
MacDonald AA, Herbison GP, Showell M and Farquhar CM. The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. *Hum Reprod Update* 2010; **16**: 293-311.
Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N and Donath MY. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes* 2001; **50**: 69-76.
Marei WF, Wathes DC and Fouladi-Nashta AA. The Effect of Linolenic Acid on Bovine Oocyte Maturation and Development. *Biol Reprod* 2009; **81**: 1064-1072.
Marei WF, Wathes DC and Fouladi-Nashta AA. Impact of linoleic acid on bovine oocyte maturation and embryo development. *Reproduction* 2010; **139**: 979-988.
Matorras R, Ruiz JI, Mendoza R, Ruiz N, Sanjurjo P and Rodriguez-Escudero FJ. Fatty acid composition of fertilization-failed human oocytes. *Hum Reprod* 1998; **13**: 2227-2230.
McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JSM and Speake BK. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *J Reprod Fertil* 2000; **118**: 163-170.
McPherson NO, Fullston T, Aitken RJ, Lane M. Paternal obesity, interventions, and mechanistic pathways to impaired health in offspring. *Ann Nutr Metab* 2014; **64**: 231-238.
Michalakis K, Mintziori G, Kaprara A, Tarlatzis BC and Goulis DG. The complex interaction between obesity, metabolic syndrome and reproductive axis: a narrative review. *Metabolism* 2013; **62**: 457-478.
Miettinen HE, Rayburn H and Krieger M. Abnormal lipoprotein metabolism and reversible female infertility in HDL receptor (SR-BI)-deficient mice. *J Clin Invest* 2001; **108**: 1717-1722.
Mihm M, Baker PJ, Fleming LM, Monteiro AM and O'Shaughnessy PJ. Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. *Reproduction* 2008; **135**: 253-265.
Miller TA, LeBrasseur NK, Cote GM, Trucillo MP, Pimentel DR, Ido Y, Ruderman NB and Sawyer DB. Oleate prevents palmitate-induced cytotoxic stress in cardiac myocytes. *Biochem Biophys Res Commun* 2005; **336**: 309-315.
Moallem U, Katz M, Lehrer H, Livshitz L and Yakoby S. Role of peripartum dietary propylene glycol or protected fats on metabolism and early postpartum ovarian follicles. *Journal of Dairy Science* 2007; **90**: 1243-1254.

Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K et al. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. *Endocrinology* 2001; **142**: 3590-3597.

Nicholas LM, Rattanatray L, Morrison JL, Kleemann DO, Walker SK, Zhang S, MacLaughlin S and McMillen IC. Maternal obesity or weight loss around conception impacts hepatic fatty acid metabolism in the offspring. *Obesity (Silver Spring)* 2014; **22**: 1685-1693.

Nolan CJ and Larter CZ. Lipotoxicity: why do saturated fatty acids cause and monounsaturates protect against it? *J Gastroenterol Hepatol* 2009; **24**: 703-706.

Nonogaki T, Noda Y, Goto Y, Kishi J and Mori T. Developmental blockage of mouse embryos caused by fatty acids. *J Assist Reprod Genet* 1994; **11**: 482-488.

Norman RJ, Noakes M, Wu RJ, Davies MJ, Moran L and Wang JX. Improving reproductive performance in overweight/obese women with effective weight management. *Hum Reprod Update* 2004; **10**: 267-280.

Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH and Hotamisligil GS. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 2004; **306**: 457-461.

Paczkowski M, Schoolcraft W and Krisher R. Fatty Acid Metabolism during Maturation Affects Glucose Uptake and is Essential to Oocyte Competence. *Reproduction* 2014.

Paczkowski M, Silva E, Schoolcraft WB and Krisher RL. Comparative importance of fatty acid beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes. *Biol Reprod* 2013; **88**: 111.

Perez-Crespo M, Ramirez MA, Fernandez-Gonzalez R, Rizos D, Lonergan P, Pintado B and Gutierrez-Adan A. Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. *Mol Reprod Dev* 2005; **72**: 502-510.

Petro EM, Leroy JL, Covaci A, Fransen E, De Neubourg D, Dirtu AC, De Pauw I and Bols PE. Endocrine-disrupting chemicals in human follicular fluid impair in vitro oocyte developmental competence. *Hum Reprod* 2012.

Ramos-Ibeas P, Calle A, Fernandez-Gonzalez R, Laguna-Barraza R, Pericuesta E, Calero A, Ramirez MA and Gutierrez-Adan A. Intracytoplasmic Sperm Injection Using DNA-Fragmented Sperm in Mice Negatively Affects Embryo-Derived Embryonic Stem Cells, Reduces the Fertility of Male Offspring and Induces Heritable Changes in Epialleles. *Plos One* 2014; **9**.

Renaville B, Bacciu N, Comin A, Motta M, Poli I, Vanini G and Prandi A. Plasma and Follicular Fluid Fatty Acid Profiles in Dairy Cows. *Reprod Domest Anim* 2010; **45**: 118-121.

Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M and Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrin* 2009; **4**: 40.

Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S et al. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J Gastroenterol Hepatol* 2009; **24**: 830-840.

Rizos D, Gutierrez-Adan A, Perez-Garnelo S, de la Fuente J, Boland MP and Lonergan P. Bovine embryo culture in the presence or absence of serum: Implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biol Reprod* 2003; **68**: 236-243.

Robker RL. Evidence that obesity alters the quality of oocytes and embryos. *Pathophysiology* 2008; **15**: 115-121.

Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M and Norman RJ. Obese Women Exhibit Differences in Ovarian Metabolites, Hormones, and Gene Expression Compared with Moderate-Weight Women. *J Clin Endocr Metab* 2009; **94**: 1533-1540.

Rooke J, Ewen M, Reis A and McEvoy TG. Comparison of the fatty acid contents of bovine follicular fluid, granulose cells and cumulus ensclosed or denuded oocytes. *Reprod Domest Anim* 2006; **41**: 306.

Simpson ER, Rochelle DB, Carr BR and MacDonald PC. Plasma lipoproteins in follicular fluid of human ovaries. *J Clin Endocrinol Metab* 1980; **51**: 1469-1471.

Smith DG and Sturmey RG. Parallels between embryo and cancer cell metabolism. *Biochem Soc Trans* 2013; **41**: 664-669.

Staiger K, Staiger H, Weigert C, Haas C, Haring HU and Kellerer M. Saturated, but not unsaturated, fatty acids induce apoptosis of human coronary artery endothelial cells via nuclear factor-kappaB activation. *Diabetes* 2006; **55**: 3121-3126.

Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ and Lonergan P. Amino Acid Metabolism of Bovine Blastocysts: A Biomarker of Sex and Viability. *Mol Reprod Dev* 2010; **77**: 285-296.
Sturmey RG, Brison DR and Leese HJ. Assessing embryo viability by measurement of amino acid turnover. *Reprod Biomed Online* 2008a; **17**: 486-496.
Sturmey RG, Hawkhead JA, Barker EA and Leese HJ. DNA damage and metabolic activity in the preimplantation embryo. *Hum Reprod* 2009b; **24**: 81-91.
Sturmey RG and Leese HJ. Role of glucose and fatty acid metabolism in porcine early embryo development. *Reprod Fert Develop* 2008b; **20**: 149-149.
Sundh KJ, Henningsen AK, Kallen K, Bergh C, Romundstad LB, Gissler M, Pinborg A, Skjaerven R, Tiitinen A, Vassard D et al. Cancer in children and young adults born after assisted reproductive technology: a Nordic cohort study from the Committee of Nordic ART and Safety (CoNARTaS). *Hum Reprod* 2014.
Sutton-McDowall ML, Gilchrist RB and Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 2010; **139**: 685-695.
Sutton ML, Gilchrist RB and Thompson JG. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update* 2003; **9**: 35-48.
Turner N and Robker RL. Developmental programming of obesity and insulin resistance: does mitochondrial dysfunction in oocytes play a role? *Mol Hum Reprod* 2015; **1**: 23-30.
Valsangkar D, Downs SM. A requirement for fatty acid oxidation in the hormone-induced meiotic maturation of mouse oocytes. *Biol Reprod* 2013; **89**:43
Van Hoeck V, Leroy JL, Arias Alvarez M, Rizos D, Gutierrez-Adan A, Schnorbusch K, Bols PE, Leese HJ and Sturmey RG. Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: mechanistic insights. *Reproduction* 2013a; **145**: 33-44.
Van Hoeck V, Rizos D, Gutierrez-Adan A, Pintelon I, Jorssen E, Dufort I, Sirard MA, Verlaet A, Hermans N, Bols PE et al. Interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations. *Reprod Fertil Dev* 2013b: [Epub ahead of print].
Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE and Leroy JL. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *Plos One* 2011; **6**: e23183.
Vander Heiden MG, Cantley LC and Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009; **324**: 1029-1033.
Vanholder T, Leroy JL, Soom AV, Opsomer G, Maes D, Coryn M and de Kruif A. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci* 2005; **87**: 33-44.
Vanholder T, Leroy JL, Van Soom A, Maes D, Coryn A, Fiers T, de Kruif A and Opsomer G. Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci* 2006; **92**: 51-63.
Wagenaar K, Ceelen M, van Weissenbruch MM, Knol DL, Delemarre-van de Waal HA and Huisman J. School functioning in 8- to 18-year-old children born after in vitro fertilization. *Eur J Pediatr* 2008; **167**: 1289-1295.
Wakefield SL, Lane M, Schulz SJ, Hebart ML, Thompson JG and Mitchell M. Maternal supply of omega-3 polyunsaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse. *Am J Physiol-Endoc M* 2008; **294**: E425-E434.
Wale PL and Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod* 2012; **87**: 24, 21-28.
Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M and Brennan L. An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. *Fertil Steril* 2012; **97**: 1078-1084 e1071-1078.
Wei Y, Wang D, Topczewski F and Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *American journal of physiology Endocrinology and metabolism* 2006; **291**: E275-281.
Wolf E, Arnold GJ, Bauersachs S, Beier HM, Blum H, Einspanier R, Frohlich T, Herrler A, Hiendleder S, Kolle S et al. Embryo-maternal communication in bovine - strategies for deciphering a complex cross-talk. *Reprod Domest Anim* 2003; **38**: 276-289.

Wonnacott KE, Kwong WY, Hughes J, Salter AM, Lea RG, Garnsworthy PC and Sinclair KD. Dietary omega-3 and-6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos. *Reproduction* 2010; **139**: 57-69.

World Health Organization.Obesity and overweight. Fact sheet N° 311 (http://www.who.int/mediacentre/factsheets/fs311/en/index.html).

Wu GQ, Jia BY, Li JJ, Fu XW, Zhou GB, Hou YP and Zhu SE. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pigs. *Theriogenology* 2011; **76**: 785-793.

Wu LL, Russell DL, Norman RJ and Robker RL. Endoplasmic Reticulum (ER) Stress in Cumulus-Oocyte Complexes Impairs Pentraxin-3 Secretion, Mitochondrial Membrane Potential ({Delta}{Psi}m), and Embryo Development. *Molecular Endocrinology* 2012; **26**: 562-573.

Yang X, Wu LL, Chura LR, Liang X, Lane M, Norman RJ and Robker RL. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. *Fertil Steril* 2012.

Yding Andersen C, Westergaard LG, Figenschau Y, Bertheussen K and Forsdahl F. Endocrine composition of follicular fluid comparing human chorionic gonadotrophin to a gonadotrophin-releasing hormone agonist for ovulation induction. *Hum Reprod* 1993; **8**: 840-843.

Yi D, Zeng S and Guo Y. A diet rich in n-3 polyunsaturated fatty acids reduced prostaglandin biosynthesis, ovulation rate, and litter size in mice. *Theriogenology* 2012; **78**: 28-38.

Yoon SB, Choi SA, Sim BW, Kim JS, Mun SE, Jeong PS, Yang HJ, Lee Y, Park YH, Song BS et al. Developmental competence of bovine early embryos depends on the coupled response between oxidative and endoplasmic reticulum stress. *Biol Reprod* 2014; **90**: 104.

Zachut M, Dekel I, Lehrer H, Arieli A, Arav A, Livshitz L, Yakoby S and Moallem U. Effects of dietary fats differing in n-6:n-3 ratio fed to high-yielding dairy cows on fatty acid composition of ovarian compartments, follicular status, and oocyte quality. *Journal of Dairy Science* 2010; **93**: 529-545.

Zhang LY, Keung W, Samokhvalov V, Wang W and Lopaschuk GD. Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. *Bba-Mol Cell Biol L* 2010; **1801**: 1-22.

Zhang X and Zhang K. Endoplasmic Reticulum Stress-Associated Lipid Droplet Formation and Type II Diabetes. *Biochemistry research international* 2012; **2012**: 247275.

SUMMARY

The work described in this thesis comprised several parts of the interaction between maternal metabolic disorders, the maternal micro-environment, follicular growth, the acquisition of oocyte developmental competence and subsequent early embryo quality and metabolism. Throughout the introduction, several knowledge gaps were formulated in the form of research questions. Now, it is time to answer them.

Research question 1 - How are serum metabolic changes, related to maternal metabolic disorders, like obesity, reflected in the ovarian follicular fluid and how may this affect oocyte developmental competence and subsequent embryo quality?

In **Chapter 3**, we showed that the serum composition is well reflected within the follicular fluid of women undergoing ART. Even though concentrations in serum and follicular fluid may differ because of the blood-follicle barrier, a strong correlation exists between serum and follicular fluid for most metabolites. Interestingly, even though many serum parameters were associated with BMI, only a few were found in the follicular fluid. Furthermore, some follicular fluid parameters did affect oocyte developmental competence and embryo quality, but these parameters were not related to BMI.

In **Chapter 4**, we expand on this knowledge by showing that the composition of human follicular fluid, used in an independent bovine *in vitro* setting, is indeed able to alter oocyte developmental competence and embryo quality. The follicular fluid composition of women with an overall negative IVF outcome or from women with obesity, reduced oocyte developmental competence, while follicular fluid from women with an overall positive IVF outcome did not affect embryonic growth. Furthermore, some changes in bovine blastocyst gene expression patterns were found, indicating that those embryos that did survive, might have an altered quality.

Research question 2 - How are fatty acids distributed in the ovarian follicular fluid and how do they associate with lipolytic conditions, like obesity?

In **Chapter 5**, we found that most fatty acids in the follicular fluid belong the phospholipid and cholesterol fraction. Only triglycerides and NEFAs were associated with BMI and combined only accounted for approximately 20% of the total fatty acid concentration. Indeed, triglyceride concentrations tended to be higher in overweight women, while NEFA concentrations were higher in obese women, compared to normal weight and overweight women. Our data furthermore showed that only the NEFA fraction presented with abundant BMI-related differences in individual fatty acids (87% of all the fatty acids analysed differed in a BMI-dependent way). Most predominant fatty acids in the follicular fluid were palmitic, oleic, stearic and linoleic acid.

Research question 3 - How do prolonged elevated NEFA concentrations affect the growth and differentiation of the ovarian follicle as a whole and the maturing oocyte enclosed within? Furthermore, how are embryo development, quality and metabolism affected by adverse metabolic conditions during follicular development?

In **Chapter 6**, we investigated how the biological unit of the growing follicle and the resulting oocyte quality is affected by a long term exposure to elevated NEFA concentrations. We showed that long term elevated NEFA concentrations only moderately affect follicular growth and antrum formation, with the most pronounced effect induced by the HIGH SA treatment. Elevated NEFA concentrations altered gene expression patterns in Day 13 luteinized granulosa cells, revealing that NEFA exposure mainly affected pathways involved in apoptosis, lipid metabolism, oxidative stress and steroidogenesis, which was also evidenced by progesterone, estradiol and inhibin B analyses in spent medium. Most importantly, the oocytes originating from the NEFA exposed follicles displayed a significantly reduced developmental competence (blastocyst formation).

Chapter 7 describes that morula stage embryos, originating from oocytes isolated from follicles exposed to the HIGH SA treatment consume little or no glucose. This opposed to the embryos originating from BASAL or HIGH NEFA exposed follicles. Also, amino acid metabolism tended to be increased in HIGH NEFA embryos. We could not show a difference in intracellular lipid content. Overall, these data point out that the environment in which the follicle grows and the oocytes matures throughout follicular growth, affects the metabolism of the resultant embryo. The following question was raised: 'Is this effect due to changes throughout follicular growth or is there a time period in development that is particularly sensitive to insult?' We investigated this window of susceptibility by exposing murine follicles throughout follicular development (Day 1-13) or only during the final maturation phase (Day 12-13) to elevated NEFA concentrations. The results suggest that the prolonged NEFA exposure induces a more severe effect on oocyte developmental competence, compared to an exposure only limited to the final phase of maturation. This thus emphasizes that not only final maturation, but also the crucial follicular development preceding final oocyte maturation are important and sensitive to insult.

Overall, the results described in this thesis enforce our main hypothesis, by confirming that the serum composition is reflected in the follicular fluid and that the follicular fluid composition may affect oocyte developmental competence. One specific characteristic of metabolic disorders, namely elevated NEFA concentrations, has been shown to affect follicular physiology, ultimately resulting in a reduced oocyte developmental competence and an altered embryo metabolism.

SAMENVATTING

Metabole stoornissen, zoals obesitas en type 2 diabetes, worden aanzien als belangrijke risicofactoren voor een verminderde vruchtbaarheid. Een gedaalde eicel en embryo kwaliteit wordt steeds vaker naar voor geschoven als een potentiële link tussen maternale metabole stoornissen en subfertiliteit. Eicellen en embryo's zijn erg gevoelig aan het micro-milieu waarin ze zich bevinden. Er werd eerder aangetoond dat de serum samenstelling in koeien tijdens de negatieve energie balans, gereflecteerd wordt in the follikelvochtsamenstelling. In **Hoofdstuk 3** hebben we deze correlatie tussen de biochemische samenstelling van het serum en het follikelvocht kunnen bevestigen bij vrouwen die een IVF behandeling ondergingen. De resultaten toonden meerdere associaties tussen serum parameters en BMI, hoewel maar enkele parameters in het follikelvocht een significante associatie vertoonden met BMI. Sommige parameters in het follikelvocht waren geassocieerd met een gedaalde eicel en embryo kwaliteit. Echter, deze parameters waren niet gerelateerd aan BMI.

In **Hoofdstuk 4** hebben we verder onderzocht hoe de follikelvocht samenstelling de eicel en embryo kwaliteit kan beïnvloeden. Hiervoor hebben we humaan follikelvocht gebruikt in een geheel onafhankelijke boviene *in vitro* setting (loskoppelen van follikelvocht en intrinsieke eicel kwaliteit factor). We toonden aan dat follikelvocht, afkomstig van vrouwen met een 'negatieve' IVF uitkomst of obesitas, een negatief effect had op de boviene eicel ontwikkelingscompetentie. Echter, het follikelvocht van vrouwen met een 'goede' IVF uitkomst had geen negatief effect op de ontwikkeling tot het blastocyst stadium. Verder toonden we ook veranderingen aan in de genexpressieprofielen van de resulterende embryo's, wat suggereert dat de kwaliteit van de overlevende embryo's veranderd kan zijn.

In **Hoofdstuk 5** zijn we specifiek gaan kijken naar de aanwezigheid en hoeveelheid van vetzuren in het follikelvocht. We toonden aan dat de meeste vetzuren veresterd zijn in de fosfolipide en de cholesterol fractie. Echter, enkel de triglyceride en de NEFA fractie waren geassocieerd met BMI. Er was een trend voor een verhoogde triglyceride concentratie in het follikelvocht van vrouwen met overgewicht, terwijl NEFA concentraties hoger waren in het follikelvocht van obese vrouwen, vergeleken met vrouwen met een normaal of overgewicht. De resultaten toonden verder aan dat enkel de NEFA fractie veel BMI-gerelateerde verschillen vertoonde in individuele vetzuur concentraties (87% van alle vetzuren verschilden tussen BMI-groepen). De meest voorkomende vetzuren in alle vetfracties waren stearine-, olie- en palmitine zuur.

Het effect van deze meest voorkomende vetzuren op folliculaire groei en het verwerven van eicel ontwikkelingscompetentie, werd onderzocht in **Hoofdstuk 6**. Meer specifiek hebben we onderzocht hoe de follikel, als biologische eenheid, reageerde op een langdurige blootstelling aan verhoogde

NEFA concentraties. De resultaten toonden aan dat de folliculaire ontwikkeling en antrum vorming slechts minimaal aangetast werden door langdurig verhoogde NEFA concentraties. De HIGH SA behandeling induceerde het meest uitgesproken effect. Verhoogde NEFA concentraties veranderde wel het genexpressie patroon van Dag 13 geluteïniseerde granulosa cellen, wat suggereerde dat verhoogde NEFA concentraties voornamelijk de processen betrokken bij apoptose, vet metabolisme, oxidatieve stress en steroidogenese aantast. Dit laatste werd ook bevestigd door progesteron, estradiol en inhibine B metingen in het geconditioneerd medium. Uiteindelijk resulteerde de folliculaire groei onder verhoogde NEFA concentraties in een extreem verlaagde eicel ontwikkelingscompetentie (blastocyst vorming). Echter, wanneer we kijken naar de embryo's die wel overleefden (**Hoofdstuk 7**), konden we vaststellen dat HIGH SA embryo's (morula stadium), weinig of geen glucose verbruikten, in vergelijking met embryo's afkomstig van BASAL en HIGH NEFA blootgestelde follikels. Verder vertoonden HIGH NEFA embryo's een trend tot een verhoogde aminozuur productie. We konden we geen verschil aantonen in intracellulair vetgehalte tussen de verschillende behandelingsgroepen. Samengevat suggereren deze data dat de omgeving, waarin een follikel groeit en de eicel matureert, het metabolisme van het resulterend embryo kan veranderen. Maar, wordt dit effect geïnduceerd door veranderingen doorheen de folliculaire groei, of is er een specifiek tijdspunt in de ontwikkeling dat meer gevoelig is voor een insult? We onderzochten dit 'gevoeligheids-tijdsraam' door muriene follikels bloot te stellen aan verhoogde NEFA concentraties tijdens de gehele folliculaire groei (Dag1-13) of enkel tijdens de finale eicel maturatie (Dag 12-13). De resultaten suggereren dat een langdurige NEFA blootstelling een meer nefast effect heeft op de eicel ontwikkelingscompetentie, vergeleken met een kortdurende NEFA blootstelling. Deze resultaten benadrukken dat niet enkel de finale eicel maturatie, maar ook de folliculaire groeiperiode die hieraan voorgaat, gevoelig zijn voor een insult, met een potentieel accumulerend negatief effect bij een langere blootstelling.

Concluderend, de resultaten beschreven in deze thesis onderbouwen onze gestelde hypothese, door te bevestigen dat de serum samenstelling gereflecteerd wordt in het follikelvocht van vrouwen die een IVF behandeling ondergaan en dat de follikelvocht samenstelling de eicel en embryo kwaliteit kan beïnvloeden. Verhoogde NEFA concentraties, als een typisch kenmerk van metabole stoornissen, veranderen de folliculaire fysiologie, wat resulteert in een verminderde eicel ontwikkelingscompetentie en een veranderde embryo kwaliteit.

CURRICULUM VITAE

Sara Donni Martine Valckx werd geboren op 26 februari 1987 te Malle. Na het behalen van het diploma hoger secundair onderwijs (Wetenschappen-Wiskunde) aan het Annuntia Instituut in Wijnegem in 2005, begon ze aan haar studies Biomedische Wetenschappen aan de Universiteit Antwerpen. Ze behaalde het diploma Bachelor in de Biomedische Wetenschappen met onderscheiding in 2008 en het diploma Master in de Biomedische Wetenschappen met grote onderscheiding in 2010. Op 1 oktober 2010 trad Sara in dienst als doctoraatsbursaal op het Laboratorium voor de Fysiologie en Biochemie van de Huisdieren aan de Universiteit Antwerpen, waar ze onderzoek verrichte naar de relatie tussen maternale metabole gezondheid en de kwaliteit van follikels, eicellen en embryo's. In 2014 behaalde ze het getuigschrift van de doctoraatsopleiding.

Sara Valckx is auteur van verschillende publicaties in internationale tijdschriften en was spreker op meerdere nationale en internationale congressen.

BIBLIOGRAPHY

1. International publications

Leroy JLMR, Rizos D, Sturmey R, Bossaert P, Gutierrez-Adan A, Van Hoeck V, **Valckx SDM**, Bols PEJ (2011): Intrafollicular conditions as a major link between maternal metabolic disorders and oocyte quality: a focus on dairy cow fertility. Reproduction Fertility and Development 24(1): 1-12

Valckx SDM, De Pauw I, De Neubourg D, Inion I, Berth M, Fransen E, Bols PEJ, Leroy JLMR (2012): BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. Human Reproduction 27(12): 3531-3539

Leroy JLMR, **Valckx SDM**, Sturmey R, Bossaert P, Van Hoeck V, Bols PEJ (2012): Maternal metabolic health and oocyte quality: the role of the intrafollicular environment. Animal Reproduction 9(4): 777-788

Valckx SDM, Arias-Alvarez M, De Pauw I, Fievez V, Vlaeminck B, Fransen E, Bols PEJ, Leroy JLMR (2014): Fatty acid composition of the follicular fluid of normal weight, overweight and obese women undergoing assisted reproductive treatment: a descriptive cross-sectional study. Reproductive Biology and Endocrinology 2014, 12:13

Valckx SDM, Van Hoeck V, Arias-Alvarez M, Gutierrez-Adan A, Berth M, Cortvrindt R, Bols PEJ, Leroy JLMR (2014): Long-term exposure of murine secondary follicles to elevated NEFA concentrations impairs folliculogenesis, follicle quality and oocyte developmental competence in vitro. Accepted for publication in Fertility and Sterility

Valckx SDM, De Bie J, Michiels E, Goovaerts I, Punjabi U, Ramos-Ibeas P, Gutierrez-Adan A, Bols PEJ, Leroy JLMR (2014): The effect of human follicular fluid on bovine oocyte developmental competence and embryo quality. Accepted for publication in Reproductive BioMedicine Online

Leroy JLMR, **Valckx SDM**, Jordaens L, De Bie J, Desmet KLJ, Van Hoeck V, Britt JH, Marei WF, Bols PEJ (2014): Nutrition and maternal metabolic health in relation to oocyte and embryo quality; critical views on what we learned from the dairy cow model? Accepted for publication in Reproduction, Fertility and Development

Valckx SDM, Leroy JLMR (2015): The effect of maternal metabolic health and diet on the follicular fluid composition and potential consequences for oocyte and embryo quality. **In**: Handbook of Fertility nutrition, diet, lifestyle and reproductive health. *Elsevier Inc*. **ISBN**: 978-0-12-800872-0

2. International abstracts

European Cooperation in Science and Technology (COST), GEMINI, 1-3 October 2010, Souston, France – Awarded travel grant of 500 euros

S. Valckx, P.E.J. Bols, D. De Neubourg, I. De Pauw, M. Berth, J.L.M.R. Leroy: Maternal metabolism and the influence on fertility: a study of the follicular microenvironment (Poster)

Y. Dezhkam, V. Van Hoeck, E. Merckx, S. Andries, P.E.J. Bols, **S. Valckx**, J.L.M.R. Leroy: Elevated non-esterified fatty acid concentrations affect bovine oviductal epithelial cell viability and sperm binding (Poster)

Association Européenne de Transfert Embryonnaire (AETE), 9-10 September 2011, Chester, England

S. Valckx, V. Van Hoeck, S. Andries, M. Arias-Alvarez, K. Lemeire, R. Cortvrindt, P.E.J. Bols, J.L.M.R. Leroy: Comparison between warm and cold transport conditions of murine early secondary follicles (Poster)

European Society for Domestic Animal Reproduction (ESDAR), 28 august – 1 September 2012, Dublin, Ireland

S. Valckx, V. Van Hoeck, M. Arias-Alvarez, S. Andries, E. Merckx, R. Cortvrindt, P. Bols, J. Leroy (2012): Exposure of murine early secondary follicles to elevated NEFA concentrations: implications for follicular development. Reproduction in Domestic Animals 47 (suppl 5): 68 (Oral communication)

V. Van Hoeck, K. Schnorbusch, **S. Valckx**, S. Andries, R. Sturmey, P. Bols, J. Leroy (2012): The impact of elevated NEFA concentrations on mitochondrial function during bovine oocyte maturation. Reproduction in Domestic Animals 47 (suppl 5): 112 (Poster)

E. Jorssen, **S. Valckx**, J. Leroy, E. Merckx, S. Andries, P. Bols (2012): Optimization of two serum-free bovine 'single' in vitro embryo production methods. Reproduction in Domestic Animals 47 (suppl 5): 90 (Poster)

AETE, 6 September – 8 September 2012, St Malo, France

L. Jordaens, V. Van Hoeck, **S. Valckx**, M. Uytterhoeven, R.G. Sturmey, P.E.J. Bols, J.L.M.R. Leroy: Effects of elevated non-esterified fatty acid concentrations on the bovine oviductal micro-environment (Poster)

E. Jorssen, A. Langbeen, **S. Valckx**, J.L.M.R. Leroy, P.E.J. Bols: Neutral red (NR) as a tool to assess pre-antral follicle survival in bovine ovarian cortical biopsies cultured in vitro (Poster)

International Embryo Transfer Society (IETS), 19 January – 23 January 2013, Hannover, Germany

S. Valckx, V. Van Hoeck, L. Jordaens, E. Merckx, R. Cortvrindt, P.E.J. Bols, J.L.M.R. Leroy (2013): The effect of elevated non-esterified fatty acid concentrations on murine in vitro folliculogenesis and subsequent oocyte developmental competence. Reproduction, Fertility and Development 25 (1): 150 (student competition)

L. Jordaens, **S. Valckx**, V. Van Hoeck, M. Berth, P.E.J. Bols, J.L.M.R. Leroy (2013): A polarized cell culture system to study the effects of elevated serum non-esterified fatty acid concentrations on the bovine oviductal micro-environment *in vitro*. Reproduction, Fertility and Development 25 (1): 195 (Poster)

AETE, 6 September – 7 September 2013, Istanbul, Turkey

S. Valckx, E. Michiels, E. Jorssen, V. Van Hoeck, U. Punjabi, I. Goovaerts, E. Merckx, S. Andries, P.E.J. Bols, J.L.M.R. Leroy: The effect of human follicular fluid from women with differential ART outcomes on bovine in vitro oocyte maturation, fertilization and blastocyst formation (Oral communication)

AETE, 12 September – 13 September 2014, Dresden, Germany

S. Valckx, C. Simintiras, P. McKeegan, R. Sturmey, R. Cortvrindt, P.E.J. Bols, J.L.M.R. Leroy: Long term elevated NEFA concentrations during in vitro murine follicle growth reduce oocyte developmental competence and alter subsequent embryo metabolism (Oral communication – Student competition)

L. Jordaens, **S. Valckx**, E. Merckx, S. Andries, P.E.J. Bols, J.L.M.R. Leroy: Non esterified fatty acids affect sperm binding capacity of Bovine Oviduct Epithelial Cells in two in vitro culture systems (Oral communication – Student competition)

IETS, 10 January – 13 January, Versailles, France

S. Valckx, L Jordaens, R Cortvrindt, PEJ Bols, JLMR Leroy: The effect of short versus long term elevated non-esterified fatty acid concentrations during murine *in vitro* follicle growth on oocyte developmental competence (Poster)

L. Jordaens, **S. Valckx**, P.E.J. Bols, J.L.M.R. Leroy: Elevated Non-Esterified Fatty Acid concentrations hamper in vitro Bovine Oviductal Epithelial Cell Physiology (Student competition)

3. International research activities

Short Term Scientific Mission, 04 January – 30 January 2012

Topic: Pre-implantation rabbit embryo exposure to elevated NEFA concentrations and gene expression patterns
Host: Dr. Véronique Duranthon and Prof. Pascale Chavatte-Palmer, Biologie du Développement et de la Reproduction, INRA, Jouy en Josas, France
COST reference number: COST-STSM-FA0702-8967 granted 1200euros

Short term training, 22 October – 24 October 2013

Topic: HPLC analyses of embryo culture medium samples
Host: Prof. Roger Sturmey, Centre for Cardiovascular and Metabolic Research, Hull York Medical School, Hull, UK
Finance granted (OJO 2013/4/021)

ACKNOWLEDGEMENTS

No one who achieves success does so without acknowledging the help of others. The wise and confident acknowledge this help with gratitude.

Er is niemand die een doctoraat succesvol kan afronden zonder de ongelooflijke steun van begeleiders, collega's, familie en vrienden. In dat opzicht was ik net als een eicel, die nauw contact met haar omliggende cumulus cellen nodig heeft om te kunnen matureren.

Graag bedank ik als eerste mijn promotor **Jo**. Je hebt me het onderzoek leren kennen en gemotiveerd om aan een doctoraat te beginnen. Ik ben je heel erg dankbaar voor de mogelijkheden die je me geboden hebt, maar ook voor de vrijheid die je me liet om hierin verder te gaan. Bedankt voor de kennis, ervaring en wijsheid die je met mij deelde. Ik geloof dat ze geleid hebben tot een beter inzicht en een kritische kijk op het onderzoek en het leven. Ik kijk naar jou op, als baas, als promotor, maar ook als man en vader. Ik kan me geen betere promotor wensen!

Peter, als diensthoofd van het Gamete Research Centre was jij mijn eerste contactpersoon binnen de groep. Ik was super gemotiveerd als masterstudent en die motivatie heb ik kunnen vasthouden doorheen mijn doctoraatsopleiding door terecht te komen in een geweldig team. Een team dat, onder jouw leiding, floreert in samenhorigheid. Natuurlijk valt er ook iets te zeggen over de teambuildingsactiviteiten. De Morvan… Wat een onvergetelijke momenten hebben we daar beleefd: de gorge, partini, discussies over het lot en het voorbestemd zijn van de dingen… Het Gamete Research Centre op zen best! Bedankt om die momenten samen te delen.

Als bureau-maatjes zijn we echte vriendinnen geworden, **Jessie**. In tranen en geluk, met frustraties en opluchting, in ziekte en gezondheid en ook het ultieme succes en doorzetting, we hebben alles gedeeld. Soms waren woorden zelfs niet nodig… Net als een in een goed huwelijk, bedankt voor alles!

Je bent een van de sterkste personen die ik ken en waar ik naar op kijk, **Lies**, met een geweldig warm hart. Jouw doorzettingsvermogen is ongelooflijk. Daarom ben ik er ook zeker van dat ik binnenkort nog eens op bezoek kan komen voor jouw verdediging! Bedankt voor je geweldige verhalen en alle momenten van plezier, steun en toeverlaat.

Ellen, we hebben zo goed als alle stappen van onze doctoraatsopleiding samen doorlopen. We hebben alle positieve en negatieve ervaringen gedeeld. Dat heeft een sterke band gesmeed tussen ons! Bedankt om er steeds voor mij te zijn, bedankt voor alles!

Je first in, last out gewoonte op de buro zal maken dat je haalt, **Karolien**! Met een glimlach trek je de wereld rond, van het ene labo naar het andere. Geen uitdaging ga je uit de weg! Geweldig van je op de buro te hebben!

An, we zijn lang buro-maatjes geweest en hebben lief en leed gedeeld. 'De koe, ik heb er iets mee' was op je buro geplakt en ik heb dan ook veel van mijn koe-ervaringen aan jou te danken. En ja, dat betekent ook rectaal onderzoek! Bedankt voor de leuke jaren samen!

Lieve **Veerle**, girlfriend, ook wij hebben lief en leed gedeeld. Bedankt voor het delen van je kennis, ervaring en visie. En, welkom terug!

Eline, **Anniek**, hoewel ik nog niet lang met jullie heb kunnen samenwerken, toonden jullie in mijn ogen alle motivatie, doorzettingsvermogen en karakter om goed in de groep te passen. Veel succes!

Evi and **Waleed**, what an additive value to have you both in the lab! You radiate motivation, expertise and the will to learn. You are a perfect match for the Gamete Research Centre. Thank you for sharing your ideas and motivation.

Je leerde me alles in het labo, **Els**. Van de afwas doen tot embryo's groeien. Je was als een mama voor mij in het labo terwijl ik zwanger was van Jade. Ik hoop dat jullie kindje jullie evenveel plezier en liefde zal brengen als Jade bij ons! **Silke**, uren hebben we samen gescoord en follikels overgestoken. Hoe blij we waren met die eerste muis embryo's, ik zal het nooit vergeten! Jullie doen ons labo draaien, bedankt voor alles, lab chicks!

Britt, hoe jij de orde terug vindt tussen al dat administratieve werk is bewonderenswaardig! Bedankt voor de heel uitgebreide hulp bij het afronden van mijn thesis en voor alle leuke momenten die we samen beleefden!

Lieve **Petra**, ik zal nooit jouw mopje 'hopje hopje hopje' vergeten! Bedankt voor alle leuke momenten en herinneringen samen.

Gilbert, Chef, ik zal je positieve noot op de gang nooit vergeten. Bedankt voor elke glimlach.

Also, **Maria**, thank you for sharing your ideas and expertise. Our 'mouse on the run' experience still makes me smile. I wish you, as always, the best!

Ook veel dank gaat naar **Isabel Pintelon** en **Dominique De Rijck** voor urenlange sessies, in het donker, achter de microscoop!

Jouw kritische kijk op het onderzoek, **Rita**, heeft ook mij kritischer leren zijn. Bedankt voor alle vruchtvolle discussies!

Aan al mijn lieve collega's, ex-collega's (Ilse en Evi) en studenten, uren hebben we gepraat en gebrainstormd… Samen hebben we protocollen uitgedacht en uitgevoerd, tot in de perfectie. Dankzij jullie heb ik het gehaald en daar zijn niet genoeg dankwoorden voor!

Ook veel dank gaat naar de **slachthuizen** van Heist-op-den-Berg en Hoogstraten en de medewerkers van **EggCentris**. Ik bedank ook graag de medewerkers van het **AML** (Mario Berth), **StatUA** (Erik Fransen) en de **centra voor reproductieve geneeskunde** van het UZA en het ZNA Middelheim voor de leuke, productieve samenwerking en de vruchtvolle discussies! Zonder jullie bijdrage had ik mijn onderzoek niet kunnen uitvoeren.

I specifically want to thank **Véronique Duranthon** and **Roger Sturmey** for allowing me to visit their lab and share their scientific ideas with me. Many thanks also to the colleagues of the **INIA**, Spain for the numerous gene expression analyses.

To the **members of my jury and IDC**, it is both an honour and a privilege to be given the opportunity to defend my work in public. Thank you for critically reading my thesis and discussing your ideas with me. I wish you all the best.

L'essentiel est invisible pour les yeux, il faut voir avec le cœur.

Jerich, jij bent al jaar en dag mijn beste vriend waar ik lief en leed mee deel. Bedankt om er voor mij te zijn! **Lynn** en **Els**, we kennen elkaar nu al zo lang en nog steeds zijn we 'thuis' bij elkaar. Ook een dikke merci aan al mijn andere vrienden voor alle leuke momenten!

Aan mijn lieve **familie en schoonfamilie**, bedankt om deel uit te maken van mijn leven.

Lieve **Carolien**, **Laura**, **Wouter** en **Kristof**, wat is het geweldig jullie te hebben! Ik zou het niet gekund hebben zonder uit zo'n liefdevolle thuis te komen. Ik zie jullie graag!
Lieve **mama** en **papa**, ik kan eerlijk waar zeggen dat ik hier niet zou staan als het niet voor jullie was. Bedankt voor jullie onvoorwaardelijke steun en toeverlaat! Ik zie jullie doodgraag!
Weinig woorden zeggen vaak het meest. Daarom, **Jeroen** en **Jade**, aan jullie draag ik mijn doctoraat op. Jullie zijn mijn thuis, mijn leven. Ik kan niet gelukkiger zijn, dan aan jullie zijde.

Nu, op het einde van mijn doctoraatsopleiding, voel ik me als een mature eicel, ge-ovuleerd en klaar om bevrucht te worden door een nieuwe uitdaging die mijn kant op komt. Maar ook los van de eens zo essentiële cumulus cellen die me gevormd hebben. Bedankt dus, allemaal!