The impact of adipokines on human male fertility

Dissertation

zur Erlangung des

Doktorgrades der Ernährungswissenschaften (Dr. troph.)

der

Naturwissenschaftlichen Fakultät III

Institut für Agrar- und Ernährungswissenschaften,

Geowissenschaften und Informatik

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Verteidigung am: 26.01.2015

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List of abbreviations

AA	Acrylamide
a.u.	Arbitrary units
ABC	ATP-binding-cassette
AdipoQ	Adiponectin
AdipoR	Adiponectin receptor
ADP	Adenosine diphosphate
ALH	Amplitude of lateral head displacement
Ambra 1	Activating molecule in Bec1-regulated autophagy
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
APPL	Adaptor protein containing a pleckstrin homology domain, a phosphotyrosine binding domain and a leucine zipper motif
APS	Ammonium persulfate
Atg	Autophagy-related gene
Atg14L	Atg14-like protein
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCF	Beat cross frequency
Bec1	Beclin-1
BH3	Bcl-2 homology-3
Bis	Bisacrylamide
BMI	Body mass index
Bp	Base pairs
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CASA	Computer aided sperm motion analysis
cDNA	Complentary DNA
CMKLR1	Chemokine-like receptor 1
CQ	Chloroquine diphosphate salt
CRH	Cytokine receptor homology
CRP	C-reactive protein
CV	Coeffcient of variation
Cys	Cysteine
DAB	3-3’-Diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindole

DEPC	Diethylpyrocarbonate
DFI	DNA fragmentation index
DGC	Density gradient centrifugation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitol
EBSS	Earle's Balanced Salt Solution
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNAMPT	Extracellular NAMPT
ERK 1/2	Extracellular-signal regulated kinase 1/2
F-actin	Filamentous actin
FITC	Fluorescein isothiocyanate
FIZZ	Found in inflammatory zone
FLICA	Fluorochrome Inhibitor of Caspases
FN III	Fibronectin type III
FSH	Follicle stimulating hormone
Fw	Forward
G-actin	Globular actin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H_2O	Water
HbA_{1c}	Hemoglobin A_{1c}
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HMW	High molecular weight
HRP	Horse raddish peroxidase
HTF	Human tubal fluid
Ig	Immunoglobulin
IL-6	Interleukin-6
iNAMPT	Intracellular NAMPT
IP3	Inositol triphosphate
JAK	Janus kinase
LC3	Microtubule associated protein 1 light chain
LDL	Low-density lipoprotein
LETO	Long-Evans Tokushima Otsuka

LH	Luteinising hormone
LIN	Linearity
MAPK	Mitogen-activated protein kinase
Min	Minutes
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NMN	Nicotinamide mononucleotide
NTC	No Template Control
ObR	Leptin receptor
ODF	Outer dense fibers
OLETF	Otsuka Long-Evans Tokushima Fatty
PAQR	Progestin and adipoQ receptor
PARP-1	Poly(ADP-ribose) polymerase-1
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PPARα	Peroxisome proliferator-activated receptor-α
RELM	Resistin like molecule
Rev	Reverse
RIA	Radioimmunoassay
RIPA	Radio Immuno Precipitation Assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Room temperature
Rubicon	RUN domain protein as Bec1 interacting and cysteine-rich containing
SDS	Sodium dodecyl sulfate
Sec	Seconds
Serpin	Serine protease inhibitor
SGBS	Simpson-Golabi-Behmel Syndrome

SHP-2	Src-homology 2 domain-containing phosphatase 2
SIRT	Sirtuin
SNP	Single nucleotide polymorphism
SP	Seminal plasma
STAT	Signal transducers and activators of transcription
STR	Straightness
T2Dm	Type 2 diabetes mellitus
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TNF-α	Tumor necrosis factor alpha
TTBS	Tween with TBS
UVRAG	UV radiation resistance-associated gene
VAP	Medium average path velocity
Vaspin	Visceral adipose tissue-derived serine protease inhibitor
VCL	Curvilinear velocity
Vps	Vacuolar protein sorting
VSL	Straight-line velocity
WHO	World Health Organization
WHR	Waist to hip ratio
ZP	Zona pellucida
$\Delta\Psi_m$	Mitochondrial membrane potential

1 Introduction

"Infertility is defined as the inability of a sexually active, non-contracepting couple to achieve pregnancy in one year", according to World Health Organization (WHO) definition (1). In Germany, 10-15 % of the couples are unable to conceive children (2). 30 % of infertility cases are caused by male, 30 % by female, 30 % by both partners and the remaining 10 % being idiopathic. Reasons for male infertility are various, such as abnormalities of spermatozoa, defects in testes, hormonal alterations, varicocele and testicular cancer (2). Overall, 7 % of men suffer from the impairment of their fertility (3). Compromising risk factors are X-radiation, chemotherapy, heat, infections, smoking, alcohol, drugs, pesticides and obesity. Predominantly in western countries, obesity is an increasing health problem because of its high number of long-term consequences, such as sleep apnoea, depression, orthopaedic diseases, atherosclerosis and type 2 diabetes mellitus. The link between obesity and male infertility by impaired semen quality has been intensively discussed (4, 5). Obese men had lower sperm concentrations and counts (4, 6, 7), less normal motile spermatozoa (8) and a higher DNA fragmentation index (8) than normal weight men, although not all investigators have confirmed such associations (6, 9, 10). Proteins secreted mainly by adipose tissue referred to as adipokines, might be involved in the molecular mechanisms of obesity-related male infertility (11-13). Adipokines are secreted into the peripheral blood by adipocytes as well as lymphocytes (14), leucocytes (15), trophoblasts (16) and fibroblasts (17). Besides, adipokines affect carbohydrate metabolism, appetite, satiation and subclinical inflammation (18). In the last several years adiponectin, chemerin, vaspin, leptin, resistin, progranulin, and visfatin/NAMPT have been revealed to be important adipose tissue-related players for central and peripheral actions (13, 19-22). Leptin and resistin were demonstrated to be present in the male reproductive tract and may be linked to the pathogenesis of impaired spermatogenesis (23-26). This study aimed to investigate the presence and function of adipokines in the male reproductive tract depending on body mass index.

2 Detailed introduction to the topic

In the following chapter, the physiology and function of human spermatozoa is explained. Additionally, the investigated adipokines adiponectin, chemerin, vaspin, leptin, resistin, progranulin and visfatin are described in detail as well as the process of autophagy.

2.1 Male reproduction - spermatozoa

2.1.1 Structure of spermatozoa

The morphology is one criterion of fertility evaluation and therefore plays an important role for sperm function. The spermatozoon is structured in a head (4.0-5.5 µm x 2.5-3.5 µm), a tail with connecting piece (1 µm), midpiece (5-7 µm), principal piece (45 µm) and an end piece (5-7 µm) (27). The primary function of a spermatozoon is the transport of the haploid chromatin to the oocyte. Accordingly, the head is mostly occupied by the nucleus and only a thin layer of cytoplasm is left. The nucleus is covered with the perinuclear theca. The cytoskeletal structure is divided into the subacrosomal region and the postacrosomal segment (28). The cap shaped acrosome is localised on the apical site of the spermatozoon's head and accounts for 40-70 % of the head. The acrosome is a large lysosome (29, 30) derived from the Golgi apparatus, which enables the spermatozoon to penetrate the oocyte due to the presence of proteases. The acrosome consists of a dense matrix, rich in proteases, surrounded by an inner and outer cytoplasmic sheath (27). Moreover, the connecting piece as a flexible link between a spermatozoon's head and the midpiece of the tail, includes the proximal centriole (31). Perpendicular located to the proximal centriole, the distal centriole alignes with the axis of the flagellum (31). These centrioles are hollow cylindrical structures with their walls composed of nine triplet microtubules (32). During generation of cilia and flagella, centrosomes migrate to the cell periphery where distal centrioles dock to the cell membrane to become basal bodies from which ciliary and flagellar axonemes originate (32). After the growing of the sperm axoneme, the distal centriole vanishes and only remnants remain in mature spermatozoa (32). The proximal centriole is crucial for the assembly of the sperm aster and mitotic spindle in the zygote after fertilisation (32). Mitochondria are closely packed at the midpiece, helically arranged and provide the energy supply for the motility of the spermatozoon. Approximately 50-75 mitochondria are enclosed in the mammalian sperm midpiece (33). Overall, the midpiece and tail are structured with a central axoneme, which is surrounded by outer dense fibers (ODF), mitochondria and plasma membrane at the midpiece. At the principal piece of the sperm flagellum, the central axoneme is surrounded by accessory structures - ODF, fibrous sheath and plasma membrane. ODF have been suggested to play a role in the modulation of the flagellar curvature and therefore the motility pattern (34). Microtubules of the axoneme have their origin in the distal centriole. The axoneme is structurally similar

to a cinocillium, a pattern of microtubules consisting of a pair of central singlet microtubules encircled by 9 doublet microtubules (35). Moreover, dynein is found in the doublet microtubule arms and is equipped with ATPase activity. Dynein arms mediate the sliding between the adjacent outer doublet microtubules (36). They contribute to the flagellar and ciliary movement and result in sperm motility (36).

2.1.2 Nuclear chromatin remodelling

The haploid round spermatids develop to spermatozoa with an extremely condensed nucleus during spermiogenesis (37). Hence, the cytoplasm and the chromatin have to be remodelled (38, 39). In the end, chromatin of spermatozoa differs in structure from somatic chromatin.

The DNA of the somatic cell nucleus is coiled around nucleosomes (40), an octamer of histones. Nevertheless, nucleosome removal requires release of torsional stress (supercoiling) performed by the formation of DNA strand breaks (41). During spermatogenesis, the nuclear remodelling occurs in the testis, whereby somatic histone proteins are replaced by transition proteins and afterwards by testis-specific protamines (42, 43) to ensure the highly compact packaging of the sperm chromatin (44). In the end, only 15 % of the chromatin of human ejaculated spermatozoa contains histones (45). Therefore, chromatin is condensed with a 6-fold decrease of metaphase chromosomes (46), which ensures the stability of the chromatin in the nucleus. Moreover, protamines are half the size of core histones (5-8 kDa) and are strongly alkaline due to the amino acid arginine, which represents 55-79 % of the amino acids. This is also the reason for the strong binding to the DNA (46) and transferring chromatin to a crystalline structure. In the second phase of sperm chromatin condensation during the passage through the epididymis, a large number of intermolecular and intramolecular covalent disulfide bonds between the cysteine residues of the protamines are formed, stabilising the chromatin (47-50). Due to the highly compact chromatin condensation, sperms are resistant to agents, like strong acids, proteases, DNAse, and detergents (51) and are referred to as transcriptionally inactive. During fertilisation, the sperm chromatin is decondensed by breaking the disulfide bonds due to glutathione of the oocyte as a reducing agent (52).

2.1.3 Transcription and translation

Due to the nuclear remodelling during spermatogenesis, transcription is attenuated, because the chromatin is tightly packed and the transcription can no longer be performed at the level of elongated spermatids (53). Additionally, human ejaculated spermatozoa are not able to perform transcription (54), whereas human sperm express messenger RNAs (mRNA). Furthermore, spermatozoa contain a minimal amount of 7.07±1.05 ng total RNA/10^6 spermatozoa (54). The function of mRNAs in human ejaculated spermatozoa is

discussed controversially, but RNAs might be important for the oocyte to decondensate sperm chromatin and activate embryonic genes (55). After the fertilisation with a spermatozoon, the oocyte is able to translate the sperm mRNA (56).
The translation in human spermatozoa is controversially discussed, as no 18S and 28S ribosomal RNA (rRNA) were detectable (54, 57). On the other hand, methionine and cysteine were incorporated into newly synthesized polypeptides during capacitation of human spermatozoa (58). Thereby, translation was inhibited by mitochondrial translation inhibitors, indicating that translation occurs in sperm mitochondria (58). As mentioned before, mRNA is not newly synthesised by mature spermatozoa (54), which suggests that mRNAs contained in spermatozoa are very stable (58).

2.1.4 Capacitation

Spermatozoa, released by the male reproductive tract, are not able to fertilise an oocyte. The complex and varied protein complement in fluids of the epididymis, accessory glands and the oviduct prepares the sperm surface for fertilisation. In the female reproductive tract, spermatozoa undergo biochemical and membranous changes, which are required to prepare spermatozoa for the fertilisation and are defined as capacitation. This mechanism enables spermatozoa to bind and fuse with an oocyte. During this process the plasma membrane profile changes, enzyme activities alter, intracellular components are modified, and membrane surface proteins alter their distribution, including loss, unmasking or rearrangements of various proteins (59). The first sign of capacitation is a hyperactivation. Thereby, the motility of a spermatozoon changes from a linear movement to asymmetrical flagellar beating of the flagellum (60, 61). Movement patterns of spermatozoa are detected by computer aided sperm motion analysis (CASA).
The fertilisation potential of spermatozoa can be determined by the following parameters (Figure 1): medium average path velocity (VAP), amplitude of lateral head displacement (ALH), straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN = VSL/VCL x 100), beat cross frequency (BCF), straightness (STR = VSL/VAP x 100). Thereby, ALH, VCL and BCF are indicators of sperm vigour. LIN and STR are also used to describe sperm swimming pattern. During capacitation, ALH increases while linearity decreases (62, 63). The specific cause of the hyperactivation is not exactly resolved, but the reactive oxygen species (ROS) production increases, a cyclic adenosine monophosphate (cAMP)-dependent tyrosine phosphorylation of sperm membrane proteins (64-66) occurs and calcium (Ca^{2+})-selective channels contribute to a Ca^{2+} influx (67).

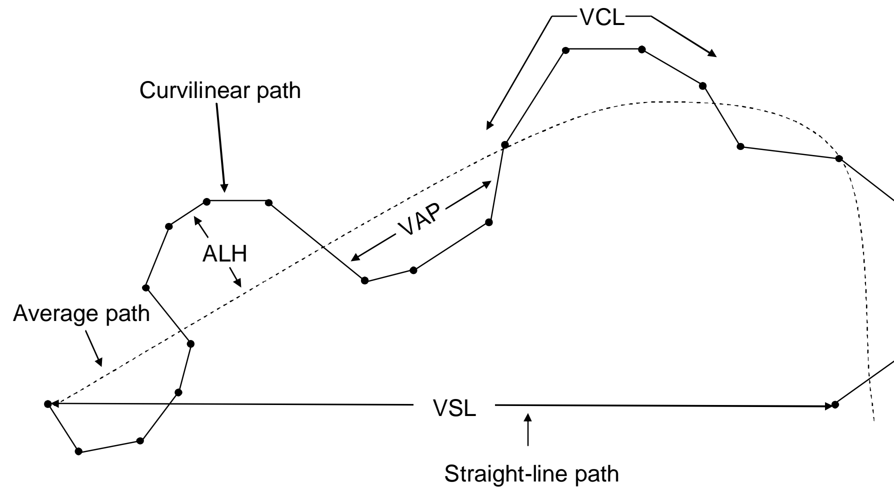


Figure 1 Motility patterns of human spermatozoa determined by computer aided sperm motion analysis (CASA). The schematic figure was drawn according to WHO (68). Medium average path velocity (VAP), amplitude of lateral head displacement (ALH), straight-line velocity (VSL), curvilinear velocity (VCL)

During capacitation, intracellular Ca^{2+} levels increase due to the release from intracellular storage. The Ca^{2+} storages are not defined as sperm lack endoplasmic reticulum. A Ca^{2+} influx contributes to an intracellular Ca^{2+} increase (69). Thereby, this intracellular Ca^{2+} increase contributes to the hyperactivation in spermatozoa (70, 71). Additionally, intracellular cAMP, HCO_3^- and pH increase, membrane permeability changes, and zona pellucida (ZP) binding receptors are exposed (66). Furthermore, cholesterin removal from the spermatozoa membrane is mediated by albumin (72). Subsequently, the membrane is destabilised, promoting the acrosome reaction and membrane fusion to the oocyte. Moreover, cholesterol efflux is associated with signalling events leading to tyrosine phosphorylation of membrane proteins (73, 74).

2.1.5 Acrosome Reaction

Acrosome intact spermatozoa pass the cumulus layer of the oocyte by a protein anchored in the plasma membrane, which includes a hyaluronidase activity (75) and induces the physiological acrosome reaction by the contact of a spermatozoon with the zona pellucida, the extracellular matrix of the oocyte (76-78). The acrosome reaction combines alterations of the membrane lipid composition occurring during capacitation. Furthermore, cytoskeletal actin components are reorganised and filamentous (F)-actin is depolymerised. Accordingly, the plasma membrane above the acrosome and the outer acrosome membrane underneath come in contact and fuse (79, 80). Vesicles develop and soluble acrosomale proteases (acrosin) and membrane-bound proteases are released (81), which enable the spermatozoon to get through the zona pellucida and fertilise the oocyte. Initially, the intracellular pH of sperm is acidic and accordingly the protease proacrosin is inactive (82). Due to the capacitation, the pH increases and becomes alkaline and subse-

quently, proacrosin is activated to acrosin and the acrosomal matrix will be proteolytically degraded (83, 84).

In contrast to the capacitation, which is ongoing for several hours and is reversible, the acrosome reaction takes only 10-15 min and is not reversible. Progesterone, a product of the cumulus cells, is the physiological inducer of the capacitation as well as the acrosome reaction. *In vitro*, the acrosome reaction can be induced by Ca^{2+} ionophore A23187 (85) or progesterone (86). The incubation with the Ca^{2+} ionophore results in an intracellular Ca^{2+} influx and a pH change. Additionally, recombinant glycosylated proteins of the ZP are able to induce the acrosome reaction (2). The spermatozoon binds to the ZP3 protein and induces a transient Ca^{2+} influx into the sperm, mediated by voltage-dependent non-selective cation channels. This leads to an activation of a pertussis toxin–sensitive trimeric $G_{i/o}$ protein (87, 88) and downstream to an activation of the adenylate cyclase and phospholipase C (PLC). The activation of adenylate cyclase results in increased cAMP levels and PLC generates inositol triphosphate (IP3) and diacylglycerol. Hence, cAMP and IP3 mobilise Ca^{2+} from the sperm's intracellular Ca^{2+} store, the acrosome, although the Ca^{2+} storing capacity of this organelle seems very limited (89).

2.2 Adipokines

2.2.1 Adiponectin

Adiponectin is a 30 kDa protein, which is secreted by adipocytes, muscle and liver cells and expressed during adipogenesis in adipocytes (90, 91). However, adiponectin circulates in plasma as a full-length protein with low molecular weight trimers, middle molecular weight hexamers and high molecular weight (HMW) 12- or 18-mers. These different isoforms have divergent biological activity (92). The HMW form is the major active form mediating the insulin-sensitizing effects of adiponectin (93, 94) and is probably a better marker than total adiponectin for later insulin resistance or metabolic syndrome (95, 96). However, globular adiponectin is generated by proteolysis of the full-length protein by neutrophil elastase or other proteases (97, 98). In comparison to HMW adiponectin, the proteolytic isoform shows lower circulating levels, which questions the biological relevance of this protein (99). Adiponectin levels in human plasma depend on body weight and range from 3 to 30 µg/ml (100, 101), which corresponds to 0.01 % of human plasma protein.

2.2.1.1 Adiponectin receptors

The effects of adiponectin are mediated by the seven transmembrane domain receptors AdipoR1 and AdipoR2 (Figure 2). In contrast to the G protein-coupled receptor, the N-terminal site of the adiponectin receptor is intracellular and the C-terminal site is extracellular (97, 102). AdipoR1 (375 amino acids, 42.4 kDa) is expressed ubiquitously, whereas

AdipoR2 (311 amino acids, 35.4 kDa) is expressed mostly in the liver (102, 103). The sequence homology of these two receptors is 67 %. AdipoR1 and AdipoR2 belong to the progestin and adipoQ receptor (PAQR) family named after the two initially described ligands, progestin and adiponectin (adipoQ) (104). AdipoR1 seems to have a high affinity for globular adiponectin and a low affinity for full-length adiponectin, whereas AdipoR2 was found to have an intermediate affinity for both forms of adiponectin (102). AdipoR1 and AdipoR2 are predicted to be located at the plasma membrane (102). Besides residing at the plasma membrane, AdipoR1 and AdipoR2 are also distributed in the cytoplasm with AdipoR1 being located in cytoplasmic puncta (105).

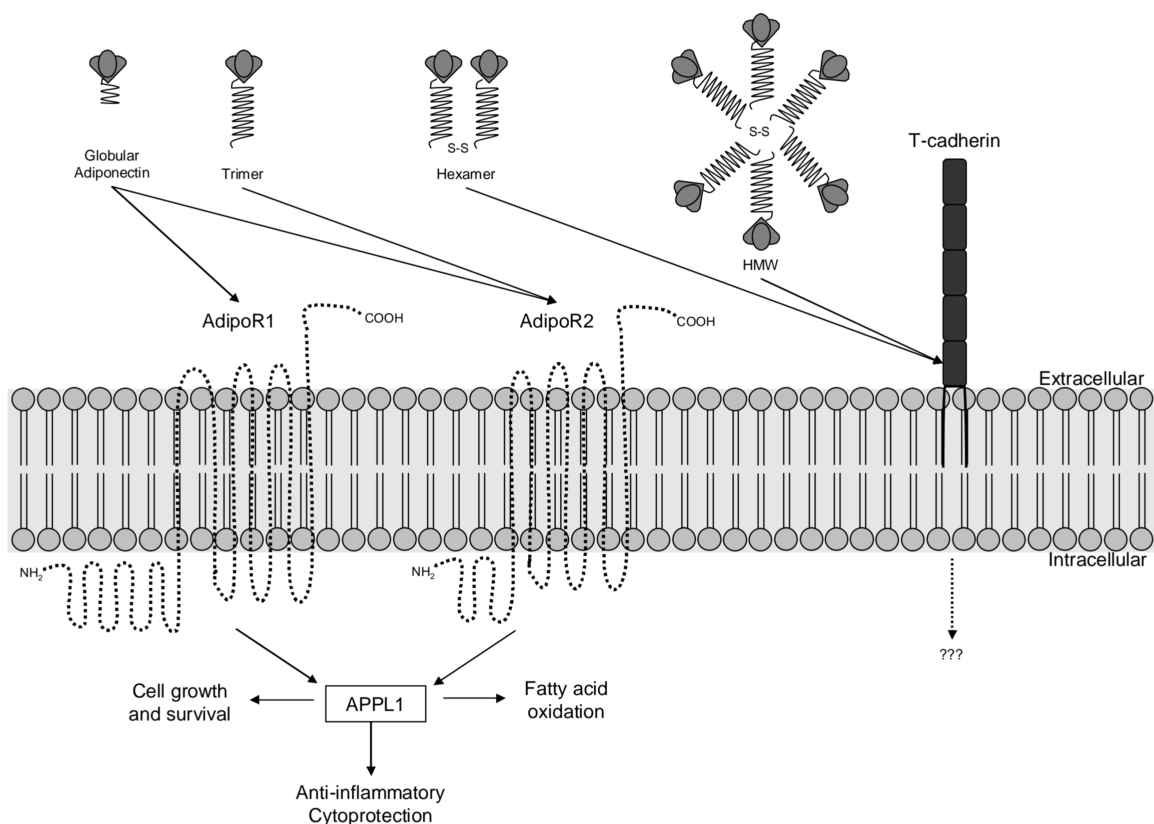


Figure 2 Adiponectin receptor signalling. Adiponectin receptor 1 (AdipoR1) has a low affinity for full-length adiponectin and a high affinity for globular adiponectin, whereas adiponectin receptor 2 (AdipoR2) binds full-length and globular adiponectin with an intermediate affinity. T-cadherin is a truncated receptor that is able to bind the hexameric and high molecular weight (HMW) oligomeric forms of adiponectin. AdipoR1 and AdipoR2 interact with the adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif (APPL1), which binds the N-terminal intracellular domains of the receptors (according to (106)).

APPL1 is known as a binding protein for adiponectin receptors AdipoR1 and AdipoR2 (107). This adaptor protein consists of a pleckstrin homology domain, a phosphotyrosine binding domain, and a leucine zipper motif (108). The formation of the AdipoR1 APPL1 complex is enhanced by adiponectin (107). Until today, 14 proteins are known to bind APPL1; they include membrane receptors (e.g. the androgen receptor) and various signal-

ling molecules with key roles in apoptosis, cell proliferation, chromatin remodelling and cell survival (108-111).

Both receptors mediate the effect of adiponectin by activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) (112, 113), p38 mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptor-α (PPARα) (108).

Adiponectin binds not only to AdipoR1 and AdipoR2, but also to T-cadherin, which is most likely an adiponectin receptor as well (114). This protein is a unique cadherin molecule that lacks the transmembrane and cytoplasmic domains, which are needed for signal transduction. T-cadherin is capable of binding hexameric and HMW forms of adiponectin but not the globular or trimeric forms (114, 115). However, it plays only a small role in signal transduction as adiponectin treatment of AdipoR1/2 double knock out mice did not improve plasma glucose levels (116).

2.2.1.2 Physiological effects of adiponectin

Plasma adiponectin levels correlate positively (117) as well as negatively (118) with age and depend on gender (119). Adiponectin plasma concentrations differ from other adipokines as they correlate negatively with obesity, body mass index (BMI), insulin resistance (91, 120-123), type 2 diabetes mellitus (T2Dm) (121, 124), and metabolic syndrome (125-127). Adiponectin is able to modulate food intake and energy expenditure during fasting and refeeding, due to its effects in the central nervous system (128). Overall, adiponectin has been suggested to have insulin sensitizing, anti-inflammatory, anti-atherogenic (129, 130), and antioxidative effects (131). Moreover, adiponectin has been reported to promote cell survival and growth, but it may also stimulate apoptosis (132-135).

Interestingly, adiponectin may play an important role in reproduction. This adipokine was found to be expressed in rat (136) and chicken testis (137), whereby the receptors AdipoR1 and AdipoR2 were also detected. Adiponectin-like molecule CTRP8 mRNA was found to be expressed in human testis (138) and androgens show an inhibiting effect on adiponectin expression (119).

2.2.2 Chemerin

The precursor protein prochemerin (163 amino acids, 18 kDa) is activated by proteolytic cleavage of several C-terminal amino acids (139) during inflammation (140, 141) into chemerin (137 amino acids, 16 kDa). This adipokine was found primarily in inflammatory fluids (139) and in peripheral circulation as a precursor protein “prochemerin/Tig-2” (139, 142, 143). The proteolytic cleavage is performed by serine proteases of coagulation, fibrinolytic and inflammatory cascades (141, 142, 144, 145), and circulating carboxy peptidases (144). Anti-inflammatory effects are dependent on cysteine protease derived cleav-

age of chemerin (146), whereas proinflammatory effects are regulated by serine proteases (141, 142).

2.2.2.1 Chemerin receptor

Chemerin exerts its function by binding to a receptor at the plasma membrane and activating intracytoplasmic cell signalling pathways. The chemerin receptor (139) is equal to human chemokine-like receptor 1 (CMKLR1) or ChemR23 (147, 148), CMKLR3 in rat (149), and DEZ in mice (150). This receptor is expressed in different cells, such as adipocytes, astrocytes, and chondrocytes (150-152). The chemerin receptor appears to be related to chemoattractant receptors (147). CMKLR1 is a 7 transmembrane G protein-coupled receptor and the chemerin C-terminus binds to CMKLR1 and shows chemotactic activity (145).

2.2.2.2 Physiological effects of chemerin

Chemerin is known to be proinflammatory (139) as well as anti-inflammatory (146), depending on cleavage of amino acids at the C-terminus. Plasma levels of chemerin correlate positively with age (153) and are also associated with BMI, plasma triglycerides, blood pressure, and fasting serum insulin (153-157). Chemerin and CMKLR1 are highly expressed in human and mouse adipocytes (152, 153, 158, 159). Chemerin expression increases with obesity, insulin resistance (153, 158), and metabolic syndrome (153). Furthermore, chemerin is involved in the regulation of adipogenesis (158). The *in vitro* expression and secretion increases drastically during differentiation of fibroblasts into mature adipocytes (152, 159). Additionally, the expression of chemerin significantly increases in adipose tissue of animals with impaired glucose tolerance or T2Dm compared to animals with normal glucose tolerance (152, 153). Chemerin also seems to have functional impact on adipocytes, for example stimulation of glucose transport (152, 159), activation of extracellular-signal regulated kinase 1/2 (ERK 1/2), and lipolysis (152). In 3T3-L1 adipocytes, chemerin promotes insulin mediated glucose uptake and insulin receptor substrate 1 tyrosine phosphorylation. Therefore, chemerin may improve insulin sensitivity in adipose tissue (159). Plasma chemerin levels were significantly higher in obese subjects compared with normal weight individuals (153). After adjusting for BMI, plasma chemerin levels were still independently associated with metabolic syndrome-related phenotypes, including systolic blood pressure and plasma triglycerides, but not measures of insulin sensitivity or glucose homeostasis (153).

Recently, chemerin and its receptor CMKLR1 were detected in Leydig cells of human testis (160). However, neither their presence in human spermatozoa nor their function was investigated.

2.2.3 Vaspin

Visceral adipose tissue-derived serine protease inhibitor (vaspin) is a member of the serine protease inhibitor (serpin) family and is constructed of 395 amino acids (161). Serpines are a super family of proteins characterised by a nuclear domain with 3 β-sheets and 9 α-helices (162, 163). Hida *et al.* isolated vaspin from visceral adipose tissue of Otsuka Long-Evans Tokushima Fatty (OLETF) rats (161). This animal model is characterised by abdominal obesity, insulin resistance, T2Dm, hypertonia, and dyslipidemia (161). Vaspin mRNA was highly expressed in white adipose tissue after 30 weeks when body weight and insulin levels reached their maximum (161). After 50 weeks, the vapsin mRNA expression decreased with worsening of diabetes, characterised by increased HbA_{1c} and declined fasting immunoreactive insulin levels (161). However, mRNA expression of vaspin was not detectable in 6 weeks old thin Long-Evans Tokushima Otsuka (LETO) rats or in subcutaneous, brown adipose tissue or non-adipose tissues of OLETF rats (161). Moreover, vaspin was detected in serum of OLETF and LETO rats and these serum levels were higher in OLETF rats than in 30 weeks old LETO rats (161). Treatment with human vaspin improved significantly insulin sensitivity and glucose tolerance (161).

Human vaspin mRNA was detected in visceral and subcutaneous white adipose tissue in a subpopulation of obese individuals but not in lean humans (BMI<25 kg/m^2) with normal glucose tolerance and visceral vaspin expression correlated significantly with BMI, and percentage of body fat (164). Subcutaneous vaspin mRNA expression correlates significantly with waist to hip ratio (WHR) and fasting plasma insulin concentrations (164). The induction of vaspin mRNA expression in human adipose tissue might be a compensatory pattern associated with obesity and insulin resistance (161, 164-166).

Vaspin serum concentrations range from 0.1 to 6.74 ng/ml (167), whereas in another study the levels in men and women were found to be 1.0±1.3 ng/ml (mean±SD) and were significantly higher in women than in men (168). The result of gender specific vaspin levels was contrasted by a study of Youn *et al.* (167). In normal glucose tolerant subjects, vaspin serum levels were significantly lower in women than in men but no gender difference was found in diabetics. Furthermore, in men no differences in serum vaspin levels were found between normal glucose tolerant individuals and diabetics (167).

Vaspin levels in serum correlated positively with obesity, impaired insulin sensitivity (167) and T2Dm (164, 167), whereas in T2Dm patients no correlation was found between circulating vaspin levels and BMI or insulin sensitivity (167). These results are contrasted by a significantly inverse correlation of vaspin serum concentrations to WHR (168). Furthermore, the authors could not demonstrate a correlation between vaspin serum levels and markers of insulin sensitivity and glucose metabolism (168). However, vaspin concentrations in serum of normal weight and overweight/obese men did not differ significantly as well as concentrations between diabetics and non-diabetics (168). Serum levels of vaspin

were lower in lean subjects and athletes with long-term training, but these levels increased with body weight loss coupled with physical training (167).
Overall, vaspin has insulin sensitizing effects in rats with T2Dm (161) and in obese and diabetic humans (164). Upregulation of vaspin might have a protective effect against insulin resistance, but it is not clear if vaspin upregulation is a cause or it might be protective in the development of obesity and metabolic dysregulation. Recently, vaspin was described to inhibit the protease kallikrein 7, which cleaves insulin into the A- and B-chain (169). Hence, vaspin might inhibit insulin degradation rather than increasing insulin sensitivity (169).
So far, only one study described the presence of vaspin in the reproductive tract of rats. Rats consuming a high fat chow had significantly higher periepididymal fat accumulation compared with control rats (170). Rats consuming high fat or basal diet for 16 weeks had higher periepididymal fat mass than subcutaneous mass. Vaspin levels in serum and periepididymal adipose tissue were much lower in rats consuming high fat diet compared with basal diet (170). In rats, serum vaspin levels correlated positively with vaspin levels in periepididymal fat tissue (170) indicating a secretion or expression independent of the type of adipose tissue.

2.2.4 Leptin

Leptin is one of best described adipokines so far. This 16 kDa protein is a product of the *ob* gene (171) and predominately regulates body weight (172), as well as immunity, haematopoiesis, angiogenesis, wound healing, reproduction, and bone homeostasis (173-177). In the brain, leptin has been shown to influence the hypothalamus controlling the appetite, thyroid axis and levels of growth hormone (178-181). Decreased leptin signalling or receptor function increases energy intake and lowers energy expenditure (182). Leptin deficiency – a cause of severe early-onset obesity, hypogonadism, hyperinsulinemia, hyperphagia, and impaired T-cell mediated immunity – is treatable with recombinant leptin (183, 184). Furthermore, leptin has peripheral effects on glucose and lipid metabolism independent of central weight regulation pattern mediated by pancreatic β-cells and hepatocytes (185-187).

2.2.4.1 Leptin receptor

Leptin binds to a single membrane spanning receptor with homology to the class I cytokine receptor family (188). The leptin receptor (ObR) also belongs to the growth hormone receptor subfamily. All of these members are activated by homodimerisation (189). Additionally, the leptin receptor undergoes homooligomerisation (190, 191). For this receptor, 5 isoforms (ObRa-e) were detected in humans and generated by alternative mRNA splicing of the *db* gene (192). The four splice variants ObRa, ObRb, ObRc, and ObRd share

an extracellular and transmembrane region. The first 29 intracellular amino acids consist of two cytokine receptor homology (CRH) domains, which are separated by an immunoglobulin (Ig) like domain and followed by two membrane proximal fibronectin type III (FN III) domains. The ObRb receptor will be described further, because this isoform is relevant for the results obtained in this project. The membrane proximal CRH2 domain is necessary for leptin binding with an affinity in the nanomolar range (193). The Ig-like domain is also critical for receptor activation since receptors lacking this domain are properly expressed on the cell surface and bind leptin comparable to the wild type receptor, but fail to activate the associated Janus kinases (JAKs) and subsequent intracellular signalling cascades (194). Only the long isoform (ObRb) has an extended intracellular domain of 301 amino acids with binding sites for downstream signalling molecules. All the receptors of class I cytokine family including ObRb lack intrinsic tyrosine kinase activity and rely on the kinase activity of constitutively associated tyrosine kinase of the Janus kinase family JAK2 (195). A conserved membrane proximal proline rich region in ObRb called Box 1 motif, is essential for the JAK2 association while the Box 2 motif also contributes to JAK activation (196, 197). The Box 2 motif is not present in short isoforms and seems to be inactive in signalling (195, 197).

The phenotype of *db/db* mice lacking ObRb is similar to that of leptin deficient *ob/ob* mice including morbid obesity, hypothermia, hyperglycaemia, hyperlipidemia, decreased insulin sensitivity and infertility (192, 198-201). The ObRb is present in several neural tissues, but is highly expressed in multiple hypothalamic regions (202-204). In addition to the brain, the ObRb is also expressed in multiple peripheral tissues, including pancreatic islets, adipose tissue, testes, ovaries, liver and immune cells as reviewed by Matarese *et al.* and Margetic *et al.* (177, 205).

Overall, leptin is able to activate three different pathways: the JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathway, the SHP-2/MAPK (Src-homology 2 domain-containing phosphatase 2/mitogen-activated protein kinase) pathway, and the PI3K (phosphatidylinositol 3-kinase) pathway (206).

2.2.4.2 Physiological effects of leptin

Obese humans have higher leptin levels in serum than normal weight individuals, but obese subjects are often refractory to a leptin treatment due to a leptin resistance (207). Moreover, serum leptin levels correlate with the amount of body fat (207). Leptin serum levels are regulated in a gender dependent manner (208, 209). Several studies suggest a proinflammatory role of leptin in the regulation of innate and adaptive immune responses. Leptin enhances the proliferation and maturation of T cells, macrophages, natural killer cells and dendritic cells and promotes the production of proinflammatory cytokines leading towards a T helper 1 immune response (176, 210). In pancreatic islets, leptin directly in-

hibits insulin expression and secretion (211, 212). In the liver and white adipose tissue, leptin inhibits lipogenesis and stimulates lipolysis (213, 214). Leptin directly promotes fatty acid oxidation in isolated adipocytes and skeletal muscles (213, 215). However, deletion of the ObRb in these peripheral tissues has no effect on energy balance, body weight or glucose homeostasis in mice, indicating that these processes are mainly directed by the central effects of leptin in the brain (216-218). The effects of leptin on reproduction (219) are well described (Figure 3), especially in females (220).

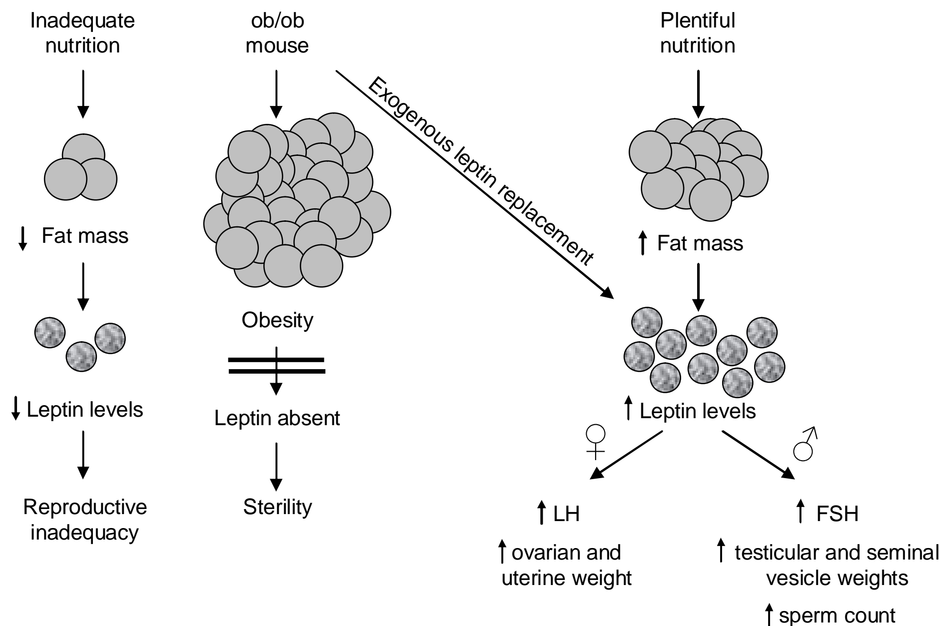


Figure 3 Leptin's function in reproduction. Nutrition as well as mutations in the *ob* gene lead to decreased serum leptin levels and therefore have a negative impact on fertility. In contrast, high leptin serum levels affect fertility in the hormonal and cellular way (according to (219)).

Ob/ob mice were investigated to get a hint of leptin's function in the reproductive tract. These animals showed significant more abnormalities in seminiferous tubules than animals treated with leptin, indicating an impact of leptin on male reproductive organs (221). Furthermore, food restriction of *ob/ob* mice did not return fertility, whereas leptin treatment was able to restore fertility in male *ob/ob* mice (222) and increased testicular weight and sperm count in *ob/ob* mice (221, 222). In humans, leptin was detected in seminal plasma (26, 223) and in seminiferous tubules (24). Leptin levels were shown to be affected by reproductive hormones. For example, leptin levels were inhibited by androgens (224-226) and estrogens increased leptin levels (227). Overall, leptin has important functions in regulation of puberty and reproduction in different species (184, 228-232).

2.2.5 Resistin

Resistin is a 12.5 kDa peptide, which belongs to the resistin like molecule (RELM) family of cysteine-rich proteins or found in inflammatory zone (FIZZ) proteins (233). This adipokine shows no homology to any known hormones or cytokines (234). Resistin is se-

creted by adipocytes of rodent white adipose tissue (234). The hormone FIZZ1 or RELMα is expressed in mononuclear cells, as well as in muscle, pancreatic cells, and adipocytes (235). Furthermore, resistin mRNA was detected in adipose tissue of rats (236) and humans (237). Resistin mostly circulates as a high molecular weight hexamer but is also present as a distinct, more active low molecular weight complex (238).

In rodents, resistin serum levels increase with obesity (234, 239). Resistin also mediates insulin resistance in liver and skeletal muscle (240-243). Moreover, administration of recombinant resistin decreased insulin sensitivity and blocking resistin activity resulted in increased insulin sensitivity (244). Resistin levels and single nucleotide polymorphism (SNPs) might be associated with obesity, insulin resistance and T2Dm (123, 245-247), whereas in other studies no such associations have been confirmed (248-251). So far, the function of resistin in glucose metabolism is not clear. Resistin is linked to insulin resistance in mice (234), but its function in the pathogenesis of human diabetes remains a matter of debate (245, 249, 252, 253). In humans, no correlation between resistin expression and obesity, insulin resistance and T2Dm was found (254), whereby other studies detected a correlation between central obesity and increased resistin expression (237, 252, 255). So far, resistin was shown to be associated with inflammation (256, 257). Resistin expression is increased in human peripheral blood mononuclear cells by inflammatory cytokines like tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (258). Furthermore, resistin induces the expression of TNF-α and IL-6 in white adipose tissue and peripheral blood mononuclear cells (256, 259, 260).

The function of resistin in male reproduction is not resolved yet, but this adipokine was detected in human seminal plasma correlating with inflammation markers, such as elastase and IL-6 (23). Moreover, resistin mRNA is expressed in Leydig and Sertoli cells of rat testis (261). The mRNA expression is regulated by PPARγ and leptin in rat testis and incubation with resistin influences testosterone secretion (261). This suggests that resistin is essential in endocrine metabolism of rat testis, whereas the function in humans is not explored yet.

2.2.6 Progranulin

Progranulin, a 593 amino acid glycoprotein belongs to the family of granulins, also called epithelins. The granulins are characterised by a unique motif of 12 cysteinyl residues (262, 263). Progranulin is proteolytically cleaved to granulins by elastase (264, 265). These granulins are proinflammatory, whereas progranulin is anti-inflammatory (265). Progranulin is characterised as a growth promoting factor (266) and an adipocyte secreted protein (267), which was found to have important functions in immune response (265, 268). It was shown to be expressed in epithelial cells, like skin, gastrointestinal tract, reproductive system and immune cells (269). Furthermore, this adipokine mediates wound

response (270, 271) and is demonstrated to have regulatory functions in sexual differentiation of the rat brain (272, 273). Progranulin serum levels in insulin resistant individuals are 1.5-fold higher compared with the insulin sensitive group (274). In patients with impaired fasting glucose or T2Dm, progranulin serum levels are significantly higher compared with normal glucose tolerant patients (275). Progranulin correlates significantly positive with waist circumference (275) and visceral obesity (276). Additionally, progranulin is identified as a marker for inflammation in obesity and T2Dm reflecting macrophage infiltration in omental adipose tissue (276). The presence of progranulin in the male reproductive tract was firstly described in guinea pig spermatozoa as an acrosomal protein, referred to as acrogranin (277, 278).

2.2.7 Visfatin/NAMPT

The visceral fat-derived hormone visfatin, also known as nicotinamide phosphoribosyltransferase (NAMPT) is a highly conserved 52 kDa protein (279) with pleiotropic biological effects. According to the nucleotide sequence it shows homologies to cytokines and chemokines (279). NAMPT mRNA is highly expressed in human visceral adipose tissue (280), liver, skeletal muscle, heart, placenta, lungs, kidney, and bone marrow (279). Moreover, this protein is ubiquitously expressed in rat tissues (281). NAMPT is secreted by mouse and human mature adipocytes, hepatocytes, macrophages, and leucocytes (15, 282-284). This secretion is mediated via an unresolved, non-classical secretory pathway (284). Two forms of NAMPT have been described in the literature: the intracellular (iNAMPT) and extracellular (eNAMPT) form (283), whereas eNAMPT secreted by adipocytes is more enzymatically active compared to iNAMPT (283). In human serum, NAMPT is enzymatically active as a dimer (283, 285). This suggests, that eNAMPT contributes to the extracellular synthesis of nicotinamide mononucleotide (NMN), which is transferred into cells by an unidentified transport and subsequently promote nicotinamide adenine dinucleotide (NAD^+) biosynthesis at a systemic level (286, 287).

Plasma levels of visfatin correlate positively in humans with age (288), obesity, visceral fat tissue mass, T2Dm, and metabolic syndrome (289-291). Furthermore, visfatin promoter SNPs are associated with fasting glucose, insulin levels and T2Dm (292, 293). Visfatin levels in plasma differ significantly in normal glucose tolerant and obese individuals (0.001±0.000 µg/ml vs. 0.037±0.008 µg/ml) (290).

NAMPT shows nicotinamide phosphoribosyltransferase activity (294) and is therefore a rate limiting enzyme in the NAD^+ salvage pathway starting from nicotinamide and biosynthesis of NMN (295). NAMPT converts nicotinamide and 5'-phosphoribosyl-1-pyrophosphate into NMN (296) and thereby regulates total NAD^+ cellular levels (297) as well as mitochondrial NAD^+ levels (298). NAD^+ plays a crucial role in energy metabolism and functions as a cofactor in more than 200 oxidation reduction reactions (299). It is a

substrate for poly(ADP-ribose) polymerase-1 (PARP-1), sirtuins and adenosine diphosphate (ADP) cyclases, which are linked to glycolysis, gluconeogenesis, lipolysis, insulin secretion, Ca^{2+} mobilisation, aging, genomic stability, and apoptosis (299-303). One of the major causes of cell death, due to genotoxic stress, is hyperactivation of the NAD^+-dependent enzyme PARP-1. It depletes nuclear and cytoplasmic NAD^+ causing the translocation of apoptosis inducing factor from the mitochondrial membrane to the nucleus (304-307). The crucial role of NAMPT in the cell survival (298, 308) is mediated by sirtuin 1 (SIRT1) (309), SIRT3 and SIRT4 (298). The sirtuins are a conserved family of NAD^+-dependent deacetylases (310).

NAMPT also regulates the insulin signalling pathway, although NAMPT does not bind to the insulin receptor (311-314). NAMPT seems to be stress and nutrient sensitive as nutrient restriction resulted in an increase of NAMPT mRNA and protein expression in human fibrosarcoma and rat fasting livers (298).

So far, NAMPT has not been detected in human spermatozoa. In contrast, NAMPT is present in different cell types of chicken testis, such as Sertoli cells, Leydig cells, primary spermatocytes, secondary spermatocytes, round spermatids, and elongated spermatids (315).

2.3 Autophagy

The ability of human spermatozoa to perform the apoptosis signalling cascade as a form of cell death has been revealed (316, 317). However, there are also other forms of programmed cell death (PCD) to be considered – autophagy/type II of PCD and necrosis/type III of PCD. Autophagy, a “self-eating” process, was firstly described by de Duve in 1963 (318). To date, its occurrence in human spermatozoa is not known. Interestingly, autophagy may not only trigger cell death, but also resembles a dynamic catabolic process for the non-specific degradation of cell compartments and maintains bio-energetic requirements (319). Damaged proteins or other cytoplasmic components are subjected to degradation via lysosomes (320) in case of severe impairment of cellular functions due to nutrient and growth factor withdrawal (319). Hence, this catabolic process occurs at a basal level in somatic cells to maintain the energy homeostasis and is involved in human diseases, including neurodegeneration, viral and bacterial infections, heart disease, and cancer (321, 322). Autophagy itself is known as a dynamic process in a cell and can be classified into three distinct stages. The first stage is described as sequestration of cytosolic components in the double-layered autophagosome. Secondly, the autophagosome fuses with a lysosome to form the autolysosome. Lastly, the autolysosomal contents are degraded by proteases (Figure 4).

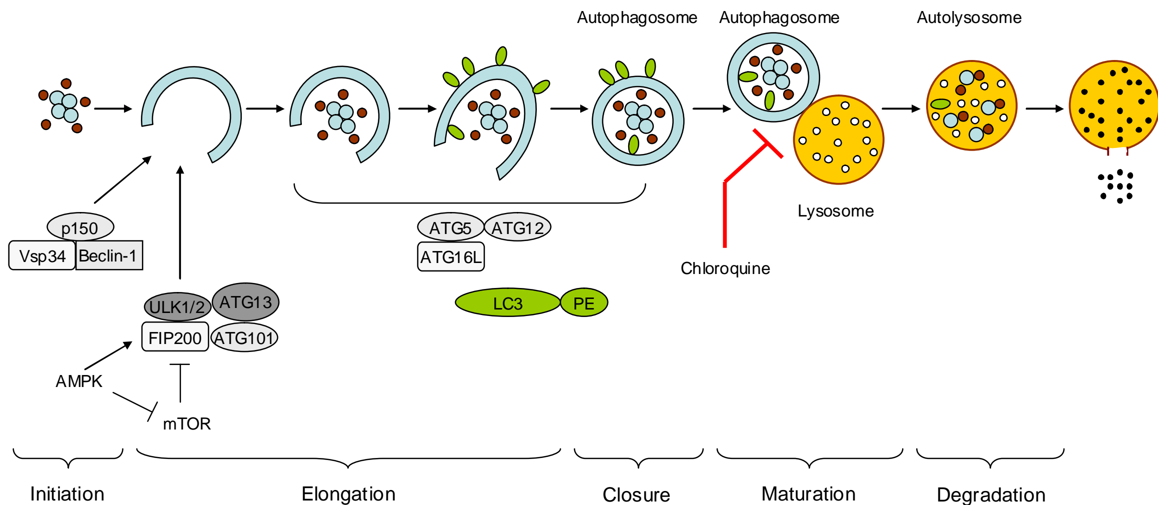


Figure 4 Schematic autophagy process. The autophagy is divided into different stages: initiation, elongation, closure, maturation and degradation (according to (323)). Chloroquine blocks the fusion of autophagosome and lysosome.

More than 30 autophagy-related gene (Atg) proteins, which are conserved from yeast to mammals, participate in autophagy at different steps throughout the process (324, 325). Mammalian Beclin-1 (Bec1), another autophagy related protein, is a structural and functional homologue of yeast Atg6/vacuolar protein sorting (Vps) 30 (326). It has been demonstrated that Beclin-1 interacts with the class III PI3K to form a complex in the trans-golgi-network (327). Furthermore, it forms a core complex (328) with Vps34, Vps15 and activating molecule in Bec1-regulated autophagy (Ambra 1) (329-331) and interacts with numerous proteins like UV radiation resistance-associated gene (UVRAG) or Atg14-like protein (Atg14L) promoting PI3K activity, activating autophagosome formation and maturation (332, 333). Rubicon (RUN domain protein as Bec1 interacting and cysteine-rich containing) is another protein binding to Beclin-1, whereas it is known as a negative regulator of autophagy (333). Moreover, Beclin-1 contains a Bcl-2 homology-3 (BH3) domain that is necessary and sufficient for binding to anti-apoptotic proteins of the Bcl-2 family (334).

A commonly used marker for autophagy is the processed form of LC3 (335, 336). It is also known as “microtubule associated protein 1 light chain” and appears as an unprocessed pro-form. It is cleaved proteolytically at the C-terminal flanking region by Atg4 protease (337). Subsequently, LC3-I is formed with a carboxyterminal exposed glycine. Due to the induction of autophagy, the glycine is conjugated to phosphatidylethanolamine by Atg7 (E1-like activating enzyme), Atg3 (E2-like conjugating enzyme) and by the Atg16L complex (E3-like ligase enzyme), generating the final LC3-II form (335, 337-341). LC3-I is localised in the cytoplasm, whereas LC3-II is associated with autophagosome membranes (340) and is therefore described as an autophagy marker (336). LC3, which is associated with the outer membrane, is liberated into the cytosol when the autophagosome matures

into an autolysosome. However, the inner membrane LC3 is trapped within the autolysosome and finally degraded by lysosomal proteases (342).
With respect to male reproduction, the presence of autophagy as measured by LC3B-II has been described only for stallion spermatozoa (343), where a subpopulation of more viable stallion spermatozoa was characterised by higher levels of LC3B-II. This may indicate a mechanism of autophagy for sperm survival. Interestingly, in this study no inducer for autophagy was used to clarify the mechanism of autophagy in spermatozoa. However, in human spermatozoa the presence and relevance of autophagy remains to be revealed.

2.4 Theses and aims

Subfertility in obese men is intensively investigated, whereas the molecular causes have not been fully elucidated. As proteins secreted by the fat tissue, so called adipokines play major role in the regulation of cell metabolism, their impact on obesity related subfertility was content of this study. Accordingly, it was hypothesised, that serum adipokines are transferred into or expressed in the male reproductive tract and have a direct or indirect influence on functionality of human spermatozoa. Also, obesity associated changes in adipokine levels are directly linked to male infertility. These hypotheses were tested in an observational study with serum and seminal plasma of normal weight, overweight, and obese donors. For this study, the associations of adipokines with the main functional parameters of spermatozoa were investigated. Additionally, it was hypothesised, that leptin and adiponectin influence the physiological processes of capacitation and acrosome reaction as well as apoptosis signalling cascade. Subsequently, human ejaculated spermatozoa were incubated with these adipokines and the motility patterns, the expression of tyrosine phosphorylated proteins involved in capacitation, the percentage of acrosome reacted spermatozoa, the acitivity of caspase 3&7 and the mitochondrial membrane activity were determined.
The high NAMPT levels in human seminal plasma indicated that NAMPT may be present in and secreted by human ejaculated spermatozoa and may have an influence on sperm motility, fertilisation capacity and sperm maturation process. As a consequence, we aimed to examine NAMPT protein expression in human testis and spermatozoa as well as in the cell supernatant. Furthermore, NAMPT enzyme activity was blocked by a specific inhibitor to investigate the effects on sperm functional parameters.
As the inhibition of NAMPT enzyme activity is accompanied by the induction of autophagy in somatic cells, it was assumed that autophagy may be an additional patho-/physiological process in human spermatozoa and testis. To answer this question, protein expression of LC3B and Beclin-1 were examined in human spermatozoa as well as in testis. In addition, the effect of an autophagic flux inhibitor was investigated in human spermatozoa to elucidate the regulation of autophagy in male reproduction.

3 Material and Methods

3.1 Study population

For the observational study, 96 voluntary men were enrolled from the Leipzig local population between 2007 and 2011. Men were recruited per notice and newspaper advertisement. The subjects were stratified according to the BMI into a normal weight (BMI 18.50-24.99 kg/m^2), an overweight (BMI 25-29.99 kg/m^2) and an obese subgroup (BMI≥30 kg/m^2) (344). Anthropometric data of study subjects are summarised in Table 1: In detail, 41 (43 %) men with normal weight served as controls, 31 (32 %) were overweight and 24 (25 %) were obese. Both phenotypes were comprised in the overweight/obese group. Normal weight men were significantly younger than overweight and obese subjects (P≤0.05). Waist circumference significantly differed between normal weight men and the overweight/obese group (P<0.001). This study was approved by the Institution Review Board of the Faculty of Medicine, University of Leipzig (vote number 216-2007). Male subjects at the age of 18-65 years were included into the study. In contrast, men with varicocele, cryptorchidism, drug abuse, vasectomy or current use of exogenous hormones (e.g. testosterone replacement therapy) were excluded from the study. Additional exclusion criteria were defined as follows: CRP > 10 mg/l and HBA_{1c} ≥ 6.5 % (345). Due to potential impairment of spermatogenesis, normal weight men with serum inhibin B < 50 ng/l and FSH > 12.4 IU/l levels were also excluded. Furthermore, normal weight men with triglycerides > 2.3 mmol/l, DNA fragmentation index (DFI) > 30 % were excluded from the study.

Table 1 Anthropometric data of normal weight (n=41) and overweight/obese men (n=55). Anthropometric data were adjusted for age using robust linear regression. Groups were compared using Mann-Whitney U-test. Data are described as median (range). * P≤0.05; ** P<0.01; *** P<0.001 compared with normal weight men.

Parameter	Normal weight	Overweight/Obese
Age (years)	27.0 (19.0-64.0)	38.0 ** (18.0-62.0)
BMI (kg/m^2)	22.9 (18.9-24.8)	29.4 *** (25.1-51.6)
Waist circumference (cm)	85.0 (67.0-100)	104 *** (87.0-148)

Spermatozoa of normal weight men were used for detection of immunofluorescence, western blot analysis, adipokine secretion and incubation experiments.

3.2 Fertility evaluation

The fertility evaluation consisted of semen analysis, blood sampling, a general questionnaire by face-to-face interview and a physical examination, including testicular ultrasound. The questionnaire included information about medical history, body weight, height, lifestyle factors, use of alcohol, cigarettes, drugs and vitamin supplements. In addition, height

and weight were measured by a nurse, because self-reports tend to overestimate height and underestimate weight (346).

3.3 Semen analysis

The semen samples were collected by masturbation into a sterile plastic container. A sexual abstinence of 3-5 days was recommended, because shorter or longer abstinence may impair semen quality (347). Each man signed an informed consent form and got a short introduction about the semen donation to guarantee a high pre-analytic standard. The number of days since last ejaculation was recorded. At least two semen analyses were performed according to WHO guidelines since the semen quality varies in a high range (348). After liquefaction of at least 15 minutes, semen samples were evaluated for sperm concentration (Neubauer chamber improved) and morphology. Sperm motility was also analysed according to WHO guidelines (68, 349) by a single examiner. All samples were characterised into 4 categories according to motility (349): a) fast progressive spermatozoa, b) slow progressive spermatozoa, c) local motile, non-progressive spermatozoa and d) non-motile spermatozoa. Based on WHO guidelines of 2010, the categories a)+b) are summarised as progressive spermatozoa, because it is difficult for the examiner to distinguish between fast and slow spermatozoa. Acrosome status was determined by fluorescein labelled *Pisum sativum* agglutinin (350). Therefore, air-dried smear preparations were fixed for 30 sec in absolute ethanol. Afterwards, smears were incubated in the dark with fluorescein isothiocyanate (FITC)-labelled lectin from *Pisum sativum* for at least 4 h at 4 °C. Lectin solution was prepared according to: 2 ml 0.2 M phosphate buffered saline (PBS)/sodium azide (pH 7.4) + 50 µl FITC-labelled lectin (stock solution 1 mg/ml). After incubation, slides were washed three times with PBS/sodium azide. The wet slide was sealed with a glycerol droplet and a coverslip (24x50 mm). Spermatozoa with intact acrosomes showed a clear, equal fluorescence at the apical site of the head. Acrosome reacted spermatozoa showed a fluorescence signal at the equatorial region. If the acrosome has already reacted, no fluorescence signal is left at the acrosome or the equatorial region. Aniline blue staining as a hint for indirect protamine content (351) and chromatin maturation was determined of air-dried smear preparations. Smears were fixed with glutaraldehyde solution (3 g of glutaraldehyde + 100 ml 0.2 M phosphate buffered NaCl solution) for 30 min. Afterwards, slides were dried at room temperature (RT) and overlayed with 5 % aqueous aniline blue solution for 2 min. Subsequently, slides were washed with aqua dest and dried at RT. For analysis, slides were covered with immersion oil and 200 spermatozoa were counted and characterised as dyed or non-dyed and lightly dyed under a 100x magnification. Spermatozoa with resting histones were detected by aniline blue as this dye binds to lysine rich sites in the nucleus, therefore a maximum of 30 % of the spermatozoa should be dyed blue. DNA fragmentation index (DFI) was determined by

acridine orange (352). Air-dried smear preparations were fixed for 24 h in carnoy solution (3 volumes methanol and 1 volume glacial acetic acid) at 4 °C. 2.5 ml of 0.1 % acridine solution and 10.6 ml buffered citric acid (0.1 M citric acid, 0.3 M Na_2HPO_4, pH 2.5) were mixed and slides were incubated for 2 min with this solution. After incubation, slides were washed with aqua dest and were covered immediately with a coverslip. Until determination, slides were stored at 4 °C or -20 °C. Analysis was performed with the FITC filter at a 100x magnification and 100 spermatozoa were counted and categorized into green, orange and red cells. DFI was calculated as follows: the number of red spermatozoa was multiplied by 100 and divided by the sum of green and red spermatozoa. Remaining portions of semen samples were centrifuged (1000 x g, 10 min). The supernatant/seminal plasma was stored at -80 °C until further measurement of adipokines. According to semen guidelines of the WHO, 200 spermatozoa were counted for evaluation of motility, vitality, morphology, DFI and acrosome status (68). Motility and vitality of all semen samples were analysed with a 40x magnification of a light microscope (Olympus CX21). Acrosome status and DFI were determined with a fluorescence microscope (Olympus BX41).

Normal weight men had normal semen parameters according to Table 2 and served as control group for the following analysis. Only 3 out of 41 normal weight men had a DFI > 13 %.

Table 2 Reference values of semen parameters. According to WHO 2010 these values are considered as the lower limit to declare a donor as fertile. a-fast progressive spermatozoa, b-slow progressive spermatozoa, c-local motile spermatozoa

Semen parameter	WHO reference values
Abstinence	2-5 d
Volume	>1.5 ml
pH	>7.2
Progressive motility	32 % a+b
Total motility	40 % a+b+c
Vitality (Eosin)	>58 %
Sperm concentration	>15x10^6/ml
Sperm count (volume x concentration)	>39x10^6
Protamine status	>70 %
Morphology	4 %
DFI (DNA Fragmentation Index)	>15 % good chance for a pregnancy >30 % decreased chance for a pregnancy
Intact acrosome	>50 %

3.4 Analysis of blood samples

All blood samples were withdrawn from cubital vein between 8 am and 11 am, because of the circadian rhythm of reproductive hormones. Blood was centrifuged (856 x g, 12 min) and supernatant/serum was collected for reproductive hormone and adipokine measurements and analysis of the full blood exam. Samples were frozen at -80 °C until analysis of cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cho-

lesterol, creatinine, urea, C-reactive protein (CRP), luteinising hormone (LH), follicle stimulating hormone (FSH) and total testosterone. These parameters were determined by the Modular System. Intra- and interassay coefficients of variation were below 11.7 %. HbA_{1c} was determined in EDTA blood of every donor to evaluate the glucose status. The analysis of these parameters was kindly accomplished by the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University of Leipzig.

3.5 Adipokine measurements in serum and seminal plasma

Levels of adiponectin, chemerin, resistin and progranulin levels were determined by enzyme-linked immunosorbent assay (ELISA). Intra- and interassay coefficients of variation were below 6.70 %, 5.16 %, 6.80 % and 8.00 %. The analytical sensitivity of these assays yielded 0.6 ng/ml, 0.005 pg/ml, 0.012 ng/ml and 0.018 ng/ml. Concentrations of vaspin and NAMPT also were also evaluated by ELISA. Intra- and interassay coefficients of variation were below 9.11 % and 9.06 %. The analytical sensitivity of these assays yielded 30 pg/ml and 12 pg/ml. The levels of leptin were determined by sensitive radioimmunoassay (RIA). The sensitivity of the RIA was 0.04 ng/ml. Intra- and interassay coefficients of variation were below 7.6 %.

The ELISA kits were not validated for measurement of adipokines in seminal plasma (SP) by the manufacturer. The validation was performed before measurements of adipokine concentrations in this fluid. A SP pool was established to find the appropriate dilution for the detection of adipokines. This pool consisted of SP from 12 normal weight men and was diluted 1:2 to get dilution series. Measurements for precision and spiking were performed, subsequently. For later experiments, adipokine concentrations in SP were determined as optimal, when concentrations were established in the lower third of the standard curve. SP samples were spiked with diverse standards and the recovery was determined, subsequently. A recovery of 100±30% was accepted. For the evaluation of the precision a 6-fold determination of adipokine concentrations was performed with the optimal dilution of the respective adipokine. The coefficient of variation was determined to get a measure of accuracy and a value less than 10 % was accepted for the precision, spiking and dilution experiments. All measurements were performed according to manufacturer's instructions.

3.6 Density gradient centrifugation of human spermatozoa

Spermatozoa concentration, count, motility, and vitality were determined from every donor by a single examiner. Afterwards, semen was centrifuged by density gradient (DGC) with ISolate® Sperm Separation Medium. This sperm preparation medium consists of a colloid suspension of silica particles, stabilised with covalently bound hydrophilic silane in a HEPES buffered human tubal fluid (HTF). This medium consists of a 50 % upper and a

90 % lower layer. Semen was overlaid on the two-layer gradient and centrifuged at 600 x g for 20 min. After the first centrifugation, the first layer consisted of seminal plasma, the second layer (50 % upper layer) as well as the third layer (90 % lower layer) consisted of immature or non-motile spermatozoa, dead spermatozoa, debris and round cells (leucocytes, epithel cells, prostate cells). At the bottom of the tube, the pellet consisted of mature (highly motile, morphologically normal) spermatozoa. The seminal plasma was immediately centrifuged at 1000 x g for 10 min, 4 °C and frozen at -80 °C. The immature spermatozoa were washed twice with Earle's Balanced Salt Solution (EBSS) medium. The mature spermatozoa were resuspended in EBSS and layered on a DGC to ensure a round cell free fraction. After the second DGC, mature spermatozoa were washed twice with EBSS as well. Leucocytes were eliminated from semen by anti-CD45 magnetic MicroBeads. Therefore, the sperm pellet was resuspended in 80 µl Annexin V Binding Buffer and 20 µl MicroBeads were added. This mixed suspension was incubated for 15 min at 4 °C. Afterwards, the spermatozoa were washed with 1 ml Annexin V Binding Buffer and centrifuged at 300 x g for 10 min at RT. The remaining pellet was resuspended in 500 µl Annexin V Binding Buffer, while the magnetic column was prepared in the magnetic field with 500 µl Annexin V Binding Buffer. After preparing the column, the sperm suspension was layered onto the column. The column was washed three times, subsequently. The spermatozoa were counted and the leucocyte negative suspension was centrifuged at 400 x g for 5 min at RT. Spermatozoa fractions were immediately prepared for the experiments or frozen at -80 °C.

3.7 RNA isolation of human spermatozoa

RNA of human spermatozoa was isolated with TRIzol® Reagent and following the manufacturer's protocol with a few alterations. Spermatozoa were solubilised in 1000 µl TRIzol®, vortexed for 1 min and incubated at RT for 10 min. Afterwards, 200 µl chloroform were added. The tubes were shaken for 15 sec and incubated 10 min at RT. The samples were centrifuged at 12000 x g for 20 min at 4 °C, subsequently. The upper layer was transferred to a new sterile tube and 500 µl isopropanol were added. The tubes were mixed carefully, followed by an incubation of 15 min at RT. Afterwards, the tubes were centrifuged at 12000 x g for 30 min at 4 °C. The supernatant was discarded carefully. Afterwards, the pelleted RNA was washed twice with 75 % ethanol at 12000 x g for 5 min at 4 °C . The pellet was dried for 10 min, resuspended in 10 µl RNase free water and shaken for 10 min at 55 °C. Afterwards, the RNA concentrations and extinction ratios $A_{260/280}$ and $A_{230/280}$ were determined immediately by NanoDrop measurement with 1.5 µl RNA solution. During RNA concentration determination, the samples were stored on ice to prevent RNA degradation. The RNA was stored at -80 °C until cDNA synthesis.

3.8 cDNA synthesis

The cDNA synthesis was performed with the SuperScript® II Reverse Transcriptase. This enzyme can perform reverse transcription with 1 ng-5 µg total RNA. Somatic cells only consist of 5 % mRNA and 95 % rRNA and tRNA. In contrast, no rRNA was detected in human spermatozoa (54). The reverse transcription was performed with at least 500 ng total RNA.

Protocol reverse transcription:

500 ng or more RNA + 1 µl Random Hexamer Primer

10 min at 68 °C

9 µl of the master mix 1x were added to the suspension of RNA + Hexamer Primer

1st strand buffer	4 µl
DTT	2 µl
dNTPs	1 µl
RNAsin	1 µl
Superscript	1 µl

1 h at 42 °C

Subsequently, samples were 1:5 diluted with Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 1 L ddH_2O, with NaOH/ HCl, pH 7.0).

3.9 Polymerase Chain Reaction (PCR) and detection of PCR products

The PCR was performed according to the following protocol:

cDNA	2.5 µl
10x PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
1.25 mM dNTPs	2.5 µl
0.4 pM primer forward	0.5 µl
0.4 pM primer reverse	0.5 µl
Taq DNA Polymerase	0.25 µl
LiChrosolv® water	13.75 µl

The master mix was prepared for 25 µl. The amplification conditions were according to:

	95 °C	3 min	initial denaturation of the cDNA-DNA hybrid
40 cycles of:	95 °C	15 sec	denaturation
	60-62 °C	20-30 sec	primer annealing
	72 °C	30 sec	primer extension
	10 °C	∞	

After 40 PCR cycles, samples were stored at -20 °C until use.

To detect the PCR products, agarose gels (3 %, UltraPure™ Agarose) were prepared. 10 µl of the samples and 2 µl of dye solution were mixed and applied to the gel, which was liquefied in a microwave. The gel chamber was attached to a power supply with 85 V for a small gel and with 110 V for a big gel. After 45-60 min, the gel was dyed with ethidiumbromide in a separate dish for 5 min. Afterwards, the gel was put back into the gel chamber for 5 min and 85-100 V. Subsequently, the PCR products were viewed with an imager G:Box EF. A lane marker was used to estimate the PCR product size (DNA Molecular Weight Marker X, 0.07-12.2 kbp).

3.10 Protein isolation

The presence of bovine serum albumin (BSA) impairs the determination of protein concentration. Therefore, spermatozoa were washed twice with PBS and afterwards were centrifuged at 5000 x g for 5 min. The supernatant was discarded and the pellet was stored at -80 °C until analysis.

Spermatozoa were lysed in Radio Immuno Precipitation Assay (RIPA) buffer (50 mM Tris/Tris-HCl pH 7.6, 150 mM NaCl, 2 % sodium dodecyl sulfate (SDS), 0.5 % (w/v) sodium deoxycholate and 1 % (v/v) NP-40 (Igepal)). Sodium deoxycholate was used for disruption of the nuclear membrane. Furthermore, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added freshly to the RIPA buffer. Sodium orthovanadate was prepared as a stock solution of 100 mM in double distilled water. Afterwards, the pH was set to 9.0 with HCl and boiled until the solution was colourless. The solution was cooled until RT was reached and the pH was again set to 9.0. The solution was boiled again and the procedure was repeated until the pH of the solution was 9.0 after boiling and cooling. The initial volume was restored with water. The aliquots were stored at -20 °C and only defrosted once. If the aliquots turned yellow, they were discarded. Overall, sodium orthovanadate inhibits protein tyrosine phosphatases, alkaline phosphatases and ATPases. PMSF inhibits serine proteases, including trypsin, chymotrypsin, thrombin and papain and was dissolved in ethanol at a concentration of 100 mM. Aliquots were stored at -20 °C. These inhibitors were added to the RIPA buffer immediately before protein isolation, because they are not stable in solutions. Spermatozoa were incubated with different volumes of RIPA buffer according to sperm count for 45 min on ice. Afterwards, spermatozoa were centrifuged at 12000 x g for 20 min at 4 °C. The supernatant was put into a new sterile tube. After isolation, the protein concentrations were determined immediately or the supernatant was stored at -80 °C.

To determine total protein concentrations in RIPA lysis buffer, a colorimetric bicinchoninic acid (BCA) Protein Assay Reagent was used. This assay relies on the biuret reaction, the reduction of Cu^{2+} to Cu^{1+} by generating a complex between peptide bonds and copper when protein is present in an alkaline system (353). Two molecules of BCA form a com-

plex with Cu^{1+} generating a purple-coloured reaction product (353). This water-soluble chromophore exhibits a absorbance maximum at 562 nm (353) that is nearly linear with increasing protein concentrations over a broad working range (20-2000 µg/ml). The macromolecular structure of proteins, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for colour formation with BCA (354). A standard curve (0-2000 µg/ml) with BSA was prepared to measure total protein concentrations. The BSA stock solution (2 mg/ml) was diluted in RIPA buffer. Subsequently, the working solution had to be prepared 50:1 reagent A + reagent B. Meanwhile, 25 µl of the standards and 25 µl of samples (1:5 diluted in RIPA buffer) were put in replicate into wells of a 96 well plate. 200 µl of working solution was added to each well. The plate was incubated for 30 sec on a plate shaker to guarantee mixing. Subsequently, the plate was covered and incubated for 30 min at 37 °C. After incubation, the plate cooled down to RT and the extinction was measured at 562 nm (Synergy 2 Reader). To get reliable values, blank was subtracted from standards and samples.

3.11 Western blot analysis

3.11.1 Electrophoresis

Electrophoresis was performed to separate proteins on the basis of their size. The separating and stacking gel were prepared according to Table 3 and Table 4. Protein samples were thawed on ice, vortexed and prepared with 4x Laemmli buffer. Samples were vortexed and centrifuged shortly and subsequently denaturated at 95 °C for 5 min.

The composition of buffers used for western blot analysis are summarised in Supplemental Table 1.

Table 3 Separating gel.

For 4 gels	7.5 %	10 %	12.5 %	15 %
Aqua dest	14.9 ml	12.4 ml	9.9 ml	7.4 ml
Lower buffer pH 8.8	7.5 ml	7.5 ml	7.5 ml	7.5 ml
Acrylamide/Bisacrylamide (AA/Bis)	7.5 ml	10 ml	12.5 ml	15 ml
Tetramethylethylenediamine(TEMED)	20 µl	20 µl	20 µl	20 µl
10 % Ammonium persulfate (APS)	90 µl	90 µl	90 µl	90 µl

Table 4 Stacking gel.

For 4 gels	
Aqua dest	6.1 ml
Upper buffer pH 6.8	2.5 ml
AA/Bis	1.3 ml
10 % SDS	0.1 ml
TEMED	50 µl
10 % APS	10 µl

Afterwards, samples were cooled down on ice and centrifuged for 5 min at 14000 rpm. Denaturated proteins (15-40 µg) and protein marker (10 µl, Spectra™ Multicolor Broad

Range Protein Ladder) were loaded in the gel pockets. The electrophoresis was accomplished at 90 V for 15-20 min until the bromophenol blue front arrived the separating gel. At this point, the voltage was increased to 160 V and the electrophoresis lasted for 60-80 min. Afterwards, the glass plates were separated carefully. The stacking gel was discarded and the separating gel was used for blotting. After blotting, the separating gel was dyed with Coomassie solution (0.25 g Coomassie Brilliant Blue R250 + 90 % methanol (1:1 diluted with aqua dest) + 10 % glacial acetic acid) for 1 h at RT. Afterwards, gel was decolourised by a decolourising solution (90 % methanol (1:1 diluted with aqua dest) + 10 % glacial acetic acid) for 3-6 h at RT. The gel was conserved by adding a conserver solution (10 % glycerine, 20 % ethanol in aqua dest) for 30 min at RT. After the incubation, 2 sheets of cellophane paper were soaked in aqua dest and the membrane was put between the sheets. About 2 ml of the conserver solution was put on the gel. The gel was dried in a self-made frame for 2-3 days.

3.11.2 Blotting

3.11.2.1 Semi-dry blot

The semi-dry blotting device was cleaned with aqua dest before use. Nitrocellulose membrane and 4 filters were put into transfer buffer for 5 min to equilibrate. The stack of 2 filters, gel, nitrocellulose membrane, and 2 filters was put in the semi-dry blotting device. The stack was pressed carefully to avoid air bubbles and transfer buffer was put on the top filters to keep the stack wet. The transfer of proteins was accomplished for 45 min at 60 mA for AdipoR1, leptin, NAMPT, Beclin-1, and LC3B detection in human spermatozoa.

3.11.2.2 Wet blot

The wet blot is a method to guarantee that proteins with high molecular weight will be blotted to the membrane. To transfer proteins with high molecular weight, a high voltage and more time is needed. The chance of drying the membrane during transfer is higher by semi-dry blot than by wet blot. To prepare the wet blot, the cooling unit was pre-cooled over night and the filter paper was pre-incubated with transfer buffer for 5-15 min. The stack of fiber pad, filter pad, membrane, gel, filter paper, and fiber pad was put into the wet blot device. The stack was pressed carefully to avoid air bubbles and prepared in a glass bowl filled with transfer buffer to prevent the membrane from drying.

The blotting device was put into a box of ice. A stir bar and the cooling unit were put into the device to ensure the cooling during the blotting procedure. The blotting was performed with 90 V for 1.5 h. The wet blot was performed for the analysis of ObR and tyrosine phosphorylation of capacitation involved proteins in human spermatozoa.

3.11.3 Blocking, antibody incubation and detection

Membranes were blocked with 3 % BSA in TTBS (0.1 % Tween 20, 20 mM Tris, 150 mM NaCl) for 1 h at RT and washed 3 times for 5 min. The primary antibody was diluted in 3 % BSA-TTBS and incubated at RT for 1 h or at 4 °C over night. The antibody incubation conditions are summarised in Supplemental Table 2. After incubation, membranes were washed three times for 5 min. Subsequently, membranes were incubated with the secondary antibody in 3 % BSA-TTBS for 1 h at RT (Supplemental Table 2). In the end, membranes were washed three times for 5 min. The chemiluminescence method (SuperSignal West Femto Chemiluminescent Substrate) was used for the detection. Therefore, equal aliquots of chemiluminescent substrate solutions 1 and 2 were put into a box and the membranes were incubated in this solution for about 1 min. After incubation, chemiluminescence was measured by an imager and analysed by the respective software (Syngene, G:Box, GeneSnap from Syngene).
Different western blot analyses were performed with respective dilutions of the antibodies (primary and secondary) and the incubation periods were evaluated by primarily experiments.

3.12 Immunofluorescence

To determine the localisation of adipokines and their receptors at human spermatozoa, the protein expression was evaluated by immunofluorescence. 10 µl of sperm suspension (nearly $1x10^6$ sperm/slide) were layered and distributed on a Superfrost® Plus slide. The suspension was dried at RT and was immediately used or stored at -20 °C to avoid degradation of proteins. The slides were fixed with ice cold 100 % methanol for 10 min at -20 °C. After fixation, slides were dried and spots were surrounded with a DakoCytomationPen to minimize antibody consumption and separating different areas on the slide to ensure control and incubation spots (different dilutions). Afterwards, slides were washed with 0.1 % Tween-20 in PBS and blocked for 45 min at RT with 1 % BSA, 1 % Triton X-100, 0.05 % Tween-20 in PBS in a humidified chamber. Triton and Tween are able to permeabilize the spermatozoa membrane. After blocking, the slides were incubated with primary antibodies diluted in PBS. Antibody incubation conditions for immunofluorescence are summarised in Supplemental Table 3. The slides were incubated over night at 4 °C in a humidified chamber. The next day, slides were washed three times with 0.1 % Tween-20 in PBS for 5 min. Secondary antibody labelled with FITC was 1:100 diluted in PBS and slides were incubated with secondary antibody for 1 h at RT in the dark to avoid photobleaching. Afterwards, slides were washed with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI) for 1 min and washed with PBS. Subsequently, slides were sealed with Mounting Medium to prevent fading. The slides were observed with a filter for FITC (excitation maximum: 494 nm, emission maximum: 521 nm) and DAPI (excitation maxi-

mum: 358 nm, emission maximum: 461 nm). Blocking peptides were tested to ensure the specifity of primary antibodies as shown in Supplemental Table 3.

3.13 Immunohistochemistry

Additionally to spermatozoa, testicular samples were examined for the presence of adipokines and their receptors. Testis of patients with Johnsen scores of 8-10 were fixed in Bouin`s solution (7.5 ml aqueous picrin acid + 2.5 ml formaldehyde + 0.5 ml glacial acetic acid), embedded in paraffin and stored at -20 °C. The frozen paraffin samples were sliced with a half automated microtome into 6 µm thick slices. These slices were put into warm water to enlarge. Afterwards, the slices were put on Superfrost® Plus slides and dried at 37 °C over night. The slides were incubated for 5-10 min at 75 °C to melt the paraffin. Subsequently, testis sections were deparaffinised in xylol three times for 5 min and rehydrated with graded ethanol solutions (100 %, 96 %, 70 %) for 5 min. Antigens were demasked, because paraffin is able to influence the epitope of antigens and therefore the binding of the primary antibody. For antigen retrieval, slides were either incubated with proteinase K (Ready-to-use) for 15 min at RT or with the Target Retrieval Solution (citrate buffer, pH 6.0) for 12 min at 95 °C and were cooled for 12 min at RT. Afterwards, slides were washed three times with TBS+0.3 % Tween-20 for 5 min and blocked with 1 % BSA in TBS for 1 h in a humidified chamber. The samples were incubated with primary antibody in a humidified chamber according to Supplemental Table 4. The primary antibodies were diluted in TBS+1 % BSA.

3.13.1 Detection of immunohistochemical staining with alkaline phosphatase

After incubation with the primary antibody, slides were washed three times for 5 min with TBS+0.3 % Tween-20. For the detection of the immunohistochemical staining, the Super-Sensitive™ Link-Label IHC Detection System kit was used. This kit includes secondary antibodies from several species labelled with multiple biotin residues. The bound secondary antibody reacts with streptavidin conjugated to the enzyme alkaline phosphatase (Figure 5). Streptavidin, consisting of 4 biotin binding sites, binds very strongly and irreversiblly to biotin of the secondary antibody resulting in a biotin-streptavidin-enzyme conjugate. This method achieves a high sensitivity, as the primary antibody is associated with multiple enzyme molecules. The multilink solution with biotinylated anti-Igs was put onto the slides and incubated for 20 min at RT in a humidity chamber. The slides were washed three times for 5 min with TBS+0.3 % Tween-20. Afterwards, the label-mix with enzyme-conjugated streptavidin was put onto the slides and incubated for 20 min at RT in a humidity chamber. The slides were washed three times for 5 min with TBS+0.3 % Tween-20. In the end, slides were washed with TBS for 5 min.

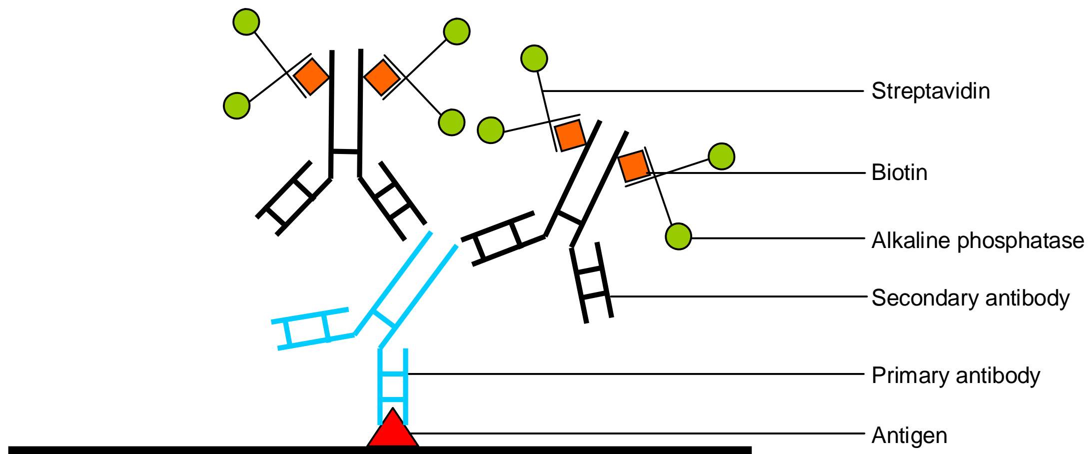


Figure 5 Alkaline phosphatase system. The primary antibody detects the antigen. Subsequently, the secondary antibody binds to the primary antibody. The secondary antibody is labelled with biotin residues. The biotin is strongly bound by streptavidin, which is conjugated to the alkaline phosphatase.

During the last washing step, the substrate solution New Fuchsin Substrate System was prepared according to the following protocol: 2 drops of Tris buffer concentrate were added into a fresh tube. Subsequently, 1800 µl aqua dest, 1 drop of substrate concentrate and 100 µl Levamisol working solution were added into this tube. The substrate solution was mixed carefully. Subsequently, the chromogen solution was prepared according to the following protocol in a separate vial: 1 drop of New fuchsin-chromogen and 1 drop of activation reagent were mixed carefully, incubated for exactly 3 min and added to the substrate solution. This solution was put onto the slides and the dying process was monitored with a light microscope. As soon as the tissue sections were intensively coloured, the reaction was stopped with aqua dest. According to Mayer, tissue sections were dyed with hemalum for 1-5 min in order to dye the nucleus. The reaction was stopped with aqua dest, slides were washed with tap water and afterwards sealed with AquaTex.

3.13.2 Detection of immunohistochemical staining with horse radish peroxidase (HRP)

Only primary antibodies from mouse and rat were detectable with the Super Sensitive™ Link-Label IHC Detection System. If primary antibodies were raised in goat or if the detection with alkaline phosphatase was too weak, the protocol and detection method were changed and HRP-labelled secondary antibodies were used. After incubation with primary antibody, slides were washed three times with TBS+0.3 % Tween 20 for 5 min. Subsequently, endogenous peroxidases were blocked with 3 % H_2O_2 in TBS for 15 min to quench endogenous peroxidase activity. The blocking was performed after the incubation with the primary antibody as epitopes are sensitive for H_2O_2. Slides were washed three times with TBS+0.3 % Tween 20 and incubated with HRP-conjugated secondary antibody

for 1 h at RT. Afterwards, slides were washed three times with TBS+0.3 % Tween 20. Subsequently, slides were stained with 3-3'-Diaminobenzidine (DAB, Liquid DAB+ Substrate Chromogen System). 1 drop (20 µl) of the DAB chromogen was added to 1 ml substrate buffer. The solution was mixed well and put on the slides for 10 min at RT. After incubation, slides were washed with aqua dest for 5 min to stop the reaction. According to Mayer, tissue sections were dyed with hemalum for 1-5 min in order to dye the nucleus. The reaction was stopped with aqua dest, slides were washed with tap water and afterwards sealed with AquaTex.

3.13.2.1 Detection of immunohistochemical staining with SignalStain® Boost IHC Detection Reagent

As the localisation of NAMPT and LC3B was barely detectable, the signal was amplified by the use of SignalStain® Boost IHC Detection Reagent (HRP). These detection reagents are based on polymers (Figure 6).

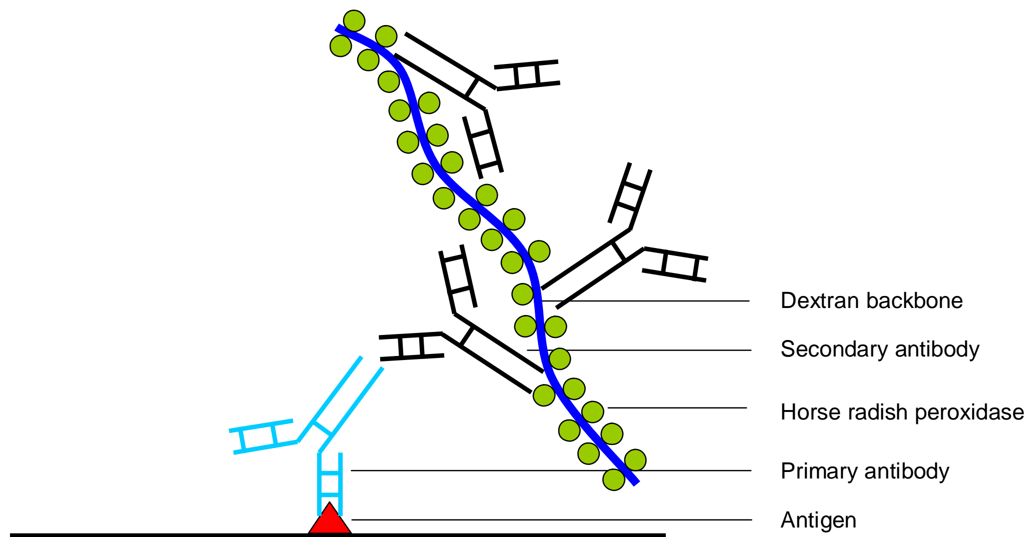


Figure 6 Schematic overview of SignalStain® Boost Detection Reagent. The antigen is detected by the primary antibody. Subsequently, the secondary antibody binds to the primary antibody. The secondary antibody is bound to a dextran backbone with the enzyme horse radish peroxidase. The multitude of bound enzymes leads to a signal amplification.

After the incubation with the primary antibody, slides were washed three times with TBS+0.3 % Tween 20 for 5 min. Subsequently, endogenous peroxidases were blocked by the incubation with 3 % H_2O_2 in TBS for 15 min at RT. Slides were washed three times with TBS+0.3 % Tween 20 for 5 min. Afterwards, the slides were incubated with 1-3 drops (30-40 µl) of SignalStain® Boost IHC Detection Reagent and incubated for 30 min at RT in a humidity chamber. Subsequently, slides were washed three times with TBS+0.3 % Tween 20 for 5 min and in the end with TBS for 5 min. Slides were stained with DAB chromogen and hemalum as mentioned before.

3.14 Measurements of adipokines in sperm supernatants

After liquefaction, semen was prepared by DGC and anti-CD45 MicroBeads to produce immature and mature spermatozoa fractions devoid of leucocytes. For experiments, $20x10^6$/ml immature and mature spermatozoa ($10x10^6$/500 µl medium) were incubated with EBSS for 3 h and 24 h at 37 °C, 5 % CO_2. At the end of incubation, suspensions were centrifuged at 500 x g for 5 min. The supernatants were frozen at -80 °C until analysis of adipokine concentrations. Adipokine levels were determined in the same way as seminal plasma samples. Supernatants were diluted 1:2 before analysis.

Additionally, NAMPT secretion was intensively examined, because high NAMPT concentrations were detected in the sperm supernatants. Semen samples were separated by DGC and checked for residual round cells. If required, leucocytes were excluded by the use of anti-CD45 MicroBeads. Afterwards, $10x10^6$ immature and mature spermatozoa were washed and resuspended in 500 µl HTF+3 % BSA and incubated at 37 °C, 5 % CO_2 for 3; 6; 12 and 24 h. At the start of incubation and after the different time periods, vitality and motility were determined by a single examiner and by CASA. After incubation, the sperm suspensions were centrifuged (700 x g, 5 min, 4 °C) and undiluted supernatants were stored at -80 °C until determination with a NAMPT ELISA kit performed according to manufacturer's instructions.

3.15 Incubation of spermatozoa with adipokines

3.15.1 Determination of motility

After semen analysis, the ejaculate was split into 2 aliquots. The first aliquot of semen was washed twice with EBSS+3 % BSA (400 x g, 4 min). The second aliquot was prepared for swim up, which is a method to isolate highly motile spermatozoa. 1 ml semen was put into a 15 ml tube, 1.2 ml EBSS+3 % BSA was carefully loaded on top and put into the incubator at 37 °C, 5 % CO_2 for 1 h in an angle of 45°. After 1 h of incubation, the tube was carefully set vertical. The upper layer was pipetted into a new sterile tube, centrifuged (400 x g, 4 min), washed and resuspended in EBSS+3 % BSA. $5x10^6$ washed or swim up spermatozoa/ml ($2.5x10^6$/500 µl) were suspended in EBSS+3 % BSA and incubated with 0; 1; 10 and 100 ng/ml recombinant human leptin or 0; 10; 100 and 1000 ng/ml recombinant human chemerin (kindly provided by S. Schultz/A.G. Beck-Sickinger, Institute of Biochemistry, University of Leipzig) or 0; 10; 100 and 1000 ng/ml adiponectin (native, from human serum) for 1; 2 and 3 h at 37 °C, 5 % CO_2. After the incubation, sperm motility was analysed by a single examiner and by CASA. The vitality was analysed with eosin. Subsequently, spermatozoa were centrifuged (400 x g, 4 min) and washed twice with PBS to remove residual BSA. Spermatozoa and the supernatants were frozen at -80 °C.

3.15.2 Determination of capacitation

Human semen was checked for pH, volume, motility, sperm concentration, sperm count and vitality. Experiments were performed with HTF+3 % BSA as capacitating medium for 3 h (81, 355) at 37 °C, 5 % CO_2. Spermatozoa ($30x10^6$-$100x10^6$/ml) were incubated either with non-capacitating medium (HTF), capacitating medium (HTF+3 % BSA) or capacitating medium supplemented with 100 ng/ml adiponectin or 100 ng/ml leptin.

3.15.3 Determination of acrosome reaction

After semen analysis, ejaculate was washed with PBS twice (400 x g, 5 min) and smears ($1x10^6$ sperm/smear) were prepared (Figure 7).

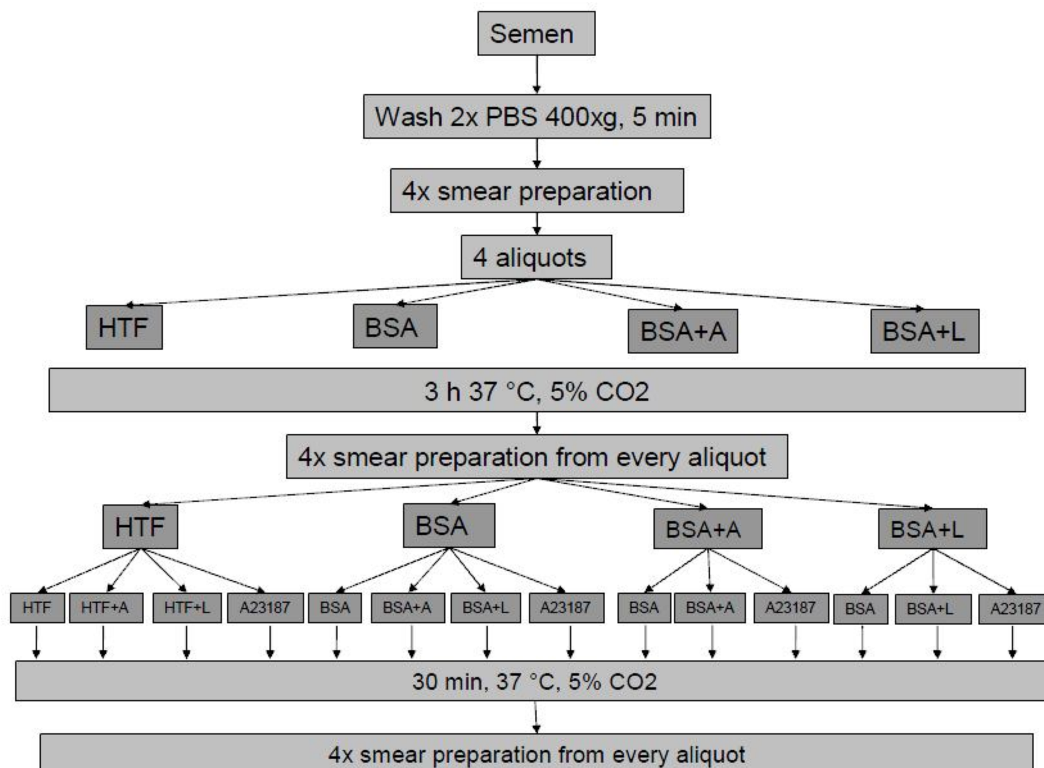


Figure 7 Determination of acrosome reaction after incubation of human capacitated spermatozoa with leptin and adiponectin. Human Tubal Fluid (HTF)–non-capacitating medium without bovine serum albumin (BSA); BSA–capacitating medium HTF+3 % BSA; BSA+A–capacitating medium HTF+3 % BSA+100 ng/ml adiponectin; BSA+L–capacitating medium HTF+3 % BSA+100 ng/ml leptin; HTF+A–non-capacitating medium HTF+100 ng/ml adiponectin; HTF+L–non-capacitating medium HTF+100 ng/ml leptin; A23187–a chemical inducer for acrosome reaction.

The remaining spermatozoa ($30x10^6$/ml per aliquot) were incubated either with a) HTF, b) HTF+3 % BSA, c) HTF+3 % BSA+100 ng/ml adiponectin or d) HTF+3 % BSA+100 ng/ml leptin for 3 h at 37 °C, 5 % CO_2. After incubation, smears were prepared from every aliquot. Spermatozoa were washed twice with PBS and incubated with the respective medium according to Figure 7 for 30 min at 37 °C, 5 % CO_2 to induce the acrosome reaction spontaneously or by the acrosome reaction inducer A23187. After the incubation, smears were prepared for the analysis of the acrosome reaction. A23187, stock solution

2.5 mg/ml in dimethyl sulfoxide (DMSO), was diluted in HTF to a concentration of 10 µM and the controls were incubated with the respective DMSO concentration.
The effects of adiponectin, leptin and A23187 were determined by FITC-labelled lectin from *Pisum sativum* as mentioned before (chapter 3.3).

3.15.4 Determination of apoptosis

3.15.4.1 Determination of caspase activity

The influence of adipokines on apoptosis was determined by the detection of caspase activity with Fluorochrome Inhibitor of Caspases (FLICA™) assay kit for caspase 3&7. The assay used the carboxyfluorescein-labelled fluoromethyl ketone peptide inhibitor of caspases. The FLICA peptide fluorescein-asparic acid-glutamic acid-valine-asparic acid (DEVD)-fluoromethyl ketone (FAM-DEVD-FMK) was used. FAM-DEVD-FMK is cell permeable and non-toxic. Once FLICA is added to the cell population, the probe enters the cell and binds covalently and irreversible to reactive cysteine residue that resides on the large subunit of the active caspase heterodimer (356), thereby inhibiting further enzymatic activity. While FLICA is bound to caspase covalently, unbound FLICA diffuses to the outside of the cells and is washed away. Therefore, the remaining green fluorescence signal is a direct measure of the number of active caspase enzymes at the time FLICA was added to the cells. Non-conserved samples were dyed with propidium iodide (PI; 250 µg/ml), which dyes necrotic, dead cells with disturbed membranes and can therefore distinct between vital and non-vital cells.
Human semen was washed twice with EBSS and $2x10^6$ spermatozoa were incubated with 1000 ng/ml adiponectin, 1000 ng/ml chemerin or 100 ng/ml leptin diluted in either EBSS or EBSS+3 % BSA. The induction of apoptosis takes 2-4 h at 37 °C, therefore sperm suspensions were incubated for 3 h at 37 °C and 5 % CO_2. Aliquots were prepared for flow cytometric analysis. Control cells were used to check for autofluorescence and for correct adjustment of the fluorescence range. To evaluate the range of caspase positive and PI positive cells, positive controls were prepared with PBS pH 7.2, 0.5 % BSA and 2 mM EDTA. Per aliquot, $1x10^6$ spermatozoa were prepared and therefore at least $18x10^6$ spermatozoa were necessary per experiment and donor. During the incubation of spermatozoa, the FLICA kit was prepared. The analysis was performed according to the manufacturer's protocol. The wash buffer was diluted 1:10 with aqua dest. The FLICA lyophilisat was carefully resolved in 50 µl DMSO to generate a 150x stock solution. This reconstituted solution was immediately used or stored in 2 µl aliquots at -20 °C. The aliquots (150x stock solution) were 1:5 diluted in PBS pH 7.4 (30x stock).
After the incubation of the spermatozoa with capacitating or non-capacitating medium for 3 h, the sperms were washed twice and resuspended with 300 µl PBS pH 7.4 (400 x g, 5 min). Subsequently, 10 µl of the 30x FLICA stock solution were resuspended in the

300 µl sperm suspension and incubated for 1 h at 37 °C, 5 % CO_2 in the dark. During incubation, spermatozoa were shaken up twice. After the incubation, 1 ml of the wash buffer was added to the sperm suspension and the samples were centrifuged at 400 x g for 5 min. The supernatant was discarded and the pellet was resuspended in 500 µl wash buffer and centrifuged at 400 x g for 5 min. Afterwards, the supernatant was discarded and the pellet was resuspended in 400 µl wash buffer. The respective aliquots were incubated with 2 µl PI and the samples were mixed and stored on ice.

The fluorescence of fluorescein/FLICA (extinction: 488-492 nm, emission: 515-535 nm) was measured at FL1 channel and PI (extinction: 488-490 nm, emission: 635 nm) at FL2 channel with a flow cytometer including a 488 nm argon laser (BD FACSCalibur). The resulting histogram consisted of log FL1/FL2 (x-axis) and sperm count (y-axis). Caspase negative cells lied in the first log decade of FL1 and caspase positive spermatozoa showed a second peak or a shoulder from the first peak.

3.15.4.2 Determination of mitochondrial membrane potential

Another method to determine effects on apoptosis is the alteration of the mitochondrial membrane potential ($\Delta\Psi_m$), which is one of the first alterations during the start of apoptosis (357). The cationic dye 5,5'6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimidazolylcarbocyanine iodide is used as a marker for the decrease of the $\Delta\Psi_m$. This dye infiltrates into the cells and fluoresces red (excitation: 585 nm, emission: 590 nm) in vital mitochondria as a multimeric form. Apoptotic mitochondria are not able to accumulate this dye, because the $\Delta\Psi_m$ is disturbed and the dye can not penetrate the mitochondria. Therefore, the dye is present in the cytoplasm in its monomeric form and shows a green fluorescence (excitation: 510 nm, emission: 527 nm). The fluorescence can be determined by the use of a fluorescence microscope or a flow cytometer. As the $\Delta\Psi_m$ is pH sensitive, the reagents or culture medium should have a pH of 7.0-8.0. According to the manufacturer, the 10x reaction buffer was diluted 1:10 with aqua dest and prewarmed at 37 °C. Per sample, 1 µl DePsipher™ solution was added to 1 ml prewarmed reaction buffer (final concentration 5 µg/ml) and vortexed. As the DePsipher™ is hardly soluble in aqueous solution, the tube was centrifuged at 13000 x g for 1 min. The supernatant was transferred into a new tube without disturbing the pellet. Subsequently, $1x10^6$ spermatozoa were centrifuged (400 x g, 4 min). The cells were resuspended in 1 ml of the diluted DePsipher™ solution and incubated for 20 min at 37 °C, 5 % CO_2. Afterwards, samples were washed twice with reaction buffer (500 µl, 400 x g, 4 min) and the cells were resuspended in 1 ml reaction buffer. The fluorescence was determined with the flow cytometer (488 nm argon laser). Until analysis (max. 30 min), the cells were stored at RT or on ice and protected from light.

3.16 Inhibition of NAMPT enzyme activity

The substance FK866 is known to inhibit NAMPT activity and therefore decreases intracellular NAD^+ levels (358). To evaluate the appropriate concentration to inhibit NAMPT activity in human spermatozoa, primarily experiments were performed. Semen samples were washed with capacitating medium (EBSS+3 % BSA) and a sperm concentration was adjusted to $20x10^6$/ml in 500 µl. Before incubation, motility, vitality and the presence of round cells were determined. FK866 (stock solution 25 mg/ml in DMSO) was diluted in EBSS+3 % BSA. Spermatozoa were incubated with 0.1; 1; 10 and 100 nM FK866 for 3; 6; 12 and 24 h at 37 °C, 5 % CO_2. After incubation, motility and vitality were determined and spermatozoa were centrifuged at 700 x g, 4 min, 4 °C and washed twice with PBS. Spermatozoa were smeared onto slides, air dried and fixed for 8 min in 100 % methanol at -20 °C. Additionally, supernatants were stored at -80 °C for further investigations.

Furthermore, washed spermatozoa were incubated in capacitating medium (HTF+3 % BSA) for 24; 48 and 72 h with different concentrations of FK866 as mentioned before in this chapter.

3.17 NAD^+ determination in human spermatozoa

Human semen was evaluated for volume, pH, motility, sperm concentration, sperm count and vitality and was separated by DGC, subsequently. The immature and mature sperm fractions were checked for round cells and if required leucocytes were excluded by the use of anti-CD45 MicroBeads. After DGC and MACS separation, the sperm fractions were washed twice with 2 ml of ice cold PBS pH 7.4 (400 x g, 5 min). Accordingly, sperm count was determined. For evaluation experiments, samples with different sperm counts were lysed in 100 µl NAD extraction buffer (EnzyChrom™ NAD+/NADH Assay Kit). The spermatozoa were stored on ice if the samples were used immediately or the samples were frozen at -80 °C for later experiments. Subsequently, NAD extraction buffer, NADH extraction buffer, assay buffer, NAD standard, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate were thawed on ice while spermatozoa were lysed additionally by sonification (3 x 5 sec, amplitude 100 %, cycle 1, on ice). The lysed extracts were incubated for 5 min at 60 °C. Thereafter, 20 µl of assay buffer and 100 µl of the NADH extraction buffers were added to neutralise the extracts. The samples were vortexed and centrifuged at 14000 rpm for 5 min at 4 °C. The supernatant was transferred to a new sterile tube and the pellet was stored at 4 °C until analysis of protein concentrations at the same day or at - 20 °C for later analysis. For the determination of the protein concentrations, 100 µl of a 2 % aqueous SDS solution was added to the sperm extract pellet and mixed and incubated for 10 min at 99 °C. After incubation, protein solutions were centrifuged at 20000 x g for 5 min and the supernatant was used for determining protein concentrations by BCA Protein Assay Reagent. The supernatants of the sperm

extracts for the NAD determination were stored on ice until the calibration curve (0-6 µM NAD) was prepared in aqua dest.

Subsequently, 40 µl of the standards and samples were added in duplicate to a 96 well plate. For each well, the working reagent was prepared in the following way: 60 µl assay buffer + 1 µl enzyme A + 1 µl enzyme B + 14 µl lactate + 14 µl MTT. Thereafter, 80 µl of working reagent were added to the standards and samples. The microplate was mixed on a shaker for 10 sec. The optical density (OD0) was measured at 565 nm (520-600 nm) at the start and after 15 min of incubation at RT (OD15). In the end, the Δ values of OD15-OD0 were determined for standards as well as samples.

3.18 Incubation of spermatozoa with chloroquine

Chloroquine is known to inhibit the fusion between the autophagosome and lysosome (359) and therefore block the autophagic flux. For the experiments, semen was checked for volume, pH, motility (determined by a single examiner and by CASA), sperm concentration, sperm count and vitality. The semen was also checked for round cells as mentioned before. The semen was divided into 2 aliquots. The first aliquot was washed with EBSS and the second with HTF. Washed spermatozoa ($20x10^6$/ml) were incubated with 10 µM chloroquine diphosphate salt (CQ). The CQ was diluted in the respective capacitating medium. The experiments were performed according to Figure 8. The control cells were incubated with the respective capacitating medium. After appropriate incubation, spermatozoa were checked for motility and vitality. After 24 h with EBSS+3 % BSA and after 72 h with HTF+3 % BSA, spermatozoa were washed twice with PBS (400 x g, 4 min) and the sperm pellet was stored at -80 °C for further western blot experiments.

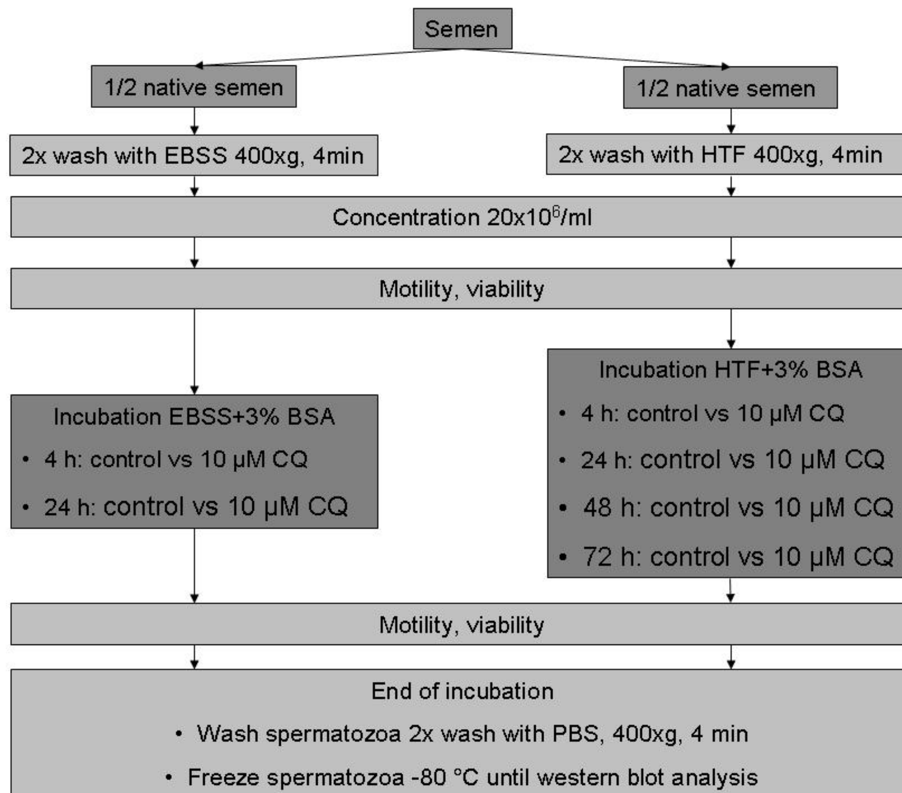


Figure 8 Incubation of human spermatozoa with chloroquine. Native semen was divided into two aliquots. The first aliquot was washed with EBSS and afterwards incubated with EBSS+3 % BSA for up to 24 h. The second aliquot was washed with HTF and incubated with HTF+3 % BSA for up to 72 h, subsequently. The spermatozoa were checked for motility and viability at the end of incubation and stored at -80 °C until western blotting.

3.19 Statistics

All variables were checked for normal distribution using Kolmogorov–Smirnov test. Correlations between adipokines in serum, SP, anthropometric data, reproductive hormones, and semen parameters were analysed using Spearman's rank correlation (Statistica software, version 7.1; StatSoft, Inc.). The adipokine concentrations, parameters of semen quality, and reproductive hormones were adjusted for the confounding factor age. This was performed by robust linear regression applying the "ImRob" function of the "robust" package of the statistical software "R" (www.r-project.org). Adjusted concentrations were compared between normal weight and overweight/obese men using the Mann-Whitney U-test. Associations of adipokines with semen parameters were again analysed by robust linear regression and with or without adjustment for age, BMI, waist circumference, as well as testosterone, FSH, and LH concentrations. Statistical analyses of the observational study were performed by M. Scholz, Institute for Medical Informatics, Statistics and Epidemiology (IMISE), University of Leipzig, Germany.

All the other calculations were performed with Statistica software using Mann-Whitney U-test. Beyond, Kruskal-Wallis test was performed as a non-parametric one-way analysis of variance. Accordingly, a Mann-Whitney U-test was performed to identify, which samples were significantly different from each other. P≤0.05 was considered statistically significant.

4 Results

4.1 The presence of adipokines in human seminal plasma and their impact on semen parameters dependent on BMI

4.1.1 Adipokine levels in serum versus seminal plasma

The median concentrations of adiponectin (370-fold), chemerin (1.6-fold), resistin (1.5-fold) and leptin (5.2-fold) were significantly higher ($P \leq 0.05$), whereas levels of progranulin (46-fold), NAMPT (81-fold) and vaspin (3.9-fold) were significantly lower in serum than in SP ($P<0.001$, n=96, Table 8). Only adiponectin concentrations correlated significantly ($r_s=0.34$, $P<0.001$) between both sample matrices (Figure 9).

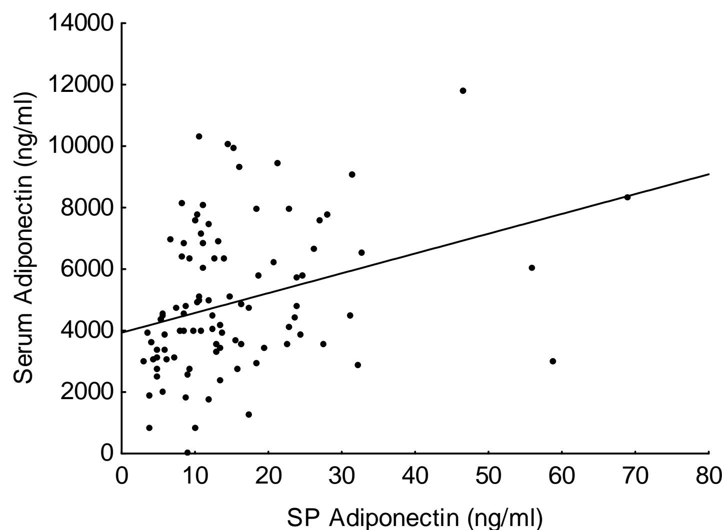


Figure 9 Correlation of adiponectin concentrations (ng/ml) in serum versus seminal plasma (SP). Data were analysed using Spearman's rank correlation (n=96). Spearman's rank correlation coefficient $r_s=0.35$, $P=0.0005$.

4.1.2 Adipokine levels in seminal plasma of vasectomised men

To evaluate the source of the measured adipokines in human male reproductive tract, concentrations of adipokines were determined in SP of vasectomised men (Table 5). The body weight of vasectomised men was not known, as in this context, the source and not the BMI dependency was important. Chemerin levels were significantly lower in SP of vasectomised men than in normal weight and overweight/obese men ($P<0.001$). Additionally, progranulin concentrations were significantly lower ($P \leq 0.05$) in SP of vasectomised men compared with normal weight men. In contrast, leptin and NAMPT levels were significantly higher ($P \leq 0.05$) in SP of vasectomised men than in men with overweight/obesity. As shown in Table 5 no further significant differences were detected.

Table 5 Adipokine levels in seminal plasma of vasectomised men (n=9) compared with normal weight (n=41) and oberweight/obese men (n=55). Statistic significant differences were analysed using Mann-Whitney U-test. The analysis was not adjusted for age. Data are described as median (range). * P≤0.05; ** P<0.001 compared with vasectomised men.

Parameter	Seminal plasma		
	Vasectomy	Normal weight	Over-weight/Obese
Adiponectin (ng/ml)	16.4 (5.96-63.9)	12.6 (3.00-69.1)	11.2 (3.87-58.8)
Chemerin (ng/ml)	24.4 (13.6-48.8)	60.9 ** (18.3-262)	74.0 ** (16.0-1481)
Vaspin (ng/ml)	0.91 (0.38-2.46)	1.36 (0.01-111)	1.20 (0.01-171)
Leptin (ng/ml)	0.88 (0.64-2.24)	0.80 (0.24-2.60)	0.76 * (0.04-4.76)
Resistin (ng/ml)	3.89 (0.72-72.2)	2.60 (0.17-194)	5.50 (0.16-149)
Progranulin (ng/ml)	1256 (758-1737)	1711 * (359-3243)	1281 (597-3114)
NAMPT (ng/ml)	293 (113-603)	146 (45.2-946)	125 * (43.9-894)

4.1.3 Comparison of semen parameters and male sexual hormones in normal weight versus overweight/obese men

Normal weight men had a significantly (P≤0.05) higher percentage of normomorph sperm, leading to a higher total number of morphological normal spermatozoa, as well as a lower DFI compared with overweight/obese men (Table 6).

Additionally, their total testosterone serum levels were significantly (P<0.001) higher compared with overweight/obese men. No significant differences were observed for LH and FSH levels and the other semen parameters. All the results were independent of the effect of the confounding factor age.

Table 6 Distribution of reproductive hormones (a) and semen parameters (b) in normal weight (n=41) and overweight/obese (n=55) men. Reproductive hormones were adjusted for age using robust linear regression. Groups were compared using Mann-Whitney U-test. Data are described as median (range). * P≤0.05; ** P<0.01; *** P<0.001 compared with normal weight men.

a) Parameter	Normal weight	Overweight/Obese
LH (U/l)	4.20 (2.00-7.80)	4.09 (1.70-7.70)
FSH (U/l)	3.37 (1.40-10.6)	3.97 (1.20-14.1)
Testosterone (nmol/l)	19.5 (9.28-31.3)	14.0 *** (7.13-28.3)

b) Parameter	Normal weight	Overweight/Obese
Volume (ml)	3.00 (0.40-7.00)	2.67 (1.00-10.0)
pH	7.83 (6.85-8.00)	7.70 (7.50-8.00)
Concentration (x10^6/ml)	89.0 (5.00-236)	75.5 (2.00-397)
Sperm count (x10^6)	215 (17.8-947)	163 (6.00-1007)
Progressive motility (%)	50.0 (38.3-65.0)	50.0 (8.33-64.6)
Total motility (%)	57.5 (47.5-77.5)	57.5 (15.0-71.3)
Normomorph spermatozoa (%)	7.00 (3.50-23.1)	4.60 *** (0.00-13.6)
Total normomorph spermatozoa (%)	17.9 (0.83-84.8)	7.57 *** (0.00-82.0)
Intact acrosome (%)	28.0 (12.0-55.0)	23.5 (4.50-56.0)
Negative aniline blue staining (%)	75.5 (53.0-87.0)	74.5 (55.0-92.0)
DNA Fragmentation Index (%)	3.45 (1.10-16.2)	5.70 * (1.10-37.0)

4.1.4 Correlation analysis for serum and seminal plasma adipokine levels with anthropometric data and reproductive hormone levels

Correlation analysis was performed within the combined group of normal weight, overweight and obese men (Table 7).

Table 7 Correlations between phenotype, reproductive hormones and adipokines in serum (S) and seminal plasma (SP) of normal weight and overweight/obese men. Correlations between adipokines in serum/SP and anthropometric data and reproductive hormones were analysed using Spearman's rank correlation (n=96). * P≤0.05; ** P<0.01; *** P<0.001 refer to a significant Spearman's rank correlation coefficient r_s. n.s.-not significant

Parameter	Matrix	Adiponectin	Chemerin	Leptin	Resistin	Progranulin
Age	**S** **SP**	n.s. 0.25 **	0.30 ** n.s.	0.27 ** -0.27 **	n.s. 0.47 **	n.s. n.s.
BMI	**S** **SP**	-0.30 ** n.s.	0.40 *** n.s.	0.72 *** n.s.	n.s. 0.26 **	n.s. -0.28 **
Waist circumference	**S** **SP**	-0.35 *** n.s.	0.42 *** n.s.	0.79 *** n.s.	n.s. 0.24 *	n.s. -0.35 ***
FSH	**S** **SP**	n.s. n.s.	n.s. n.s.	n.s. n.s.	n.s. n.s.	n.s. 0.21 *
Testosterone	**S** **SP**	0.31 ** n.s.	-0.42 *** n.s.	-0.60 *** n.s.	n.s. n.s.	n.s. 0.22 *

BMI and waist circumference correlated significantly positive (P≤0.05) with serum levels of chemerin, leptin and SP resistin concentrations, whereas serum adiponectin and SP progranulin correlated negatively with BMI and waist circumference (P<0.01). Serum FSH concentrations correlated positively (P≤0.05) with progranulin in SP. Total serum testos-

terone demonstrated a significantly (P≤0.05) positive correlation with serum adiponectin and SP progranulin but a negative correlation with serum chemerin and leptin levels (P<0.001). Adipokine levels in serum and SP were adjusted for age, because of a significant (P≤0.05) age effect on chemerin and leptin concentrations in serum as well as on adiponectin, resistin and leptin concentrations in SP (Table 7). No significant correlations were found between phenotype, reproductive hormones and vaspin or NAMPT levels in serum and seminal plasma. LH did not correlate significantly with adipokine levels in serum and SP.

4.1.5 Comparison of adipokine levels in serum and seminal plasma in normal weight and overweight/obese men

After adjustment for age, normal weight men had significantly higher serum adiponectin, lower serum chemerin and leptin levels compared with the overweight/obese group (P≤0.05, Table 8). In contrast, adiponectin and progranulin levels in SP were significantly higher in normal weight men compared with the overweight/obese group (P≤0.05).

Table 8 Comparison of adipokine levels in serum and seminal plasma (SP) between normal weight (n=41) and overweight/obese (n=55) men. Adipokines were adjusted for age using robust linear regression. Groups were compared using Mann-Whitney U-test. Data are described as median (range). * P≤0.05; ** P<0.01; *** P<0.001 compared with normal weight men.

Parameter	Serum		Seminal plasma	
	Normal weight	Overweight/ Obese	Normal weight	Overweight/ Obese
Adiponectin (ng/ml)	4820 (1267-11781)	3859 ** (809-9038)	12.6 (3.00-69.1)	11.2 * (3.87-58.8)
Chemerin (ng/ml)	97.9 (55.3-193)	111 * (63.2-174)	60.9 (18.3-262)	74.0 (16.0-1481)
Vaspin (ng/ml)	0.31 (0.04-2.96)	0.28 (0.05-4.08)	1.36 (0.01-111)	1.20 (0.01-171)
Leptin (ng/ml)	1.65 (0.30-9.70)	6.20 *** (0.03-25.9)	0.80 (0.24-2.60)	0.76 (0.04-4.76)
Resistin (ng/ml)	5.49 (2.64-12.7)	5.90 (2.51-21.2)	2.60 (0.17-194)	5.50 (0.16-149)
Progranulin (ng/ml)	32.0 (21.6-54.2)	30.3 (18.9-48.9)	1711 (359-3243)	1281 * (597-3114)
NAMPT (ng/ml)	1.66 (0.15-41.4)	1.73 (0.05-97.1)	146 (45.2-946)	125 (43.9-894)

4.1.6 Correlation of BMI and adipokine levels in serum and seminal plasma with sperm quality

Increased BMI resulted in decreased progressive motility (r_s=-0.25, P≤0.05), normomorph spermatozoa and total number of normomorph spermatozoa (r_s=-0.45, r_s=-0.32, P<0.01). Moreover, BMI was positively associated with DFI (r_s=0.32, P<0.01).

Among serum adipokine levels only vaspin correlated positively (P≤0.05) with semen volume and intact acrosomes. In contrast, leptin correlated negatively with sperm count as well as with normomorph and total number of normomorph spermatozoa (P≤0.05; Table 9). Additionally, SP vaspin and leptin were negatively correlated with semen volume

(P<0.001). Adiponectin, chemerin, leptin and progranulin in SP demonstrated a direct correlation with the sperm concentration (P≤0.05). With regard to the functional parameters of the spermatozoa, adiponectin, leptin and progranulin in SP (P≤0.05; strongest effect for progranulin P<0.001) appeared to reflect a positive relationship, whereas chemerin and vaspin revealed to be rather negative predictors of spermatozoa function. To control the potential confounder effect, significant correlations were adjusted for age, LH, FSH, total testosterone, BMI and waist circumference (data not shown). Relationships of SP vaspin and leptin with semen volume as well as of SP chemerin, vaspin and progranulin with progressive motility were found to be independent of confounding effects (P≤0.05). No significant correlations were detected between adipokine levels in serum and SP and pH or chromatin packaging (P>0.05). NAMPT and resistin levels in serum and SP did not correlate significantly with semen parameters (P>0.05).

Table 9 Correlations of adipokines in serum (S) and seminal plasma (SP) and semen parameters of normal weight and overweight/obese men. Correlations of adipokines in serum and SP and semen parameters were analysed using Spearman's rank correlation (n=96). * P≤0.05; ** P<0.01; *** P<0.001 refer to a significant Spearman's rank correlation coefficient r_s. n.s.-not significant

Parameter	Matrix	Adiponectin	Chemerin	Vaspin	Leptin	Progranulin
Volume	S SP	n.s. n.s.	n.s. n.s.	0.25 * -0.36 ***	n.s. -0.34 ***	n.s. n.s.
Concentration	S SP	n.s. 0.25 **	n.s. 0.27 **	n.s. n.s.	n.s. 0.24 *	n.s. 0.23 *
Sperm count	S SP	n.s. 0.22 *	n.s. n.s.	n.s. n.s.	-0.24 * n.s.	n.s. 0.23 *
Progressive motility	S SP	n.s. n.s.	n.s. -0.25 **	n.s. n.s.	n.s. 0.27 **	n.s. 0.32 ***
Total motility	S SP	n.s. n.s.	n.s. -0.22 *	n.s. n.s.	n.s. 0.23 *	n.s. 0.25 **
Normomorph spermatozoa	S SP	n.s. n.s.	n.s. n.s.	n.s. n.s.	-0.34 *** n.s.	n.s. n.s.
Total normomorph spermatozoa	S SP	n.s. 0.28 **	n.s. n.s.	n.s. n.s.	-0.34 *** n.s.	n.s. 0.25 **
Intact acrosome	S SP	n.s. n.s.	n.s. n.s.	0.28 ** n.s.	n.s. n.s.	n.s. n.s.
DNA Fragmentation Index	S SP	n.s. n.s.	n.s. n.s.	n.s. 0.22 *	n.s. n.s.	n.s. n.s.

4.2 Presence of adipokines and their receptors in human testis and spermatozoa

4.2.1 Adipokine and receptor protein localisation in human testis

AdipoR1 protein was detected in human testis after performing an immunohistochemical staining without antigen retrieval. The protein expression was lightly detectable in the cytoplasm of Sertoli cells and Leydig cells (Figure 10, n=1, primary antibody dilution 1:100).

The control tissue samples were incubated only with the secondary antibody and no unspecific binding was detectable (data not shown).

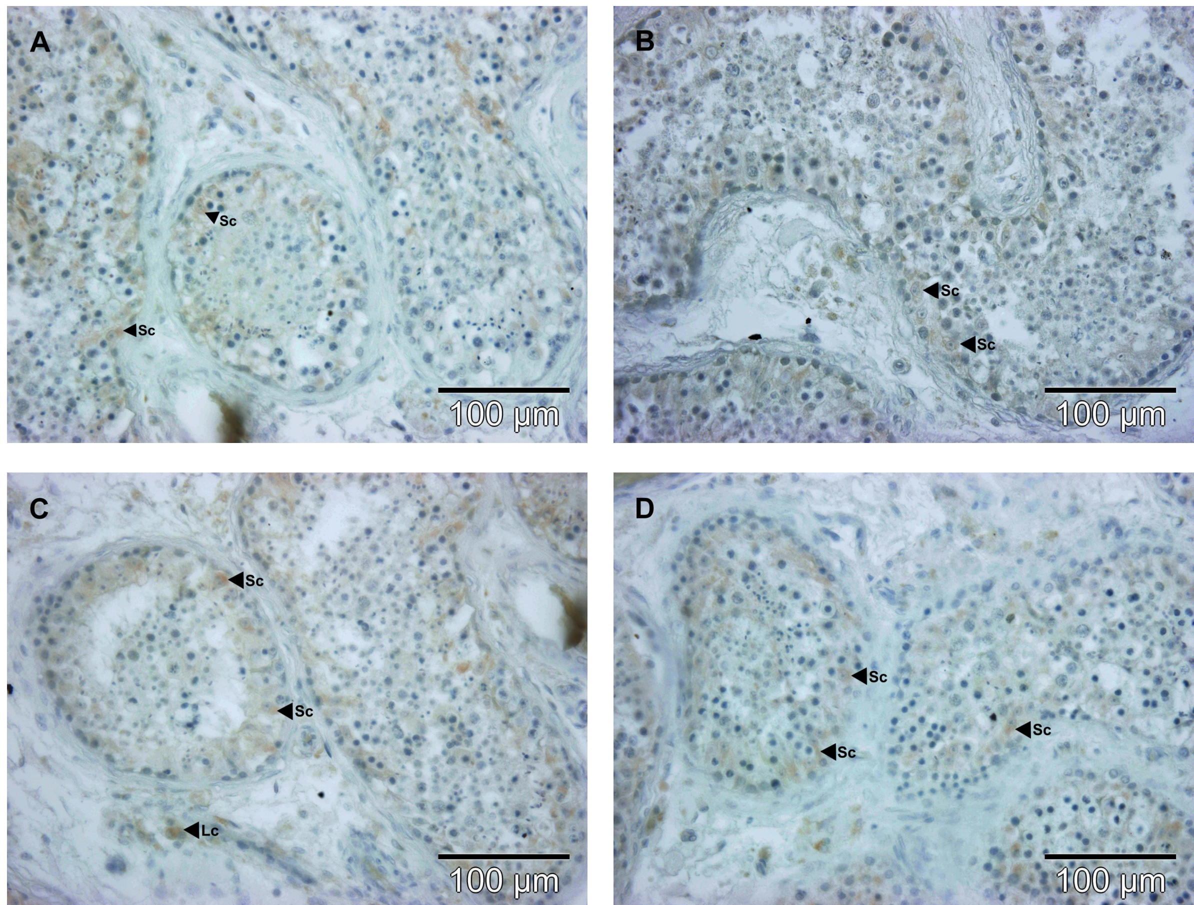


Figure 10 Immunohistochemical detection of AdipoR1 in human testis. Testis sections were not treated with proteinase K or Target Retrieval solution (n=1). Testis specimens were incubated with AdipoR1 antibody and counterstained with hemalum (A-D). AdipoR1 protein was detected in the cytoplasm of Sertoli cells (Sc) and Leydig cells (Lc).

In testis samples, which were not treated with proteinase K or target retrieval solution, a slight or no leptin immunoreaction was detectable (n=3, primary antibody dilution 1:100 and 1:200). The 1:200 dilution was not adequate for every specimen. Moreover, the immunoreaction differed between patients. Leptin protein was detectable in the cytoplasm of Sertoli cells, spermatocytes and spermatogonia and in the nucleus of Sertoli cells (Figure 11 A-D). The control tissue samples were incubated only with the secondary antibody and no unspecific binding was detectable (Figure 11 E;F)

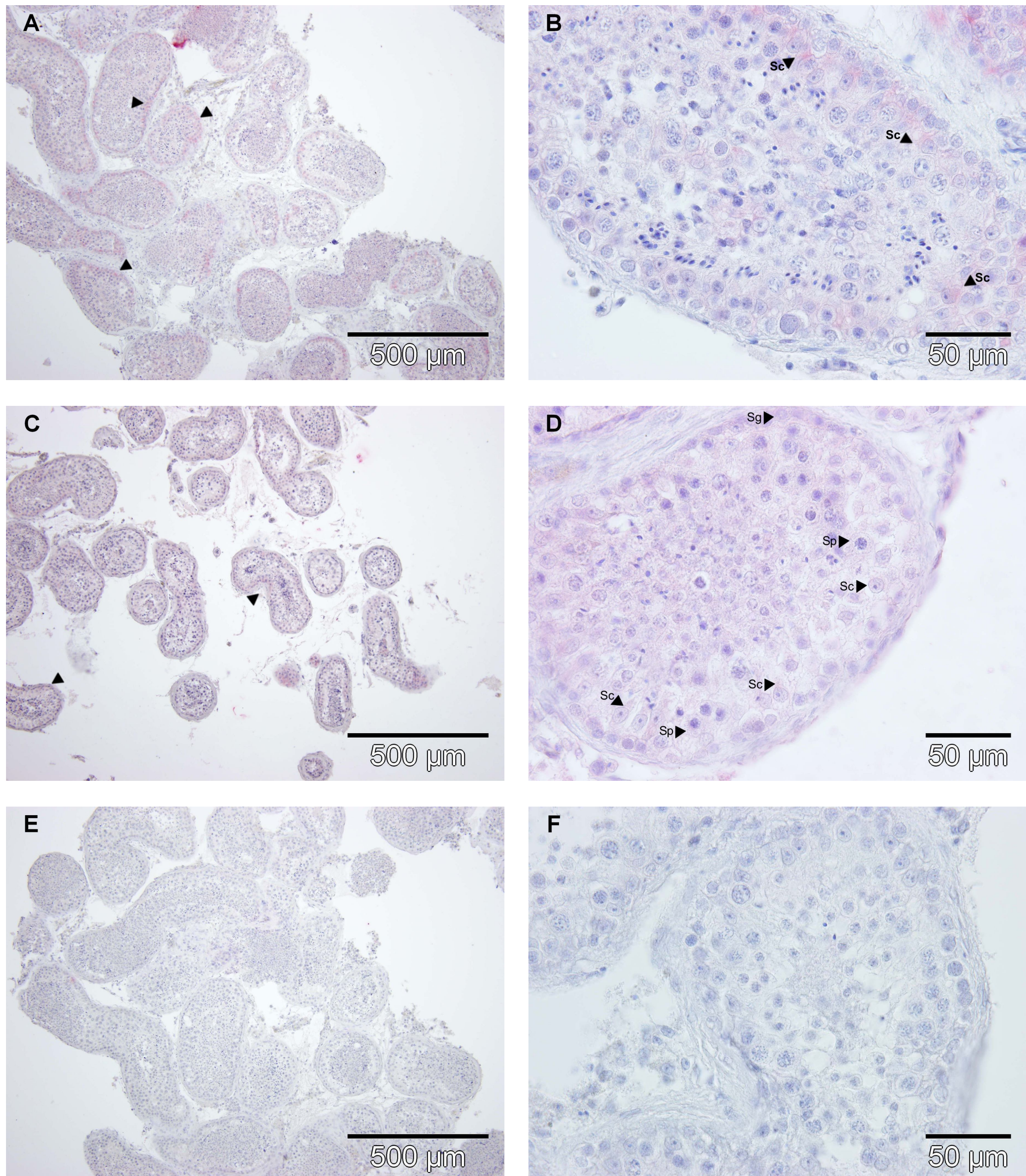


Figure 11 Immunohistochemical detection of leptin in human testis. Specimens were not treated with proteinase K or Target Retrieval solution (n=3). Testis specimens were incubated with leptin antibody. Arrow heads point to immunoreactive sites in the germinal epithelium of the tubuli seminiferi. Leptin protein was detectable in the cytoplasm of Sertoli cells (Sc), spermatocytes (Sp) and spermatogonia (Sg) and in the nucleus of Sc (A-D). Testis sections were counterstained with hemalum (A-F). Testis specimens were incubated without the primary antibody against leptin (E;F).

The treatment with proteinase K revealed no leptin expression in human testis samples (n=1, Figure 12 A;B).

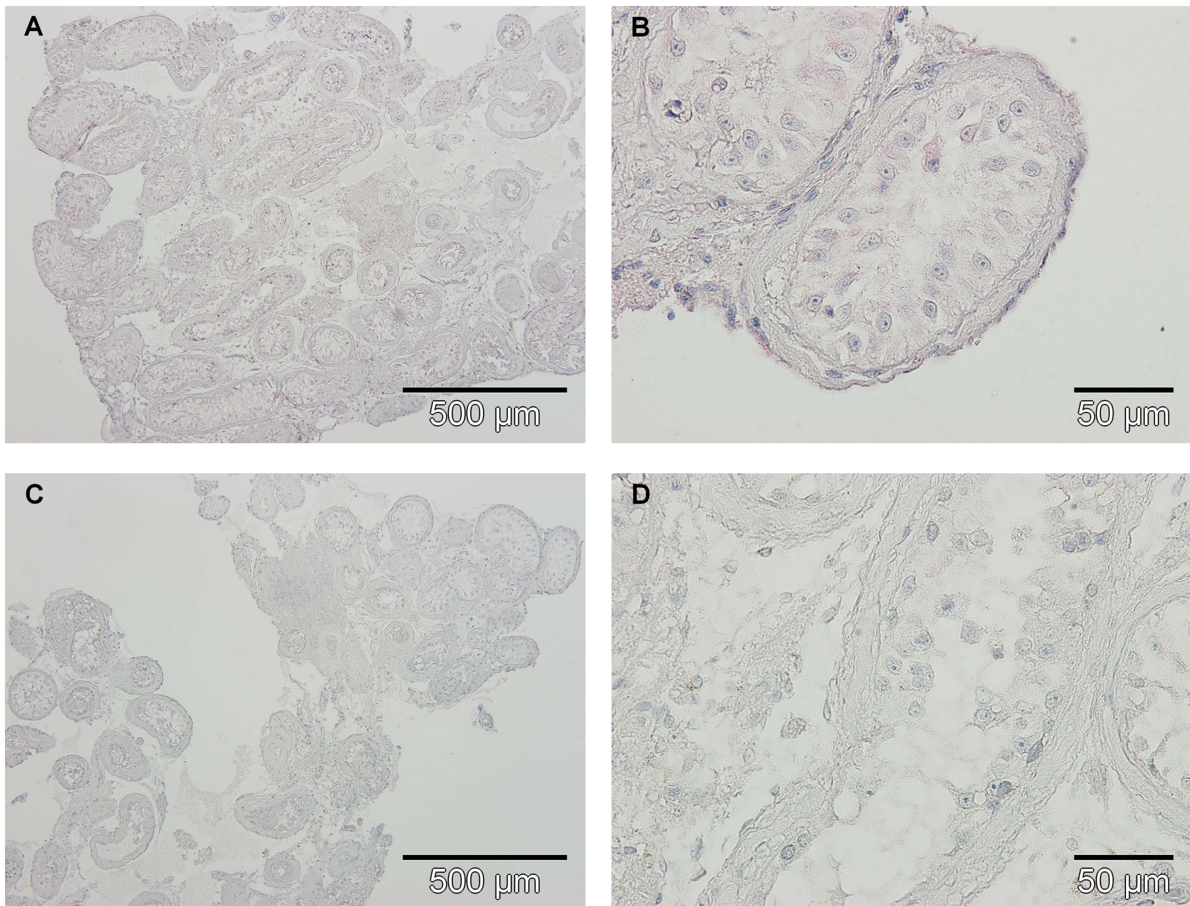


Figure 12 Immunohistochemical detection of leptin in human testis. Specimens were treated with proteinase K (n=1). Testis sections were counterstained with hemalum (A-D). Testis specimens were incubated with leptin antibody (A;B). Testis specimens were incubated without the primary antibody against leptin (C;D).

The antigen retrieval with a commercial citrate buffer resulted in a strong immunoreaction for leptin in the tested sample (n=1). Leptin was detected in the tubuli seminiferi (Figure 13 A). In detail, leptin was localised not only in the cytoplasm but also in the nucleus of Sertoli cells and in the cytoplasm of spermatogonia (Figure 13 B).

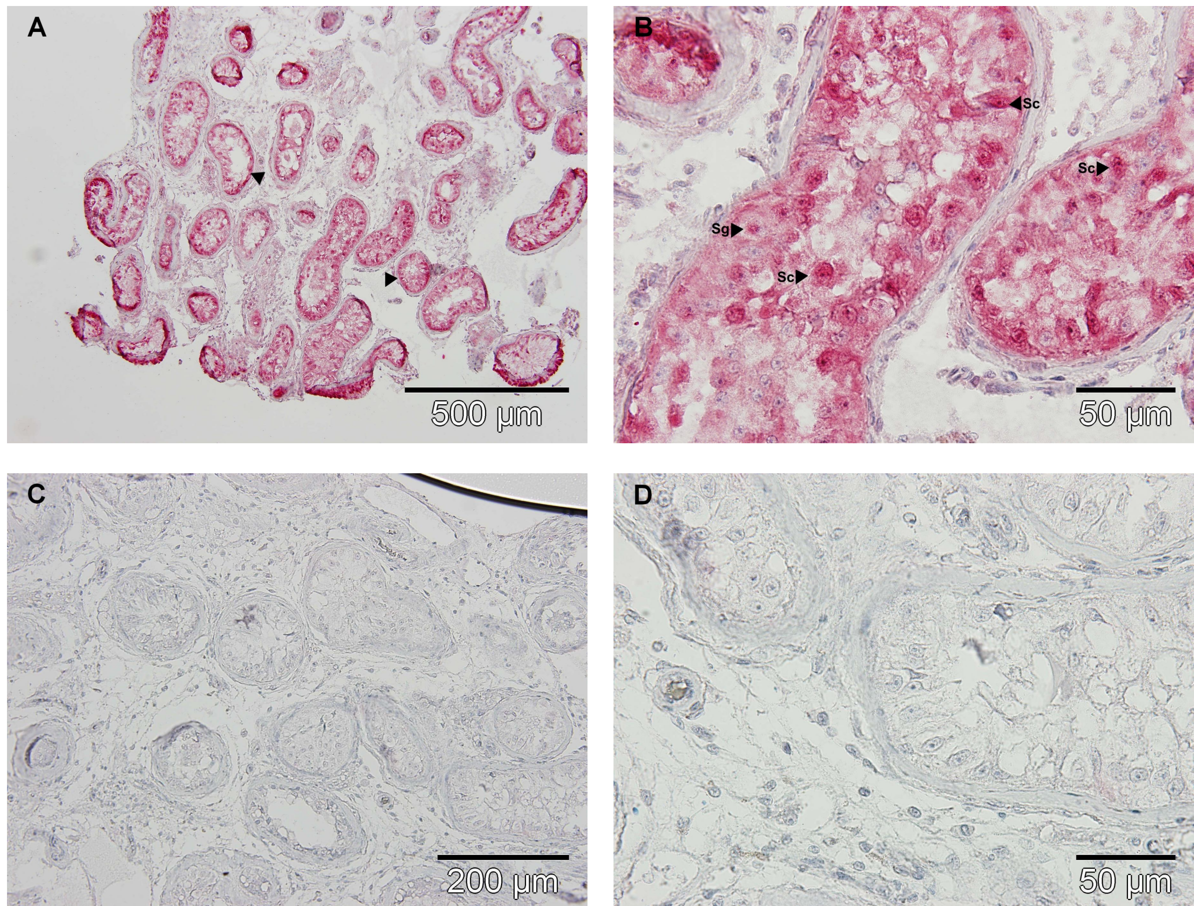


Figure 13 Immunohistochemical detection of leptin in human testis. Specimens were treated with Target Retrieval Solution (A-D, n=1). Testis sections were counterstained with hemalum (A-D). Arrow heads point to immunoreactive sites in the germinal epithelium of the tubuli seminiferi. Testis specimens were incubated with leptin antibody. Leptin protein was expressed in Sertoli cells (Sc), spermatogonia (Sg) (A;B). Testis specimens were incubated without the primary antibody against leptin (C;D).

To proof that leptin was expressed in Sertoli cells, we performed an immunohistochemical staining in samples of one patient with a Sertoli-Only-Syndrome. In this sample, leptin was expressed in the cytoplasm and the nucleus of the Sertoli cells (Figure 14 A;B).

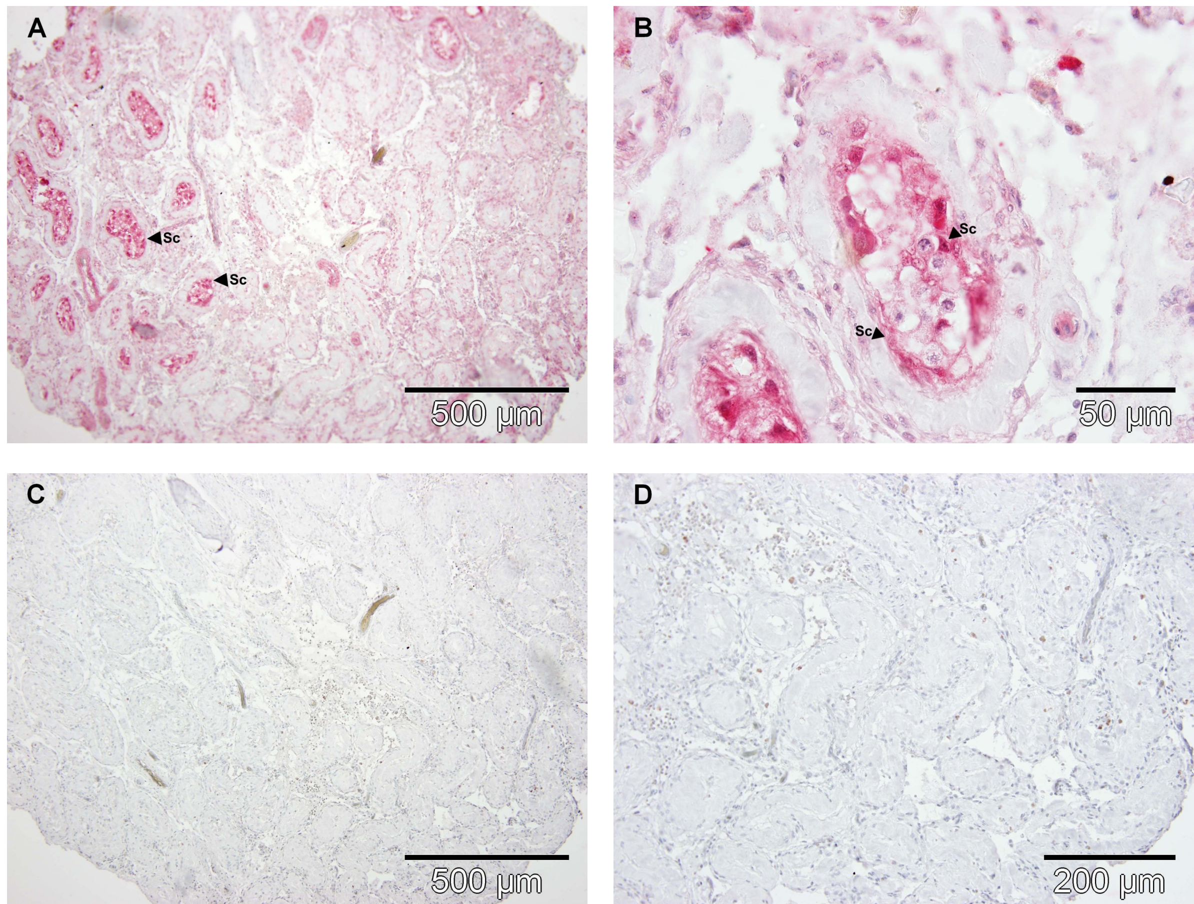


Figure 14 Immunohistochemical detection of leptin in a testis specimen from a patient with Sertoli-Only-Syndrome. Specimens were treated with Target Retrieval Solution (n=1). Testis sections were counterstained with hemalum (A-D). Testis specimens were incubated with leptin antibody. Leptin protein was detectable in the cytoplasm and nucleus of Sertoli cells (Sc) (A;B). Testis specimens were incubated without the primary antibody against leptin (C;D).

Furthermore, NAMPT was detected in biopsies of human testis (n=5, Figure 15 A-D). In detail, NAMPT expression was revealed in the cytoplasm of spermatogonia, spermatocytes, Sertoli cells and Leydig cells. Additionally, NAMPT was present in the nucleus of Sertoli cells, Leydig cells and several spermatocytes. No staining against NAMPT was visible in spermatids. NAMPT expression appeared to differ between individuals, as the staining intensity was variable throughout the specimens. The control tissue samples were incubated only with the secondary antibody and no unspecific binding was detectable (data not shown).

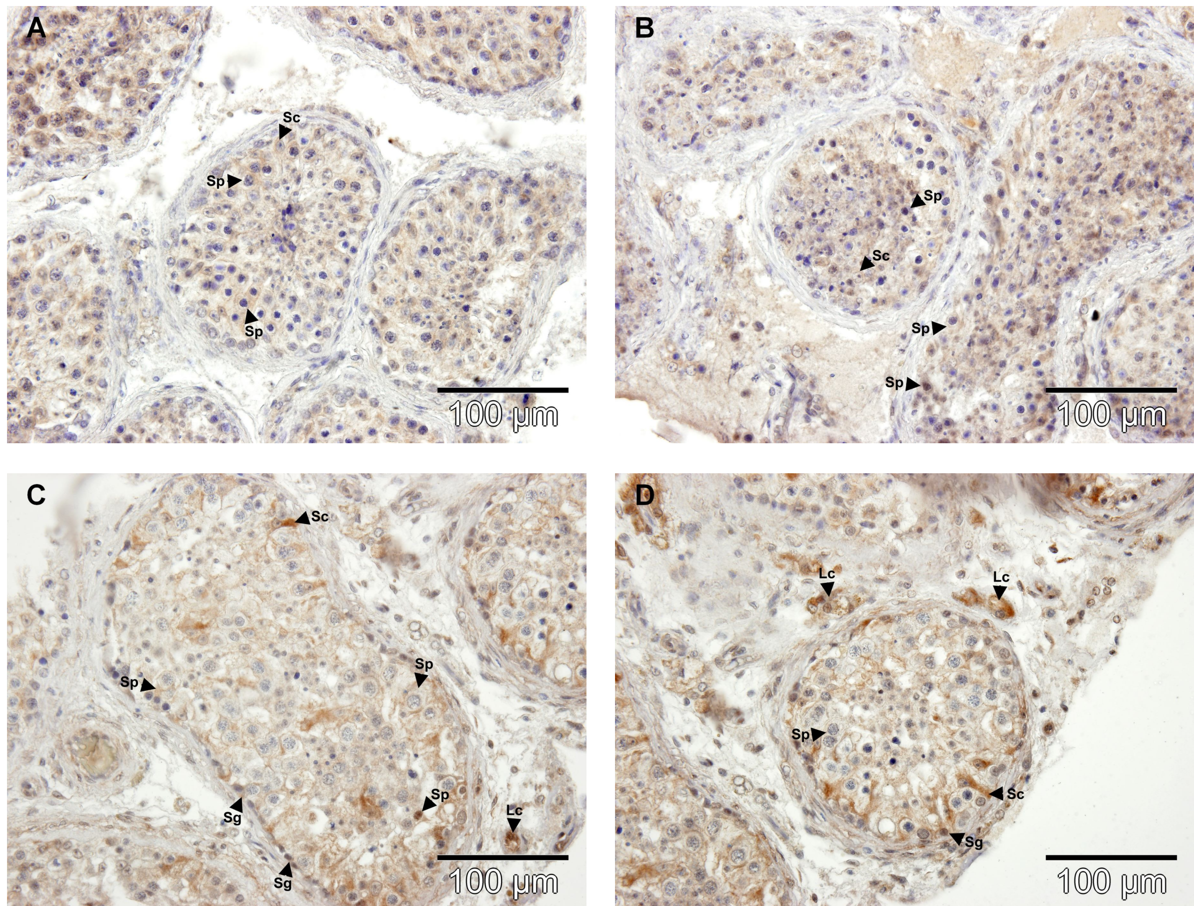


Figure 15 Immunohistochemical detection of NAMPT in human testis. Specimens were not treated with proteinase K or antigen retrieval solution (n=5). Testis sections were counterstained with hemalum. Testis specimens were incubated with NAMPT antibody (A-D). NAMPT was expressed in spermatocytes (Sp), Sertoli cells (Sc), Leydig cells (Lc), and spermatogonia (Sg).

4.2.2 Adipokine and respective receptor mRNA expression in human spermatozoa

4.2.2.1 Validation of RNA isolation method

First of all, the number of spermatozoa, which were necessary to perform a cDNA synthesis, had to be determined. Therefore, different amounts of CD45-negative spermatozoa were lysed by TRIzol®. After performing several RNA isolations, a RNA content of 10-60 ng/10^6 spermatozoa was detected. Although RNA was resuspended only in 10 µl diethylpyrocarbonate (DEPC)-H_2O, the RNA concentrations were often to low to perform a cDNA synthesis with 500 ng of RNA. Overall, 50-70x10^6 sperms were necessary to perform a cDNA synthesis with at least 500 ng total RNA. Furthermore, spermatozoa have a 100-fold higher DNA content compared with RNA. Therefore, spermatozoa lysates had to be handled with care.

The ratio $A_{260/280}$ was determined to evaluate the purity of RNA and DNA. Normally, this ratio is 1.8 for pure DNA and 2.0 for pure RNA. A decrease of the ratio indicates a contamination with protein, phenol or other contaminants absorbing at 280 nm. Furthermore, the $A_{260/230}$ ratio is determined for purity and is normally 2.0-2.3. The ratio decreases in

presence of contaminants (EDTA, carbohydrates, phenol like TRIzol®), which absorb at 230 nm. In the isolation experiments with TRIzol®, the ratios were all lower than described before (Table 10).

Table 10 Representative RNA output and purity of human spermatozoa. $A_{260/280}$ and $A_{260/230}$ were determined to evaluate the purity of the RNA solution. iS-immature sperm, mS-mature sperm, conc.-concentration

Preparation	$A_{260/230}$	$A_{260/280}$	Conc. (ng/µl)	Resuspended in x µl	RNA (ng) /10^6 sperm	Total RNA (ng)
iS TRIzol®	0.14	1.79	91.1	10	76	912
mS TRIzol®	0.94	1.71	206	20	18	4126

4.2.2.2 Validation of primer annealing and their respective mRNA expression

For cDNA synthesis and subsequent PCR, human spermatozoa RNA suspension had to be devoid of DNA. Sperm samples were checked for possible DNA contamination by performing a PCR with primers specific for protamine 2. A RNA sample without DNA contamination had a PCR product length of 182 bp. If the sample was contaminated with DNA, the PCR product was 345 bp long. Human spermatozoa DNA was used as a positive control (kindly provided by A. Paradowska, Urology, Children Urology, and Andrology, University Hospital Giessen).

Table 11 Primer sequences, corresponding PCR product length and annealing temperature. ObR-leptin receptor, AdipoR1-Adiponectin receptor 1, AdipoR2-Adiponectin receptor 2

Gene	**Primer sequence** (5’→3’, forward (fw), reverse (rev))	PCR product length	Annealing temperature
Protamine 2	TCGAGGTCTACGAGAGGACC (fw) CCTTCTGCATGTTCTCTTCCT (rev)	182 bp	62 °C
CD45	CATTCACTGCAGGGATGGATC (fw) GTCCATTCTGAGCAGGGTAGG (rev)	206 bp	65 °C
Adiponectin	GGCCGTGATGGCAGAGAT (fw) CCTTCAGCCCCGGGTACT (rev)	110 bp	57 °C
AdipoR1	ACCGGTTTGCCACTCCTAAG (fw) GGCACGACGCCACTCAA (rev)	76 bp	63 °C
AdipoR2	CTGGGCATTGCAGCCATTAT (fw) AGTGCAAGGTAGGAATGATTCCAC (rev)	121 bp	60 °C
ObR	AAGAGGCTAGATGGACTGGGATATT (fw) ATTCTCCAAAATTCAGGTCCTCTCA (rev)	104 bp	64 °C

Semen can also be contaminated with leucocytes. CD45 was used as a marker for leucocytes in isolated RNA. Blood leucocytes were used as a positive control. After designing the primers, they were checked for different annealing temperatures with a gradient cycler. The final primer sequences are listed in Table 11.

According to Figure 16, a band for protamine 2 with the highest density was detected at an annealing temperature of 61.7 °C at 182 bp. Furthermore, a light band was detected at 345 bp, indicating that this sample was lightly contaminated with spermatozoa DNA.

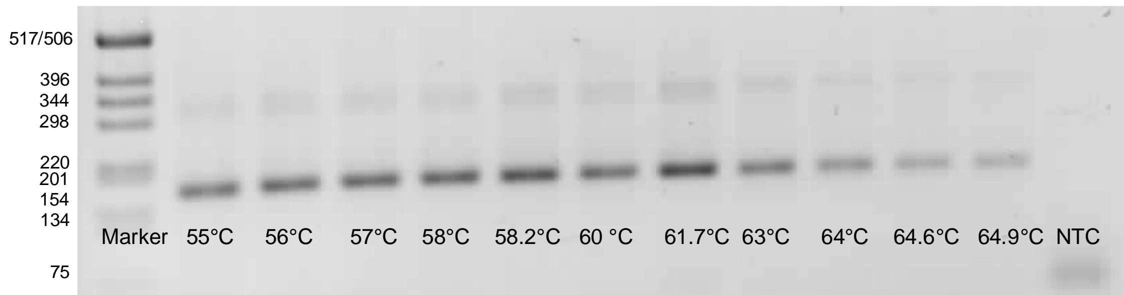


Figure 16 Protamine 2 mRNA expression in human spermatozoa after performing a gradient PCR. NTC-NoTemplate Control

In further experiments, spermatozoa samples (n=70) were tested for DNA contamination with an annealing temperature of 62 °C (40 cycles). 3 out of 70 samples were contaminated with spermatozoa DNA as a light band at 345 bp was detectable (Figure 17). Sperm DNA served as a positive control.

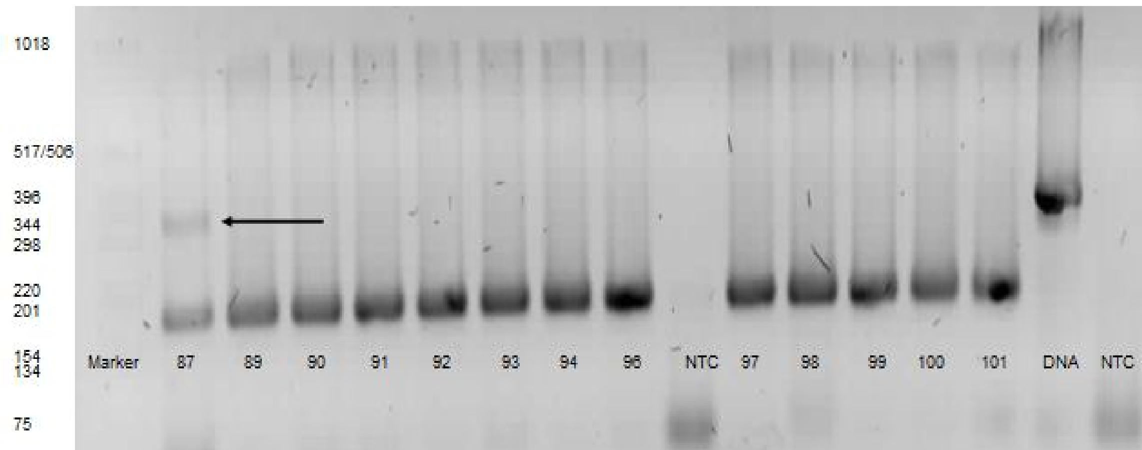


Figure 17 Representative agarose gel for protamine 2 mRNA expression in human spermatozoa after performing a PCR. The arrow head points to the genomic DNA contaminated specimen. NTC-No Template Control

Moreover, the mRNA expression of protamine 2 was evaluated in immature and mature spermatozoa. As different amounts of spermatozoa were lysed and the whole amount of isolated mRNA was transcribed into cDNA, the mRNA expression between immature and mature spermatozoa was not comparable. According to Figure 18 all samples were devoid of genomic DNA.

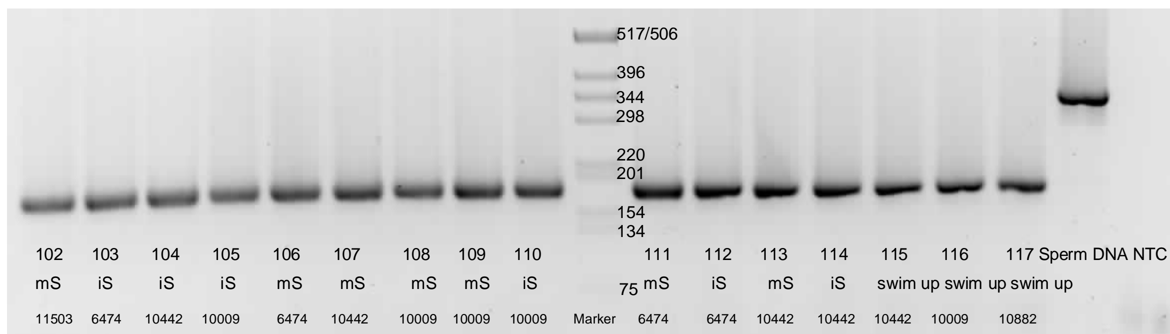


Figure 18 Protamine 2 mRNA expression in human spermatozoa after performing a PCR. Spermatozoa DNA served as positive control. iS-immature spermatozoa, mS-mature spermatozoa, swim up-spermatozoa acquired by swim-up technique, NTC-No Template Control

According to Figure 19, a valid annealing temperature for the detection of CD45 trancripts is 64.9 °C (40 cycles).

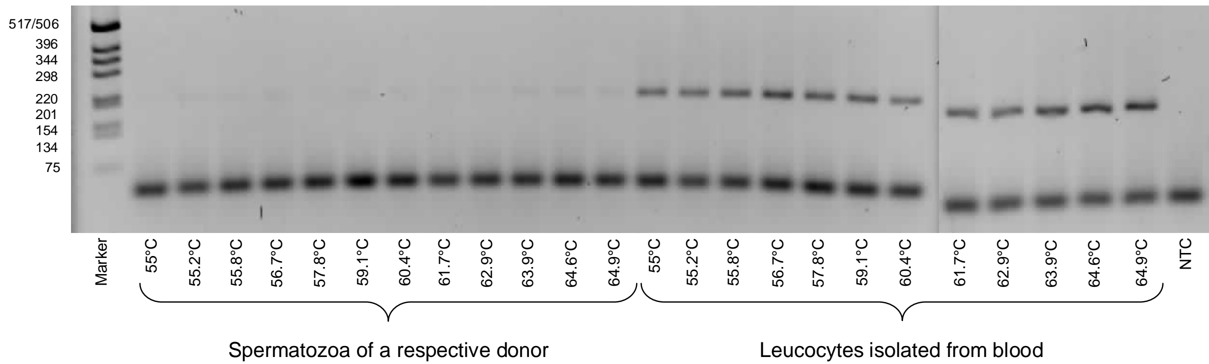


Figure 19 CD45 mRNA expression in human spermatozoa and leucocytes after performing a gradient PCR. NTC-No Template Control

In further experiments, spermatozoa samples (n=70) were tested for leucocyte contamination with an annealing temperature of 65 °C (40 cycles). About half of the samples were contaminated with leucocytes, because a band at 206 bp was detectable (Figure 20). Leucocytes isolated from blood served as positive control.

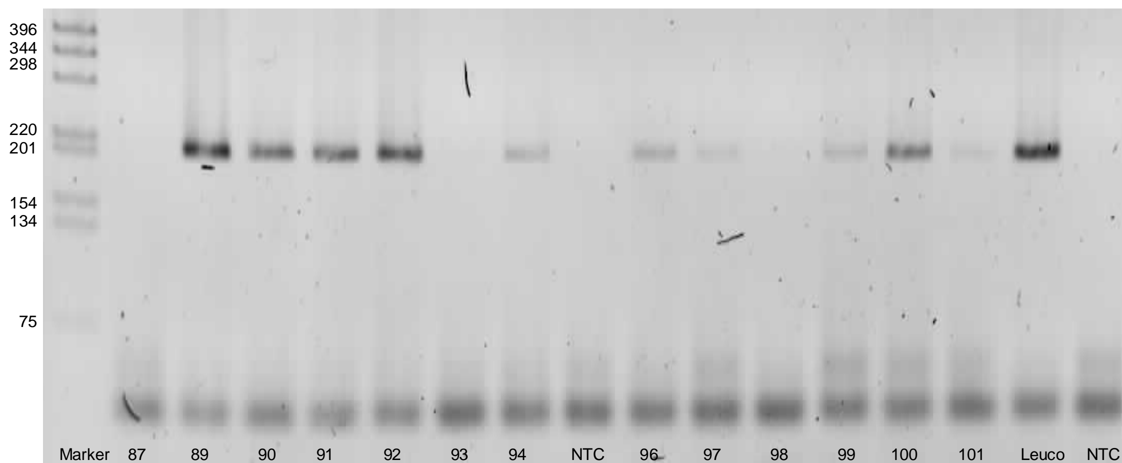


Figure 20 Representative agarose gel for CD45 mRNA expression in human spermatozoa after performing a PCR. A band at 206 bp indicated a leucocyte contaminated specimen, Leucocyte cDNA served as positive control. Leuco-leucocyte, NTC-No Template Control

Moreover, the mRNA expression of CD45 was evaluated in immature and mature spermatozoa. According to Figure 21 almost every sample was contaminated with leucocytes. All samples positive for leucocyte and genomic DNA contamination were excluded from further experiments.

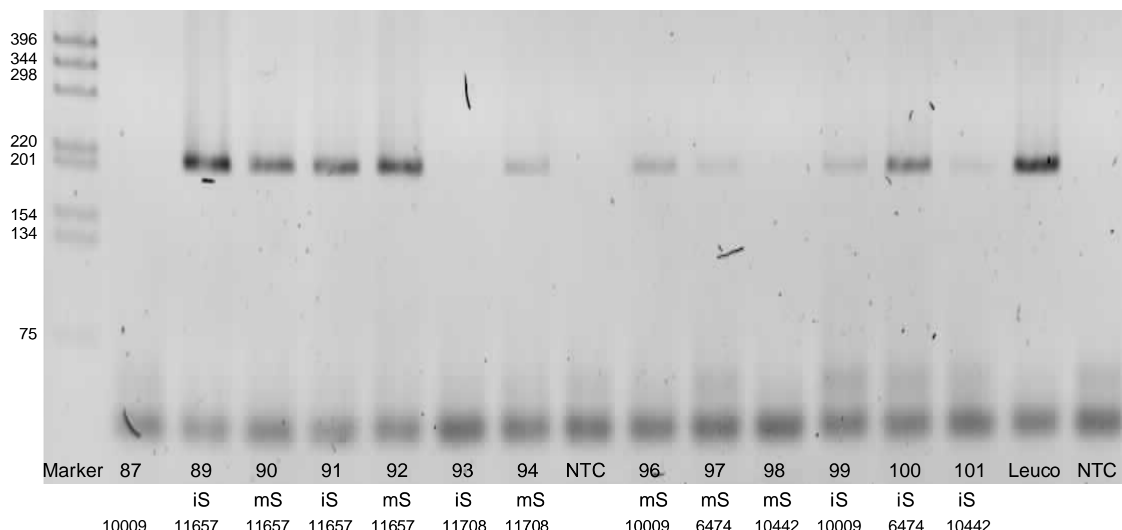


Figure 21 CD45 mRNA expression in human spermatozoa after performing a PCR. Leucocyte cDNA served as positive control. iS-immature spermatozoa, mS-mature spermatozoa, Leuco-leucocyte, NTC-No Template Control

In human spermatozoa samples, transcripts of adiponectin and adiponectin receptor 1 (AdipoR1) were detectable (annealing temperature 60 °C, 40 cycles), whereas AdipoR2 transcripts were not detected (annealing temperature 60 °C, 40 cycles). Hepatocyte cDNA was used as positive control for adiponectin receptors (Figure 22).

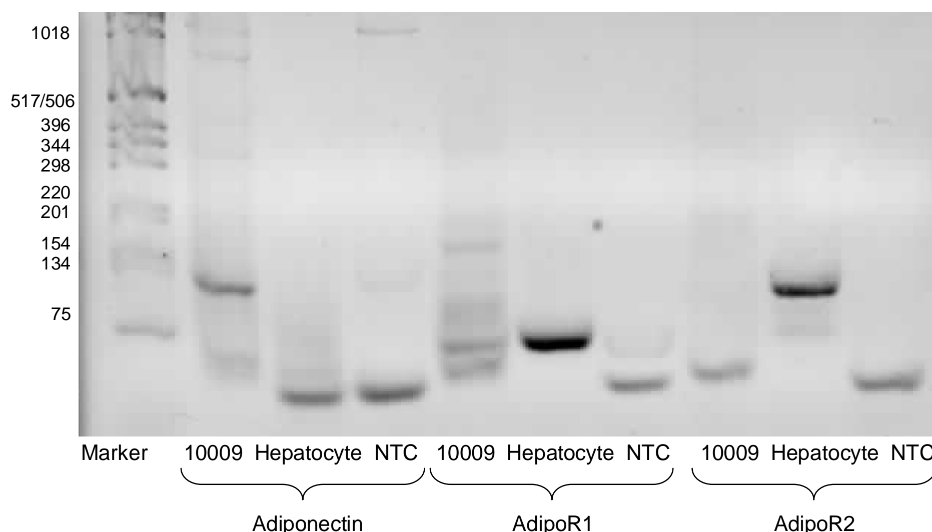


Figure 22 mRNA expression of adiponectin, AdipoR1, and AdipoR2 in human spermatozoa of a representative donor. AdipoR1-adiponectin receptor 1, AdipoR2-adiponectin receptor 2, 10009-representative spermatozoa of one donor, NTC-No Template Control

A gradient PCR was performed to improve the sensitivity of PCR for adiponectin and AdipoR1, because more than one band was detected for AdipoR1 in human spermatozoa. In the experiment shown in Figure 23, the highest expression of adiponectin mRNA in human spermatozoa was detected after performing a gradient PCR at 56.7 °C (n=3). Adiponectin cDNA was detectable at 110 bp (Figure 23). Adiponectin cDNA of adipocytes and Simpson-Golabi-Behmel Syndrome (SGBS) adipocytes served as positive controls as shown by Vaittinen *et al.* (360).

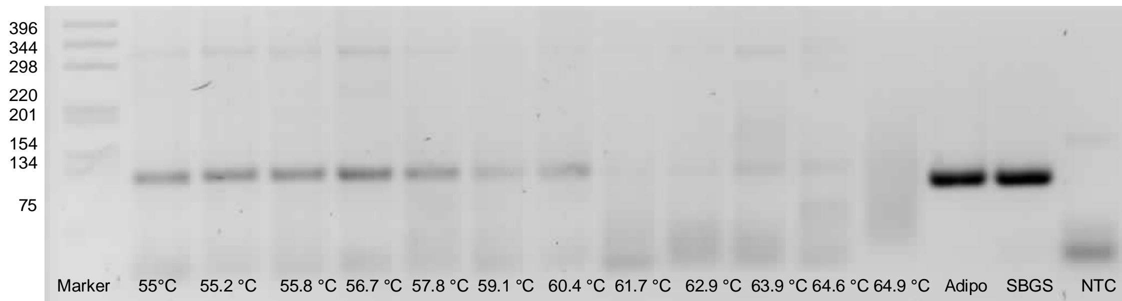


Figure 23 Adiponectin mRNA expression in human spermatozoa of a representative donor after performing a gradient PCR. Adipo-Adipocytes, SGBS-Simpson-Golabi-Behmel Syndrome cells, NTC-No Template Control

Moreover, AdipoR1 transcripts were detectable after performing a gradient PCR (76 bp, Figure 24). The band with the highest density was determined at an annealing temperature of 62.9 °C (40 cycles). Adipocyte, SGBS adipocyte and hepatocyte cDNA served as positive controls for AdipoR1 mRNA expression as demonstrated by Vaittinen *et al.* (360).

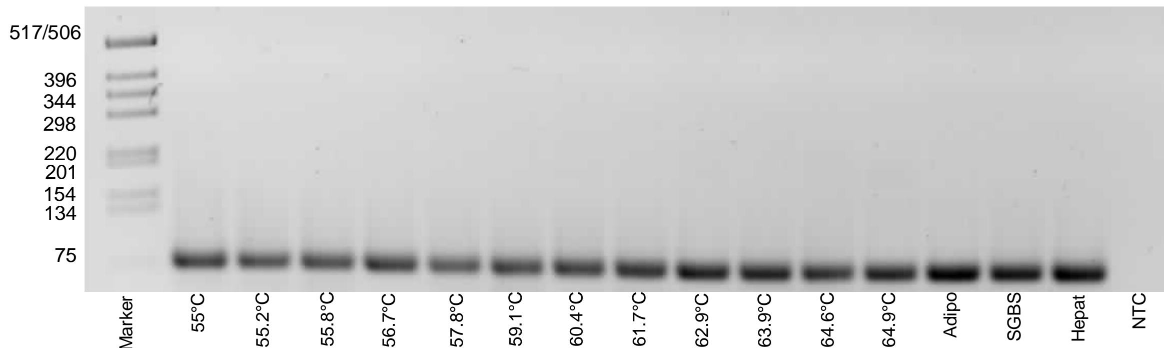


Figure 24 Adiponectin receptor 1 mRNA expression in human spermatozoa after performing a gradient PCR. Adipo-Adipocytes, Hepat-Hepatocytes, NTC-No Template Control

In 13 spermatozoa samples, AdipoR1 mRNA was detected (annealing temperature 63 °C, 40 cycles), whereas the expression differed between the individuals (Figure 25).

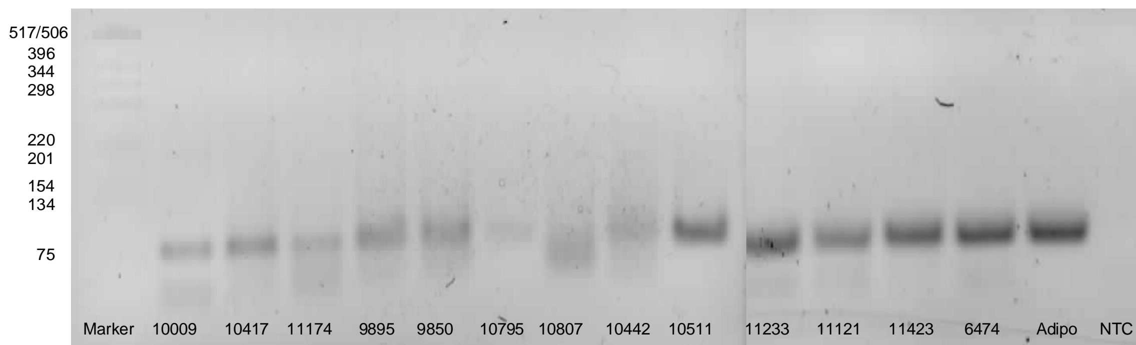


Figure 25 Representative agarose gel for adiponectin receptor 1 mRNA expression in human spermatozoa after performing a PCR. Adipo-adipocyte cDNA, NTC-No Template Control

The presence of ObR mRNA in human spermatozoa was evaluated as well (Figure 26). ObR transfected HEK cells and hepatocytes served as positive controls for ObR expression. The primers for ObR were specific for all receptor isoforms. A PCR was performed with an annealing temperature of 60 °C (40 cycles). In HEK cells and hepatocytes, a PCR

product of 104 bp was detected, whereas in human spermatozoa, the PCR product was presented with 220 bp (Figure 26).

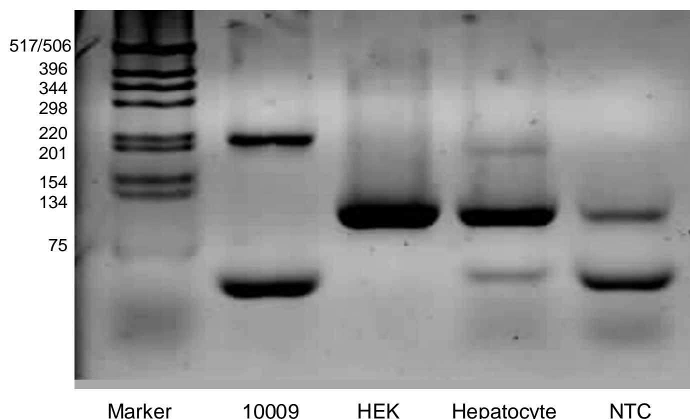


Figure 26 Leptin receptor mRNA is not expressed in human spermatozoa. 10009-spermatozoa sample, HEK-ObR transfected HEK cells, NTC-No Template Control

As the expected PCR product length of 104 bp was not detectable in human spermatozoa, a gradient PCR was performed to exclude the possibility of different PCR products. After performing the gradient PCR with human spermatozoa cDNA, a PCR product with a length of approximately 220 bp was detectable (genomic DNA 211 bp, 40 cycles, Figure 27). Due to the length of the PCR product, we assume that genomic DNA was detected and not cDNA, although samples had been checked for DNA contamination. Therefore, we suggest that human spermatozoa do not express ObR mRNA.

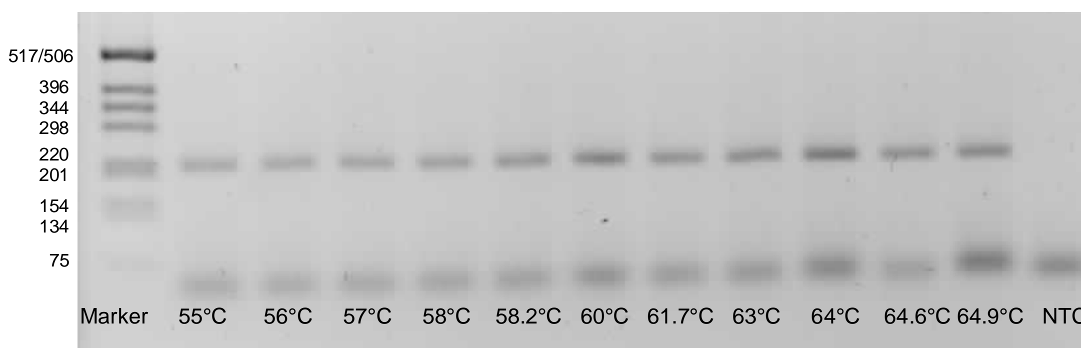


Figure 27 ObR mRNA expression in human spermatozoa after performing a gradient PCR. NTC-No Template Control

4.2.3 Adipokine and receptor protein detection in/at human spermatozoa

Additionally to testis, the presence of different adipokines and their known receptors was investigated in human spermatozoa by immunofluorescence and western blot analysis. Before smears were incubated with antibodies, they were checked for auto-fluorescence. Some specimens showed fluorescence in the FITC channel without antibody incubation (Figure 28).

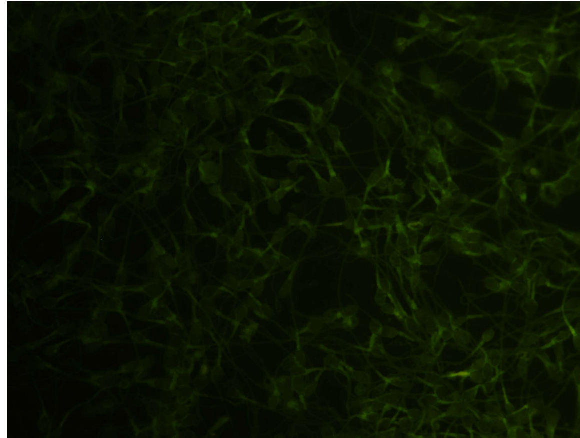


Figure 28 Auto-fluorescence of human spermatozoa detected at the FITC channel.

AdipoR1 immunofluorescence signal was detected at the connecting piece and the tail of ejaculated spermatozoa (n=12, primary antibody dilution 1:20, Figure 29 A).

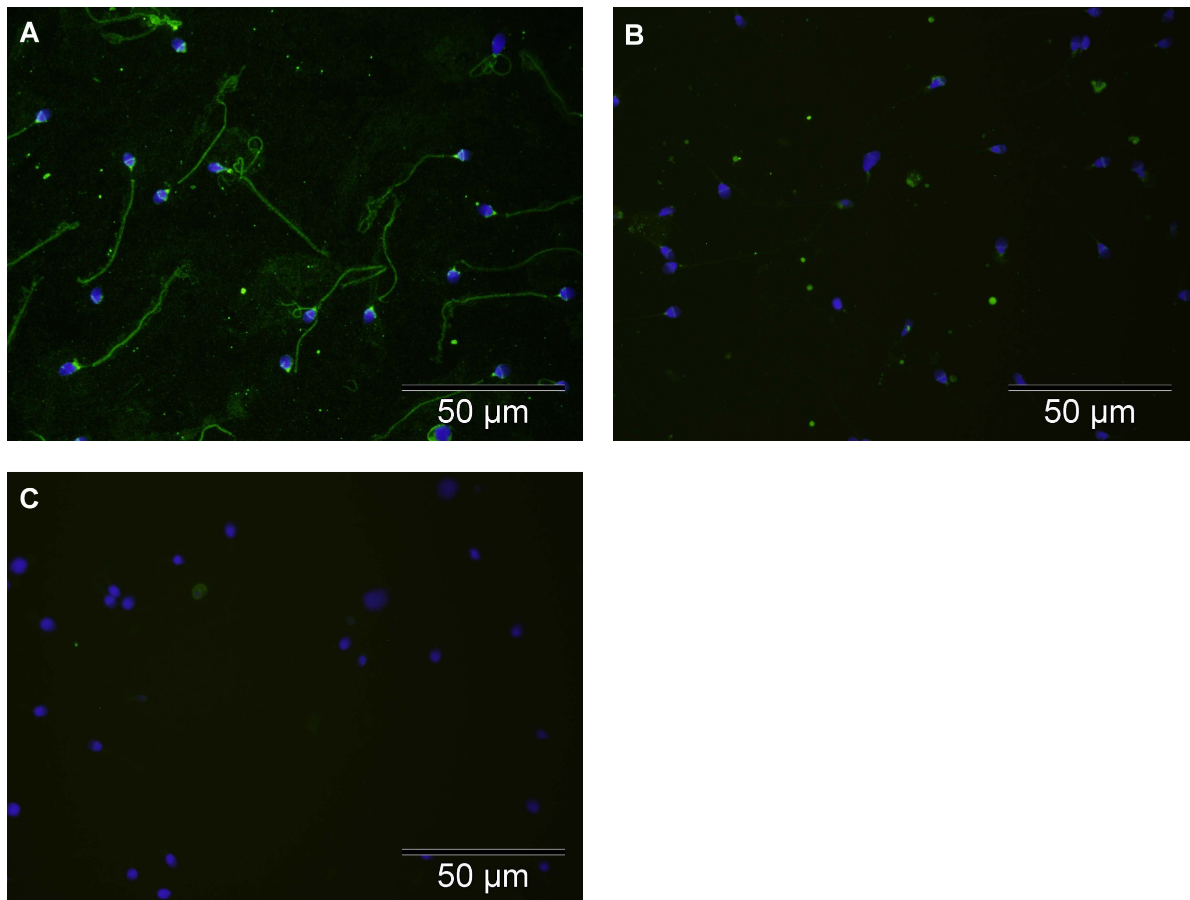


Figure 29 Adiponectin receptor 1 expression at human spermatozoa. Immunofluorescence signal was detected at the connecting piece and tail (A). Human spermatozoa incubated with anti-human adiponectin receptor 1 antibody and 4 µg adiponectin receptor 1 blocking peptide revealed a specific binding of the primary antibody at the sperm tail (B). Human spermatozoa were incubated with secondary antibody to evaluate the unspecific binding of the secondary antibody (C). Nucleus was counterstained with DAPI (n=12).

Occasionally, an immunofluorescence signal was detectable at the midpiece or the equatorial band at the spermatozoa head. To evaluate non-specific bindings of primary and secondary antibodies, spermatozoa were incubated with 4 µg AdipoR1 blocking peptide or

only with the secondary antibody. The incubation with blocking peptides revealed a significant decrease of the immunofluorescence signal at the tail of spermatozoa (Figure 29 B), suggesting that this immunofluorescence signal was specific. However, the signal obtained at the midpiece, equatorial band and occasionally at the connecting piece did not decrease in its immunofluorescence intensity, indicating that this localisation of AdipoR1 at human spermatozoa was not or less specific. The secondary antibody did not bind to unspecific sites of human spermatozoa (Figure 29 C).

In addition to immunofluorescence, the presence of AdipoR1 (43 kDa) was tested by western blot analysis. Thereby, different secondary antibodies were tested, as these antibodies showed non-specific binding (Figure 30).

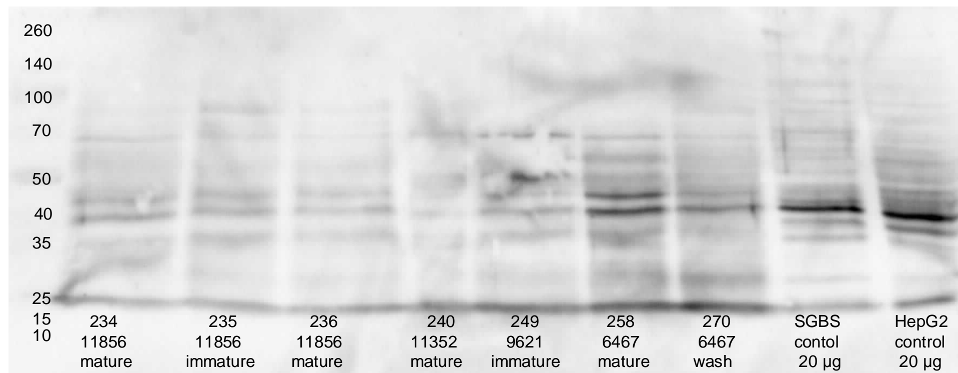


Figure 30 AdipoR1 protein expression in human spermatozoa. Primary antibody was diluted 1:2000 and the secondary antibody was diluted 1:10000 in 3 % BSA-TTBS.

SGBS adipocytes were used as a positive control (kindly provided by A. Garten, Department of Women and Child Health, Hospital for Children and Adolescents, Center for Pediatric Research Leipzig, University Hospital Leipzig). Western blot analysis was performed with different dilutions of the primary antibody. As seen in Figure 31, the 1:4000 dilution seemed to be appropriate for detecton of AdipoR1.

The secondary antibody was diluted in TTBS without BSA or dried non-fat milk in a 1:40000 dilution. A western blot with the respective blocking peptide against AdipoR1 was performed to evaluate the specifity of the primary antibody against AdipoR1. This analysis did not reveal a valid result, as the positive control did not show a band at 43 kDa. Moreover, the light bands at 43 kDa in spermatozoa did not vanish after the incubation with the blocking peptide (Figure 32).

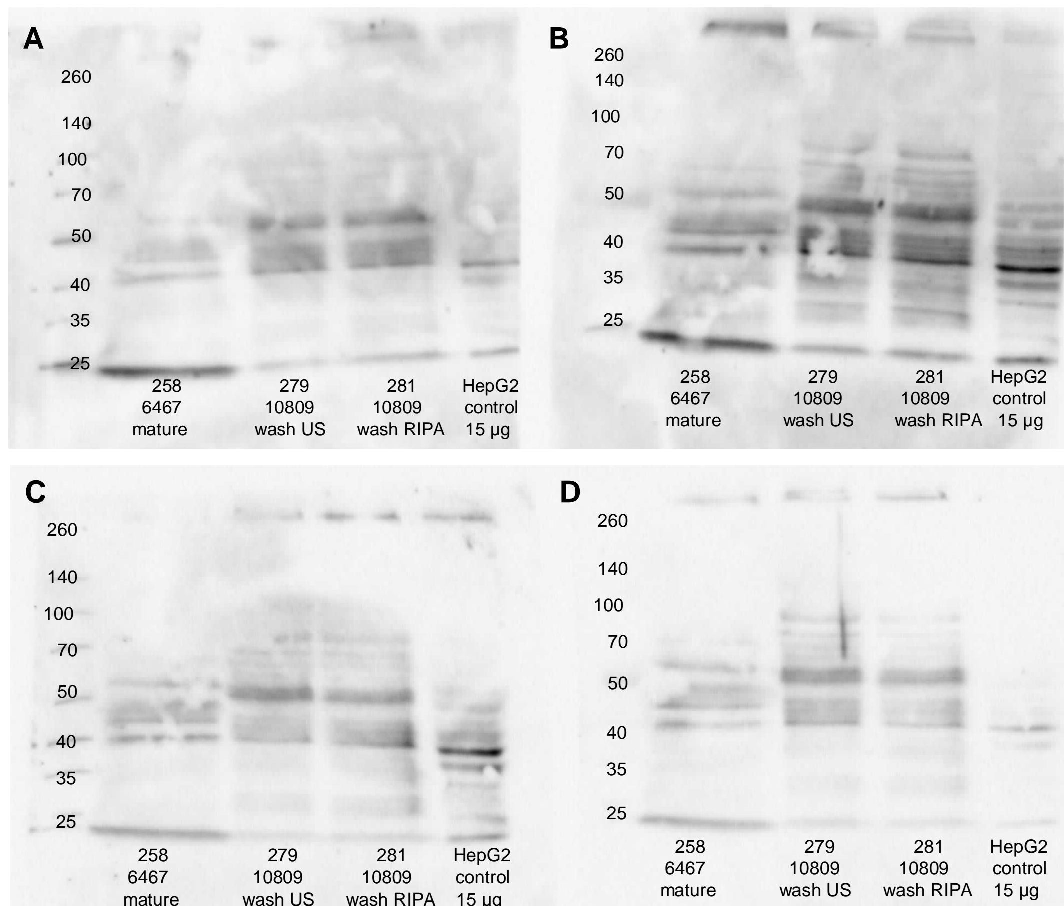


Figure 31 AdipoR1 protein expression in human spermatozoa. Nitrocellulose membranes were incubated with different primary antibody concentrations (A) 1:2000, B) 1:4000, C) 1:6000, D) no primary antibody). The secondary antibody was diluted 1:10000 in 3 % BSA-TTBS.

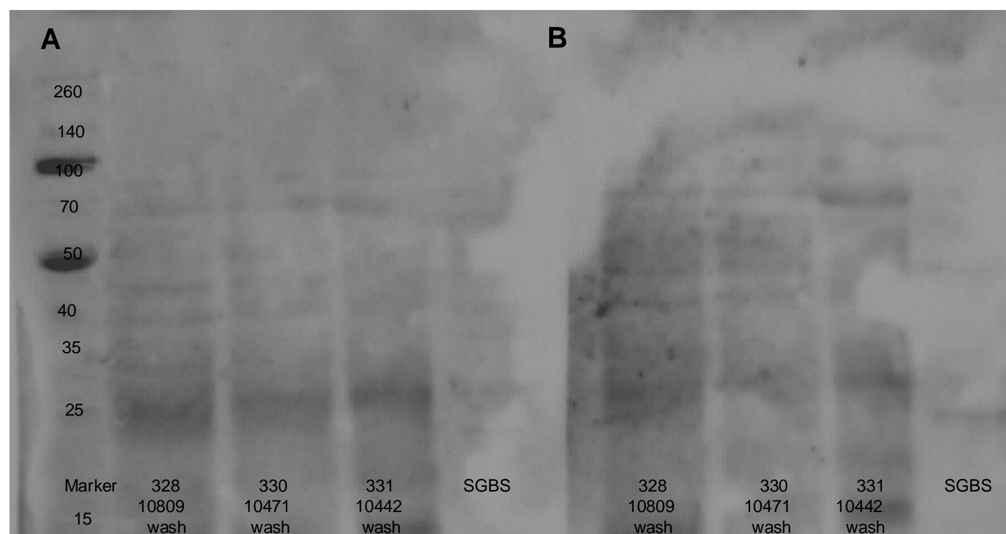


Figure 32 AdipoR1 protein expression in human spermatozoa after incubation with blocking peptide. Primary antibody was diluted 1:1000. Secondary antibody was diluted 1:40000 in TTBS (A). A 5x concentration of blocking peptide was used to evaluate unspecific binding of the primary antibody (B).

The localisation of the chemerin receptor ChemR23 was checked in human ejaculated spermatozoa (n=2). An immunofluorescence signal was detectable at the tail in different intensities between the individual spermatozoa (Figure 33 A, primary antibody dilution

1:50). Occasionally, ChemR23 was detectable at the connecting piece and the midpiece of spermatozoa (Figure 33 B). For this antibody, no blocking peptide was available. The specifity of the secondary antibody was revealed by performing an experiment without incubation of the primary antibody (Figure 33 C).

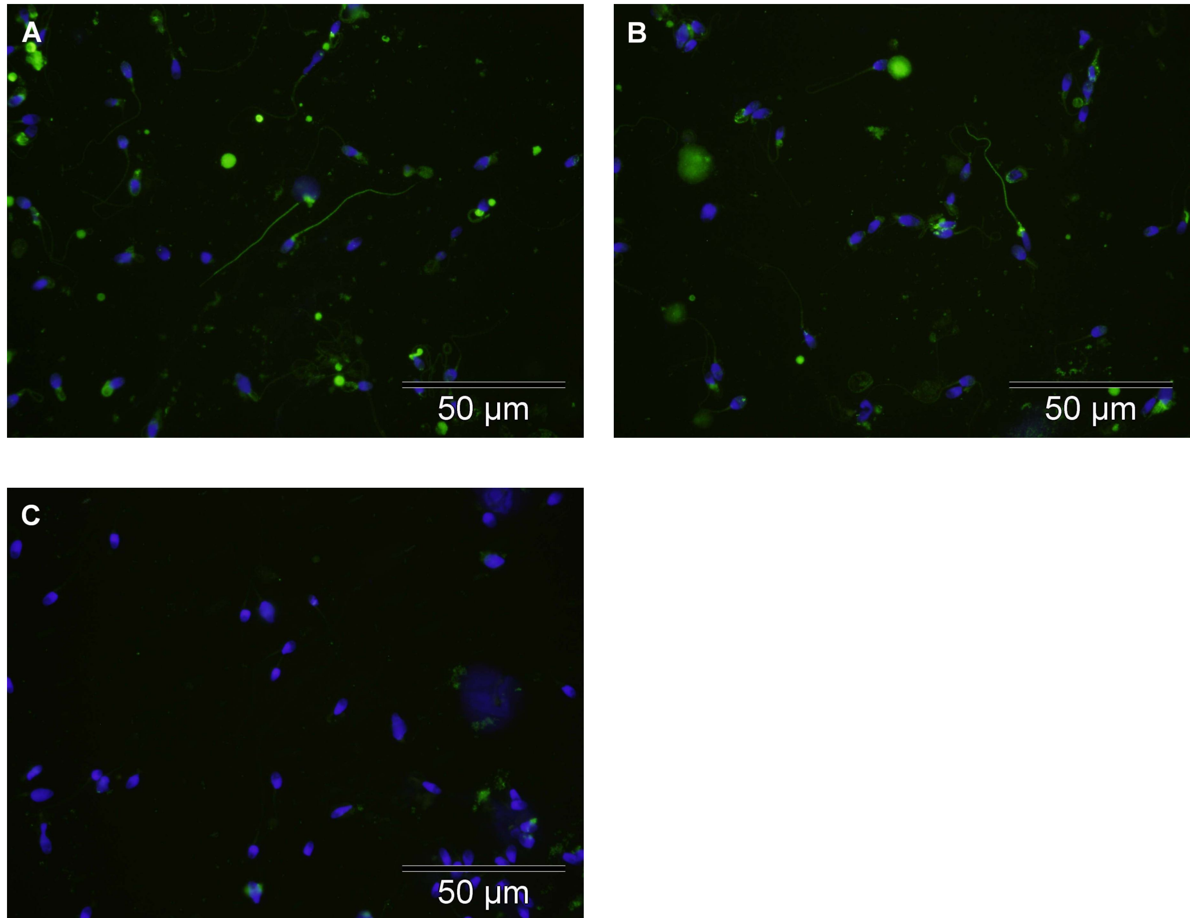


Figure 33 Chemerin receptor expression at human spermatozoa. Immunofluorescence signal was detected at the connecting piece (A;B). Human spermatozoa were incubated with secondary antibody to evaluate the unspecific binding of the secondary antibody (C). Nucleus was counter-stained with DAPI (n=2).

Leptin and its receptor were also detected at human spermatozoa. Leptin immunofluorescence signal was detectable at the connecting piece and the tail (n=12, Figure 34 A, primary antibody dilution 1:10). Occasionally, immunofluorescence signal was detectable at the midpiece or the equatorial band of the spermatozoon's head. To evaluate non-specific binding of primary and secondary antibodies, spermatozoa were incubated with 2 µg leptin blocking peptide or only with the secondary antibody. The immunofluorescence signal at the tail and connecting piece decreased significantly after the incubation with the respective blocking peptide, indicating that this binding of the primary antibody was specific (Figure 34 B). The secondary antibody did not bind to unspecific sites of human spermatozoa (Figure 34 C).

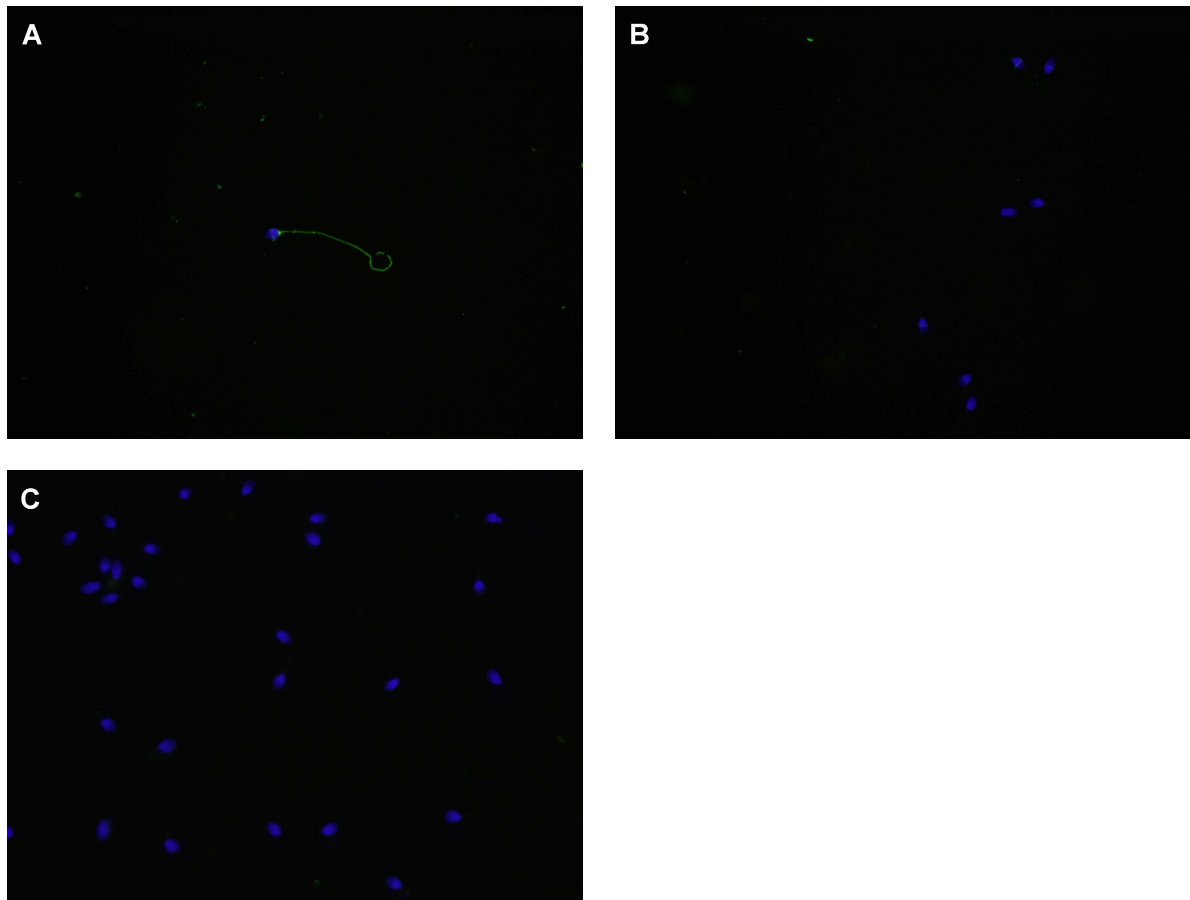


Figure 34 Leptin expression in human spermatozoa. Immunofluorescence signal was detected at the connecting piece, midpiece and tail (A). Human spermatozoa incubated with anti-human leptin antibody and 2 µg leptin blocking peptide revealed a specific binding of the primary antibody at the sperm tail and connecting piece (B). Human spermatozoa were incubated with secondary antibody to evaluate the unspecific binding of the secondary antibody (C). Nucleus was counterstained with DAPI (n=12).

Leptin protein expression was also investigated by western blot analysis. Different primary and secondary antibodies in variable dilutions were used, although no valid results were obtained. Leptin protein was not or hardly detectable by western blotting (data not shown). Moreover, the determined molecular weight did not correspond to expected molecular weight of 16 kDa.

Leptin receptor immunofluorescence was detected at the tail and occasionally at the equatorial band and connecting piece (n=11, Figure 35 A, primary antibody dilution 1:10). In contrast to leptin, no immunofluorescence signal was found at the midpiece. To evaluate non-specific bindings of primary and secondary antibodies, spermatozoa were incubated with 2 µg leptin receptor blocking peptide or only with the secondary antibodies. After the incubation with the blocking peptide against the ObR antibody, the immunofluorescence signal at the tail and connecting piece was not detectable anymore (Figure 35 B), suggesting that this primary antibody against ObR was specific. Moreover, the secondary antibody was revealed to be specific, as no immunofluorescence signal was detectable after the incubation only with the secondary antibody (Figure 35 C).

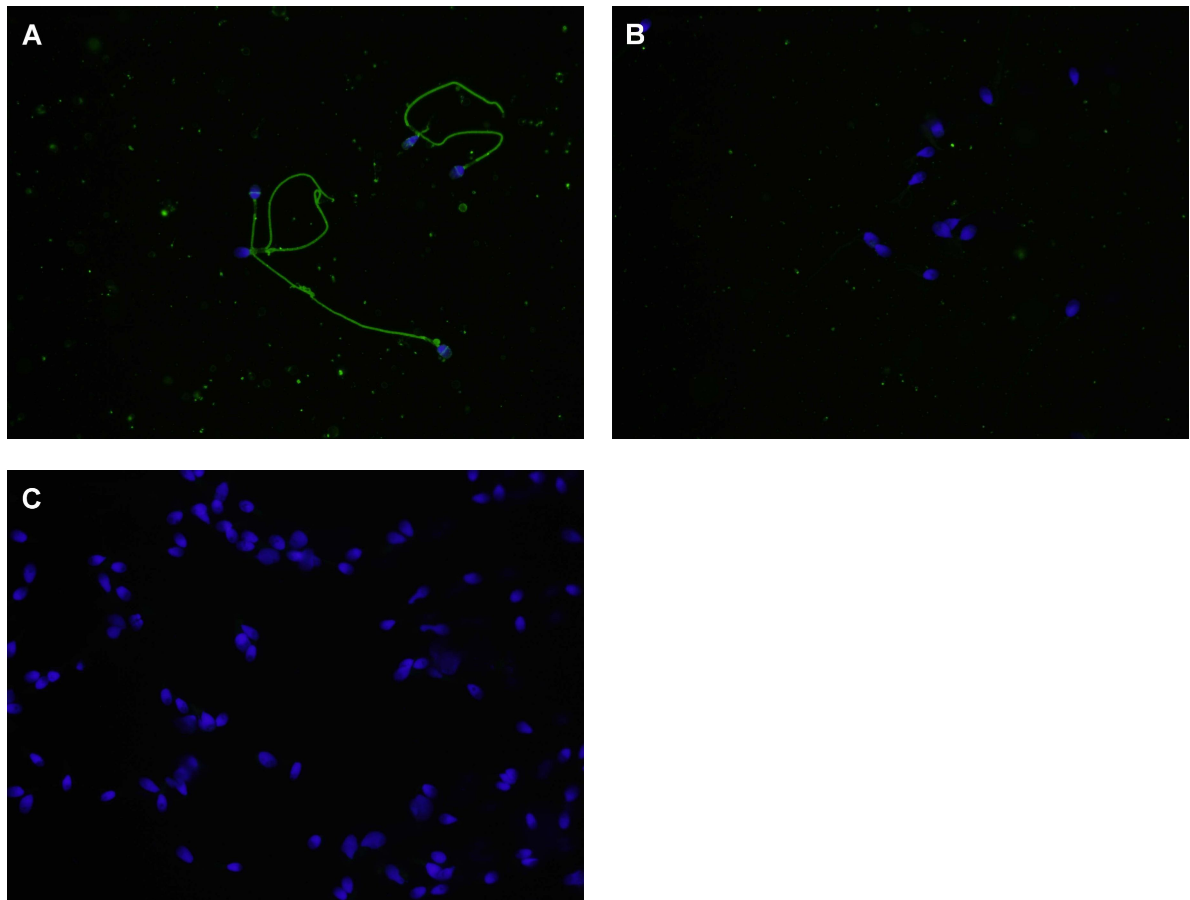


Figure 35 Leptin receptor expression at human spermatozoa. Immunofluorescence signal was detected at the connecting piece, midpiece and tail (A). Human spermatozoa were incubated with anti-human leptin receptor antibody and 2 µg leptin receptor blocking peptide revealed a specific binding of the primary antibody at the sperm tail and connecting piece (B). Human spermatozoa were incubated with secondary antibody to evaluate the unspecific binding of the secondary antibody (C). Nucleus was counterstained with DAPI (n=11).

ObR protein expression was also investigated by western blotting. SGBS adipocytes were used as positive control for ObR expression. According to the expected molecular weight of 100 kDa for the short isoform and 125 kDa for the long isoform, only the long-form was detectable in SGBS adipocytes, whereas in human spermatozoa no ObR was detectable (Figure 36).

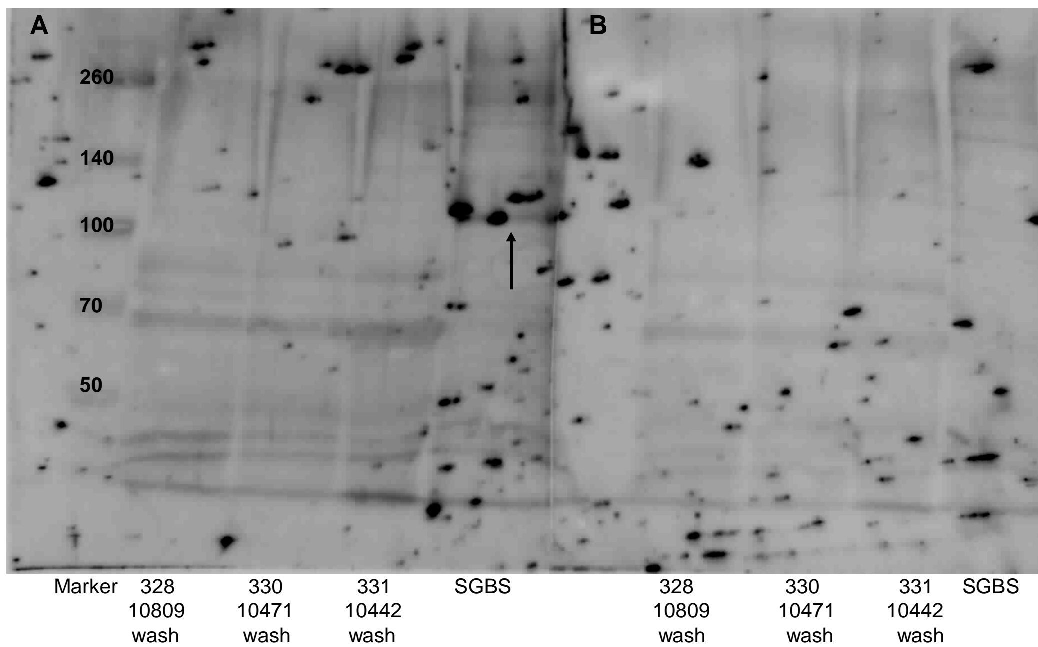


Figure 36 Leptin receptor expression after performing western blot analysis. Primary antibody was diluted 1:400 and secondary antibody was diluted 1:40000 in TTBS. SGBS cells expressed the long isoform of ObR (125 kDa; A). Primary antibody was diluted 1:400+blocking peptide and secondary antibody was diluted 1:40000 in TTBS. In human spermatozoa no ObR protein was detectable. The blocking peptide decreased the signal for the long isoform of ObR in SGBS cells (B).

The localisation of NAMPT protein at human spermatozoa was also investigated. A strong expression of NAMPT was revealed by immunofluorescence at the tail as well as at the connecting piece of human ejaculated spermatozoa, predominantly in immature spermatozoa (Figure 37, n=5, primary antibody dilution 1:50). The specifity of the antibody binding was only evaluated for the secondary antibody, as no blocking peptide was available for the primary antibody against NAMPT.

NAMPT protein expression was also investigated by western blot analysis. Firstly, non-separated spermatozoa (n=5) were checked for the presence of NAMPT, whereas HepG2 cells served as positive controls (284). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein and therefore as loading control. NAMPT protein was detectable in all spermatozoa samples (Figure 38).

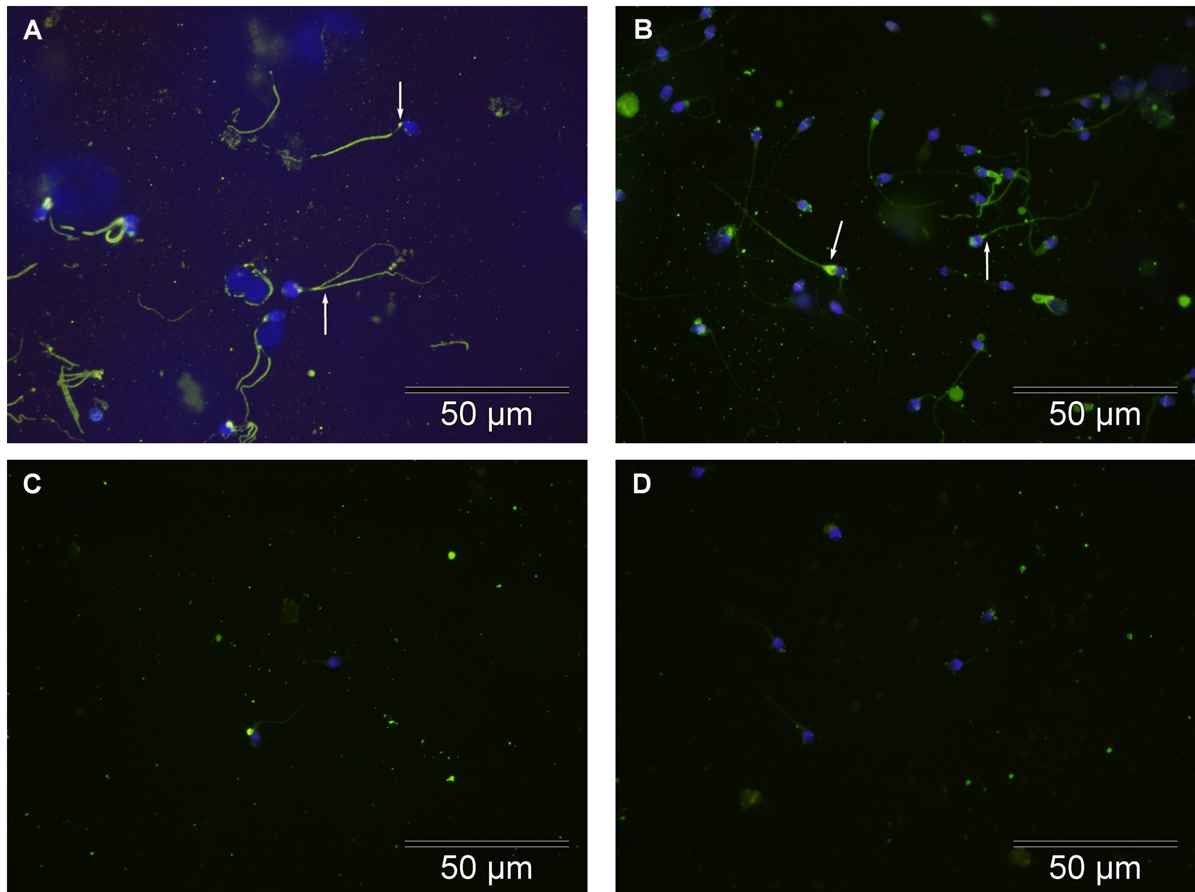


Figure 37 NAMPT expression in immature (A;B) and mature (C;D) spermatozoa. NAMPT was expressed at the connecting piece and tail of immature spermatozoa (A;B). Nucleus was counter-stained with DAPI (n=5).

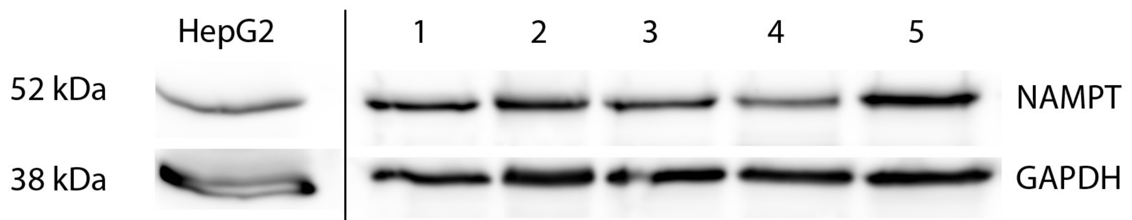


Figure 38 Western blot analysis of non-separated human spermatozoa against NAMPT protein. HepG2 cells were used as a positive control. GAPDH served as loading control. 1-5 are representative donors (n=5).

As the immunofluorescence result revealed a difference between mature and immature spermatozoa in the NAMPT expression, a western blot with these 2 fractions was performed. The NAMPT protein expression was 3.50-fold (median) higher in immature spermatozoa compared with mature spermatozoa (n=14, P=0.007, Figure 39, Figure 40).

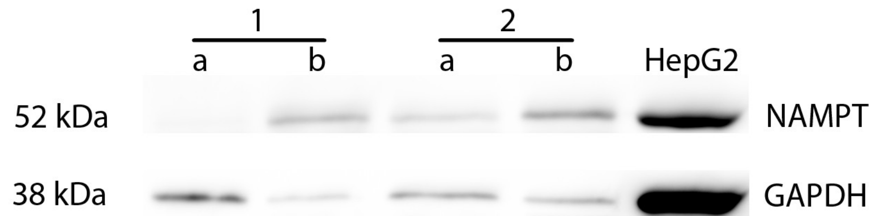


Figure 39 Representative western blot analysis against NAMPT protein of separated spermatozoa from different donors (1, 2). (a) represents mature spermatozoa (n=14) and (b) represents immature spermatozoa (n=14). GAPDH served as loading control.

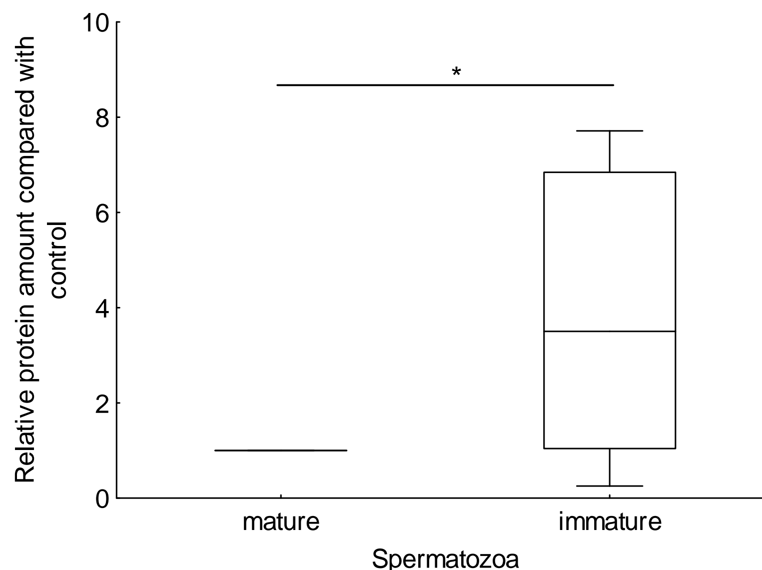


Figure 40 NAMPT protein expression in human spermatozoa depending on maturation status. Data were analysed using Mann-Whitney U-test (n=14). * P≤0.05 refers to significantly different values. — Median □ 25 %-75 % ⌶ Non-Outlier Range

4.2.4 Presence of adipokines in supernatant of human spermatozoa

After the incubation of $10x10^6$ human immature and mature spermatozoa (n=9) with EBSS as a non-capacitating medium for 3 h or 24 h, adipokine concentrations were measured in the supernatant (data not shown). In order to have enough volume available to investigate all 7 adipokine concentrations, the supernatant was 1:2 diluted.

Adiponectin protein was not detectable in the supernatant of immature and mature spermatozoa. Chemerin protein was detectable in one supernatant of 3 h incubated immature spermatozoa, whereas the other samples revealed no detectable chemerin concentrations. Vaspin was measurable in 11 out of 30 supernatants of immature and mature spermatozoa, but also in native EBSS medium. Consequently, the detection of vaspin in sperm supernatant was not valid. Leptin was detectable in the supernatant of mature spermatozoa incubated for 24 h, whereas leptin concentrations were only determined in supernatant of one donor. The resistin protein was not detectable in the supernatant of immature and mature spermatozoa, which were incubated with EBSS for 3 h or 24 h. In

two specimens of 24 h incubated immature spermatozoa, progranulin was detectable, whereas the levels were near to the detection limit. This suggests, either progranulin is secreted in very low levels or these results are false positive. In 24 out of 30 samples, NAMPT protein was detectable in supernatant of mature and immature spermatozoa incubated for 3 h or 24 h in non-capacitating medium. As NAMPT was the only adipokine detected in sperm supernatant in higher concentrations, this experiment was repeated with 10 donors and capacitating medium to ensure that spermatozoa survive 24 h of incubation.

NAMPT was detectable in the supernatant of human spermatozoa incubated with HTF+3 % BSA (n=10, Table 12). NAMPT concentrations in the supernatant of immature spermatozoa were significantly higher ($P \leq 0.05$) than in the supernatant of mature spermatozoa after 3 h (median NAMPT concentrations; 0.76 vs. 0.27 ng/ml per 10^6 sperm), 6 h (0.75 vs. 0.27 ng/ml per 10^6 sperm), 12 h (0.92 vs. 0.20 ng/ml per 10^6 sperm) and 24 h of incubation (1.62 vs. 0.42 ng/ml per 10^6 sperm), whereas the vitality of immature spermatozoa was significantly lower ($P \leq 0.05$) compared with mature spermatozoa after 3 h (77.0 vs. 85.8 %), 12 h (75.8 vs. 83.0 %) and 24 h (70.3 vs. 78.5 %) (Table 12). NAMPT levels in the supernatant of ejaculated spermatozoa did not increase significantly over time (immature: $P=0.31$, mature: $P=0.57$, data were analysed using Kruskal-Wallis test). After 3 h (median of immature and mature sperm; 0.76 and 0.27 ng/ml per 10^6 sperm), 6 h (0.75 and 0.27 ng/ml per 10^6 sperm), 12 h (0.92 and 0.20 ng/ml per 10^6 sperm) and 24 h of incubation (1.62 and 0.42 ng/ml per 10^6 sperm) NAMPT concentrations in the supernatant of immature and mature spermatozoa were significantly higher compared with the start of incubation, when NAMPT was not detected in supernatants ($P<0.001$). However, the NAMPT concentrations in the supernatant of mature and immature spermatozoa did not alter significantly after 6; 12 or 24 h of incubation compared with 3 h ($P>0.05$).

The vitality of mature spermatozoa did not decrease significantly over time (immature: $P=0.12$, mature: $P=0.58$, data were analysed using Kruskal-Wallis test). Immature spermatozoa revealed a vitality, which was stable until 12 h of incubation in capacitating medium, but the vitality was decreased significantly after 24 h compared with the start of incubation ($P=0.02$; median vitality after 24 h vs. beginning 70.3 vs. 81.3 %). Motility patterns were also determined and revealed significantly differences between immature and mature spermatozoa (Table 12).

Table 12 NAMPT secretion, vitality and motility of human spermatozoa over 24 h of incubation with HTF+3 % BSA. Data were analysed using Mann-Whitney U-test (n=10). Values are described as median (range). * P≤0.05 compared with immature spermatozoa; + P≤0.05 compared with 0 h; ‡ P≤0.05 compared with 3 h.

Incubation time		0 h	3 h	6 h	12 h	24 h
NAMPT (ng/ml)	Immature	0	0.76 $^{+}$ (0.19-8.99)	0.75 $^{+}$ (0.22-11.7)	0.92 $^{+}$ (0.26-11.6)	1.62 $^{+}$ (0.35-14.2)
	Mature	0	0.27 $^{*+}$ (0.10-1.07)	0.27 $^{*+}$ (0.08-0.77)	0.20 $^{*+}$ (0.09-0.87)	0.42 $^{*+}$ (0.12-1.44)
Vitality (%)	Immature	81.3 (34.5-86.5)	77.0 (44.0-85.0)	77.5 (30.0-85.0)	75.8 (36.0-84.0)	70.3 $^{+}$ (30.0-82.0)
	Mature	87.5 (64.5-97.5)	85.8 * (70.0-90.0)	82.0 (61.0-93.0)	83.0 * (55.0-93.0)	78.5 * (57.0-92.0)
Progressive motility (%)	Immature	52.0 (27.0-65.0)	54.8 (24.0-63.0)	48.8 (26.0-62.0)	44.8 (24.0-54.0)	41.0 (14.0-52.0)
	Mature	69.5 * (60.0-79.0)	70.3 * (55.5-84.0)	69.5 * (47.5-77.0)	67.0 * (47.5-78.0)	55.5 $^{*+‡}$ (40.5-60.0)
Total motility (%)	Immature	63.5 (33.5-74.0)	62.0 (33.0-70.5)	60.5 (31.5-70.0)	57.0 (31.0-66.0)	51.5 (18.0-63.0)
	Mature	76.3 * (62.5-84.5)	81.3 * (61.5-88.0)	75.5 * (56.0-85.0)	77.0 * (51.0-83.0)	68.5 $^{*+‡}$ (51.0-76.0)
Hyperactive (%)	Immature	10.5 (6.00-28.0)	15.0 (6.00-38.0)	18.0 (7.00-29.0)	13.5 (5.00-20.0)	8.50 (1.00-20.0)
	Mature	22.5 * (12.0-61.0)	32.5 * (8.00-64.0)	36.0 * (16.0-61.0)	28.5 * (16.0-54.0)	14.0 $^{*+‡}$ (4.00-36.0)
VAP (µm/sec)	Immature	53.5 (47.8-71.8)	60.6 (51.9-76.4)	61.8 (42.3-70.1)	47.2 ‡ (37.0-73.4)	37.9 $^{+‡}$ (32.2-56.1)
	Mature	80.8 * (64.3-109)	85.8 * (69.3-97.5)	85.4 * (52.2-97.2)	65.7 *‡ (42.7-94.4)	47.3 $^{+‡}$ (34.5-67.8)
VSL (µm/sec)	Immature	49.2 (40.7-59.1)	55.1 (46.3-66.7)	55.5 (33.9-64.8)	42.3 ‡ (27.4-67.9)	31.3 $^{+‡}$ (23.2-52.5)
	Mature	75.1 * (57.6-97.0)	78.3 * (65.1-90.8)	78.4 * (45.8-86.0)	58.8 *‡ (33.5-84.4)	40.1 $^{+‡}$ (24.4-63.6)
VCL (µm/sec)	Immature	81.8 (74.7-123)	88.6 $^{+}$ (84.6-124)	91.0 $^{+}$ (82.6-108)	76.3 ‡ (70.0-106)	69.6 $^{+‡}$ (57.2-83.4)
	Mature	112 * (89.8-169)	125 * (92.1-156)	122 * (83.5-152)	101 * (76.5-149)	80.7 $^{*+‡}$ (61.0-108)
ALH (µm)	Immature	3.45 (3.00-5.10)	3.85 (3.10-5.70)	3.90 (3.10-4.90)	3.80 (3.30-4.90)	3.50 (3.10-4.10)
	Mature	4.05 (3.30-6.30)	4.55 (3.20-6.30)	4.65 (3.40-6.20)	4.25 (3.70-5.80)	3.90 (2.90-5.10)
BCF (Hz)	Immature	31.0 (28.2-32.3)	30.9 (28.6-34.6)	29.9 (25.7-33.5)	24.9 $^{+‡}$ (23.1-31.7)	26.4 $^{+‡}$ (19.1-34.9)
	Mature	32.9 (27.0-35.1)	32.5 (29.4-36.4)	31.3 (26.4-36.8)	25.5 $^{+‡}$ (21.6-35.2)	28.0 ‡ (19.0-35.0)
STR (%)	Immature	86.0 (82.0-90.0)	87.5 (82.0-91.0)	88.0 (80.0-91.0)	84.0 (74.0-89.0)	83.0 ‡ (71.0-90.0)
	Mature	89.0 * (85.0-95.0)	90.5 * (85.0-93.0)	90.5 * (87.0-92.0)	87.5 ‡ (78.0-92.0)	84.5 $^{+‡}$ (68.0-92.0)
Lin (%)	Immature	55.5 (50.0-66.0)	58.5 (54.0-67.0)	57.5 (44.0-69.0)	53.0 ‡ (42.0-62.0)	51.0 $^{+‡}$ (39.0-59.0)
	Mature	62.0 * (58.0-71.0)	62.0 * (56.0-72.0)	61.5 (56.0-70.0)	54.5 ‡ (44.0-63-0)	51.5 $^{+‡}$ (41.0-64.0)

4.3 Effects of adipokines on human sperm function

After the presence of AdipoR1, ChemR23 and ObR at human spermatozoa was validated by immunofluorescence, the functionality of these receptors was investigated.

4.3.1 Effects on sperm motility and vitality

After incubation of swim up and washed spermatozoa (n=5) with 0; 10; 100 or 1000 ng/ml adiponectin for up to 3 h, significant differences between motility, velocities and vitality were detected. However, no significant concentration-dependent effect for motility pattern and vitality was detected (Kruskal-Wallis test, P>0.05).

The progressive motility (CASA) of swim up spermatozoa incubated with 10 ng/ml adiponectin was significantly lower compared with 100 ng/ml adiponectin incubated cells for 2 h (P=0.03, Figure 41).

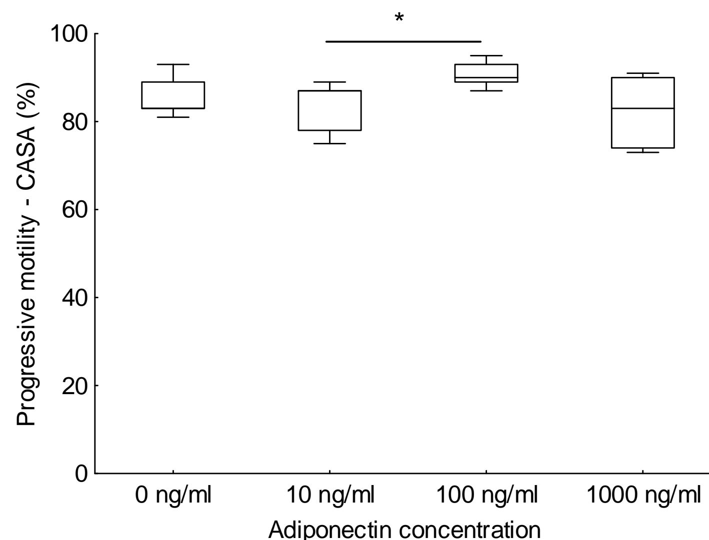


Figure 41 Progressive motility (CASA) of swim up spermatozoa after 2 h of incubation with different adiponectin concentrations. Data were analysed using Mann-Whitney U-test (n=5). * P≤0.05 refers to significantly different values. — Median ▯ 25 %-75 % ⌶ Non-Outlier Range

The progressive motility (CASA) of swim up spermatozoa incubated with 10 ng/ml adiponectin was significantly higher compared with 1000 ng/ml adiponectin incubated cells for 3 h (P=0.02, Figure 42).

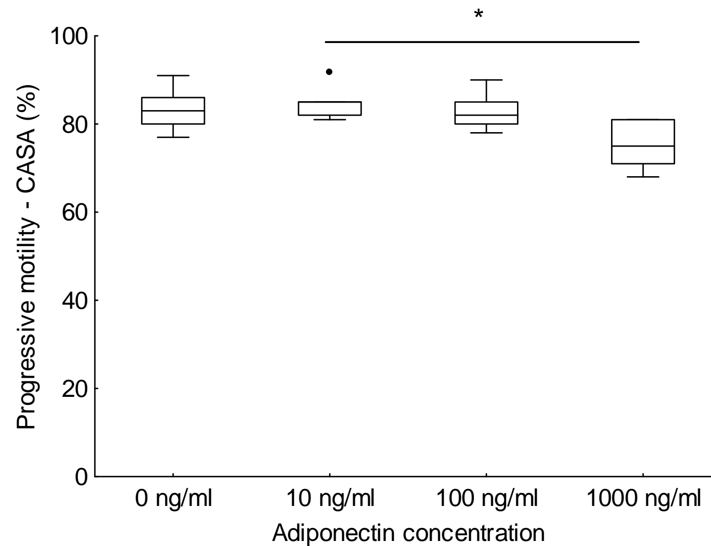


Figure 42 Progressive motility (CASA) of swim up spermatozoa after 3 h of incubation with different adiponectin concentrations. Data were analysed using Mann-Whitney U-test (n=5). * P≤0.05 refers to significantly different values. — Median ⎕ 25 %-75 % ⌶ Non-Outlier Range ● Outliers

The incubation of human spermatozoa with chemerin (n=5) did not result in a concentration-dependent significance for vitality and motility (Kruskal-Wallis test, P>0.05). After incubation of swim up spermatozoa with 10 ng/ml chemerin for 2 h, the progressive motility (CASA) was significantly higher compared with the control (Figure 43, P=0.05). In contrast, 100 or 1000 ng/ml chemerin did not alter the progressive motility (CASA) after 1; 2 and 3 h of incubation of swim up and washed spermatozoa compared with the control (P>0.05).

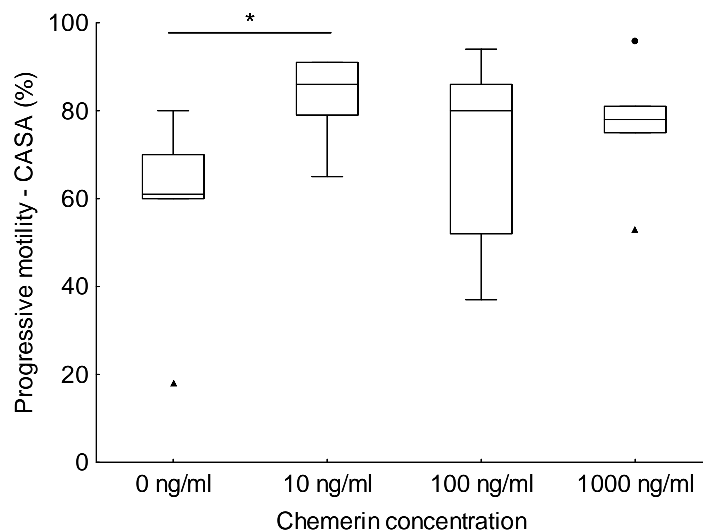


Figure 43 Progressive motility (CASA) of swim up spermatozoa after 2 h of incubation with different chemerin concentrations. Data were analysed using Mann-Whitney U-test (n=5). * P≤0.05 refers to significantly different values. — Median ⎕ 25 %-75 % ⌶ Non-Outlier Range ● Outliers ▲ Extremes

After incubation of swim up spermatozoa with 10 ng/ml chemerin for 2 h, the total motility (CASA) was significantly higher compared with the control (Figure 44, P=0.02). In con-

trast, 100 or 1000 ng/ml chemerin did not reveal a significant alteration of the total motility (CASA) after 1; 2 and 3 h of incubation of swim up and washed spermatozoa compared with control (P>0.05).

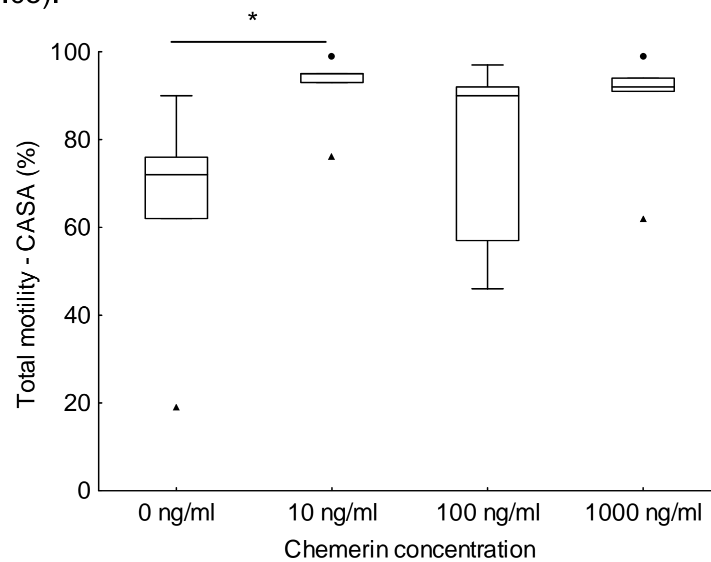


Figure 44 Total motility (CASA) of swim up spermatozoa after 2 h of incubation with different chemerin concentrations. Data were analysed using Mann-Whitney U-test (n=5). * P≤0.05 refers to significantly different values. — Median □ 25 %-75 % ⌶ Non-Outlier Range ● Outliers ▲ Extremes

After incubation of washed spermatozoa with 1000 ng/ml chemerin for 3 h, the ALH was significantly higher compared with the control (Figure 45, P=0.05). In contrast, 10 or 100 ng/ml chemerin did not alter the ALH after 1; 2 and 3 h of incubation of swim up and washed spermatozoa compared with control (P>0.05).

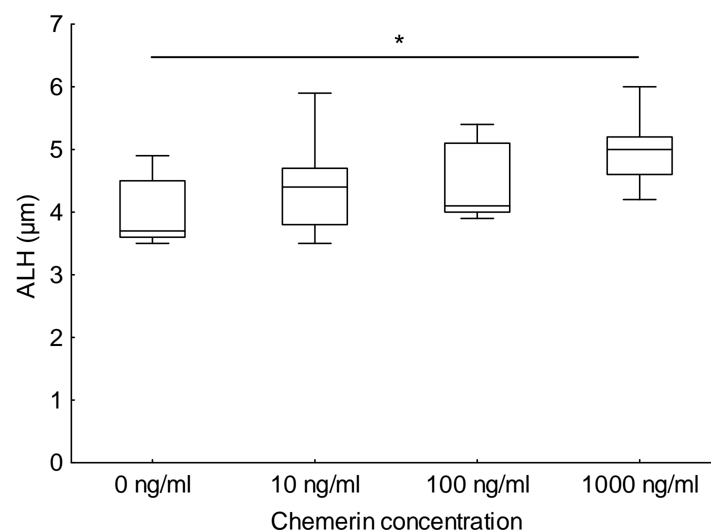


Figure 45 ALH of washed spermatozoa after 3 h of incubation with different chemerin concentrations. Data were analysed using Mann-Whitney U-test (n=5). * P≤0.05 refers to significantly different values. ALH-amplitue of lateral head displacement; — Median □ 25 %-75 % ⌶ Non-Outlier Range

Leptin incubation (n=3) did not provide a significant concentration-dependent effect for the sperm parameters motility and vitality (Kruskal-Wallis test, P>0.05). After incubation of

washed spermatozoa with 1; 10 and 100 ng/ml leptin for 1 h, the total motility (CASA) was significantly higher compared with the control (Figure 46, P=0.05).

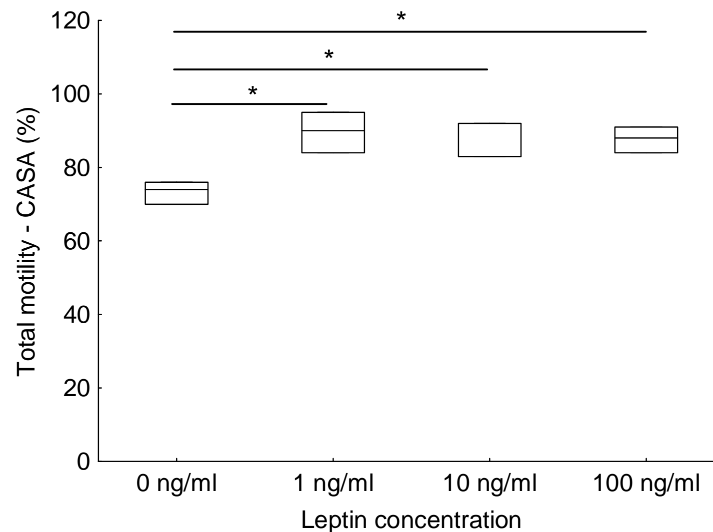


Figure 46 Total motility (CASA) of washed spermatozoa after 1 h of incubation with different leptin concentrations. Data were analysed using Mann-Whitney U-test (n=3). * P≤0.05 refers to significantly different values. — Median □ 25 %-75 % ⌶ Non-Outlier Range

Furthermore, 10 ng/ml leptin increased total motility (CASA) of washed spermatozoa after 2 h of incubation compared with control group (Figure 47, P=0.05). Washed spermatozoa incubated with 10 ng/ml leptin had a significantly higher total motility (CASA) compared with 100 ng/ml incubated spermatozoa (Figure 47, P=0.05).

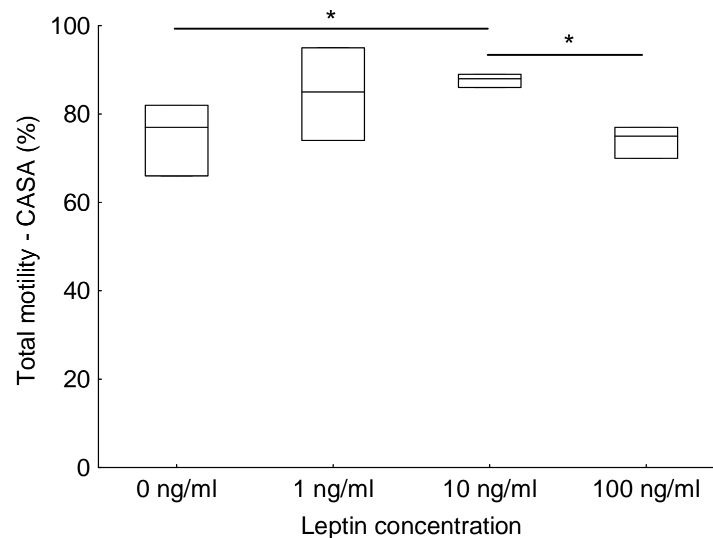


Figure 47 Total motility (CASA) of washed spermatozoa after 2 h of incubation with different leptin concentrations. Data were analysed using Mann-Whitney U-test (n=3). * P≤0.05 refers to significantly different values. — Median □ 25 %-75 % ⌶ Non-Outlier Range

In contrast, 1 or 100 ng/ml leptin did not change the total motility (CASA) after 2 h and 3 h of incubation of washed spermatozoa and after 1; 2 and 3 h of incubation of swim up spermatozoa (P>0.05).

4.3.2 Effects on capacitation

The effects of adiponectin and leptin on capacitation were determined by western blot analysis against the tyrosine phosphorylation of capacitation involved proteins (n=12). In all semen samples, the bands with a molecular weight of 43; 68 and 85 kDa were detected (Figure 48).

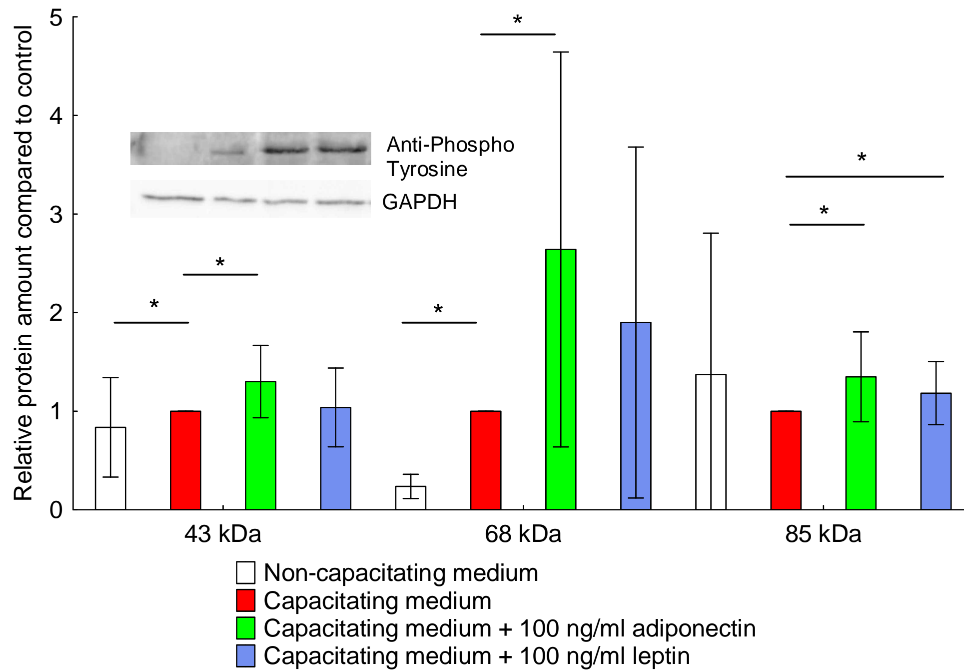


Figure 48 Effect of adiponectin and leptin on tyrosine phosphorylation of proteins involved in capacitation of human spermatozoa. Spermatozoa were incubated for 3 h with non-capacitating medium, capacitating medium, capacitating medium with 100 ng/ml adiponectin or 100 ng/ml leptin. Data were analysed using Mann-Whitney U-test (n=12). Values are described as mean±SD. GAPDH served as loading control. * P≤0.05 refers to significantly different values.

After the incubation with non-capacitating medium, the expression of tyrosine phosphorylated proteins with a molecular weight of 43 kDa (P=0.04) and 68 kDa decreased significantly (P=0.00003) compared with the incubation with capacitating medium. The expression of a 85 kDa tyrosine phosphorylated protein was not significantly different between non-capacitating and capacitating medium (P>0.05) as the SD value varied within a high range. After the incubation of spermatozoa with 100 ng/ml adiponectin in capacitating medium, the tyrosine phosphorylation increased significantly (43 kDa: P=0.006; 68 kDa: P=0.0005; 85 kDa: P=0.04) compared with capacitating medium. Moreover, after incubation of spermatozoa with 100 ng/ml leptin in capacitating medium, the tyrosine phosphorylation of the 85 kDa protein was significantly increased (P=0.04) compared with capacitating medium. However, the tyrosine phosphorylation of 43 kDa (P=0.49) and 68 kDa (P=0.17) protein did not differ between leptin incubated spermatozoa and control cells.

4.3.3 Effects on acrosome reaction

The acrosome reaction was determined in native and capacitated spermatozoa to evaluate the spontaneous acrosome reaction. In native semen (n=8) median 12.1 %, range 3.67-23.0 % of the spermatozoa underwent an acrosome reaction. After 3 h, the number of acrosome reacted spermatozoa was significantly increased in spermatozoa incubated with non-capacitating medium (HTF; 32.2 %, 21.0-53.5 %; P=0.001), capacitating medium (BSA; 31.0 %, 18.0-55.3 %; P=0.001), capacitating medium+100 ng/ml adiponectin (BSA+A; 22.0 %, 11.3-42.5 %; P=0.02) and capacitating medium+100 ng/ml leptin (BSA+L; 36.8 %, 16.0-49.7 %; P=0.002) compared with native spermatozoa (Figure 49).

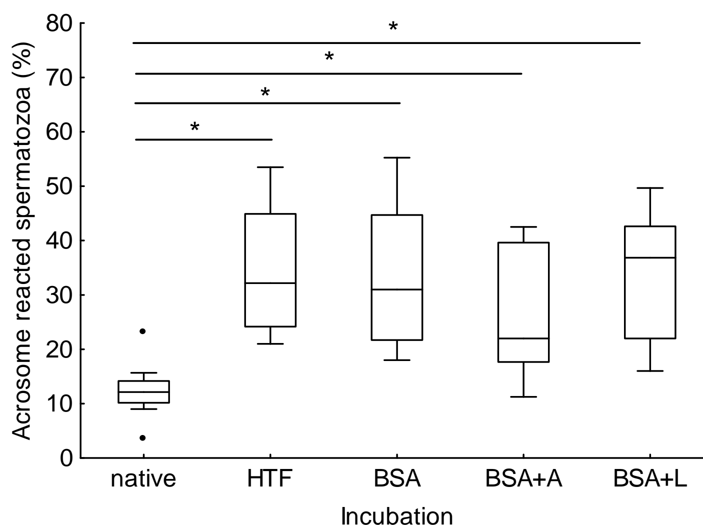


Figure 49 Acrosome reaction of native and capacitated spermatozoa determined with *Pisum sativum* agglutinin. Data were analysed using Mann-Whitney U-test (n=8). * P≤0.05 refers to significantly different values. — Median □ 25 %-75 % ⌶ Non-Outlier Range ● Outliers; native-native spermatozoa, HTF-non-capacitating medium, BSA-capacitating medium, BSA+A-capacitating medium+100 ng/ml adiponectin, BSA+L-capacitating medium+100 ng/ml leptin

Spermatozoa were incubated with different media, either without adipokines or with adipokines for 3 h. After this incubation period, spermatozoa were washed and incubated for additional 30 min with a medium containing either adipokines or no adipokines to induce the acrosome reaction.

The incubation with adiponectin (HTF HTF+A; 41.9 %, 26.0-54.0 %; P=0.29) and leptin (HTF HTF+L; 41.6 %, 27.3-53.0 %; P=0.34) for 30 min did not increase acrosome reaction in human spermatozoa compared with spermatozoa incubated with non-capacitating medium (HTF HTF; 38.8 %, 24.8-47.8 %; Figure 50). The chemical induction of the acrosome reaction by A23187 (HTF HTF+A23187) increased the number of acrosome reacted spermatozoa significantly (63.3 %, 53.0-73.7 %; P=0.01) compared with spermatozoa incubated with non-capacitating medium (HTF HTF; 38.8 %, 24.8-47.8 %), adiponectin (HTF HTF+A; 41.9 %, 26.0-54.0 %) and leptin (HTF HTF+L; 41.6 %, 27.3-53.0 %).

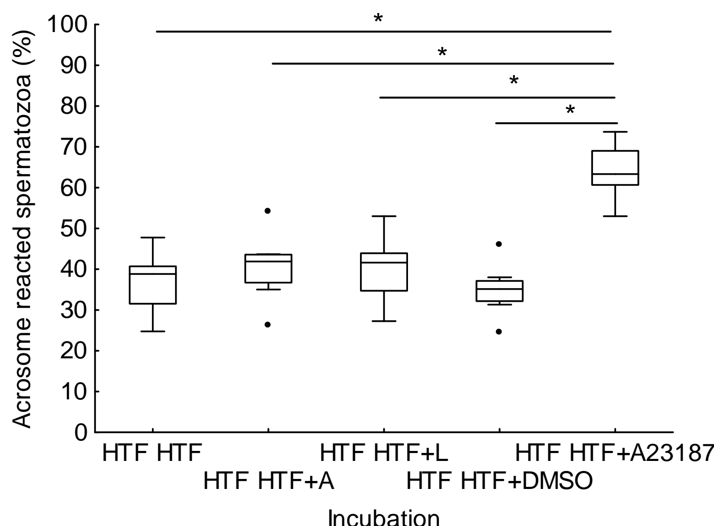


Figure 50 Acrosome reacted spermatozoa determined with *Pisum sativum* agglutinin after incubation with non-capacitating medium. Data were analysed using Mann-Whitney U-test (n=8). * P≤0.05 refers to significantly different values. — Median ⎕ 25 %-75 % ⌶ Non-Outlier Range ● Outliers; HTF-non-capacitating medium, HTF+A-non-capacitating medium+100 ng/ml adiponectin, HTF+L-non-capacitating medium+100 ng/ml leptin, HTF+DMSO-non-capacitating medium+dimethyl sulfoxide, HTF+A23187-non-capacitating medium+10 µM acrosome reaction inductor, HTF x-incubation for 3 h and x is the following incubation for 30 min.

The number of acrosome reacted spermatozoa incubated with capacitating medium (BSA BSA; 22.0 %, 14.0-38.3 %) for 3 h and additional 30 min was not significantly (P>0.05) different from spermatozoa incubated with adiponectin (BSA BSA+A; 26.3 %, 10.5-45.0 %), leptin (BSA BSA+L; 29.3 %, 13.5-35.0 %) or A23187 incubated spermatozoa (BSA BSA A23187; 36.5 %, 12.0-45.0 %) (Figure 51).

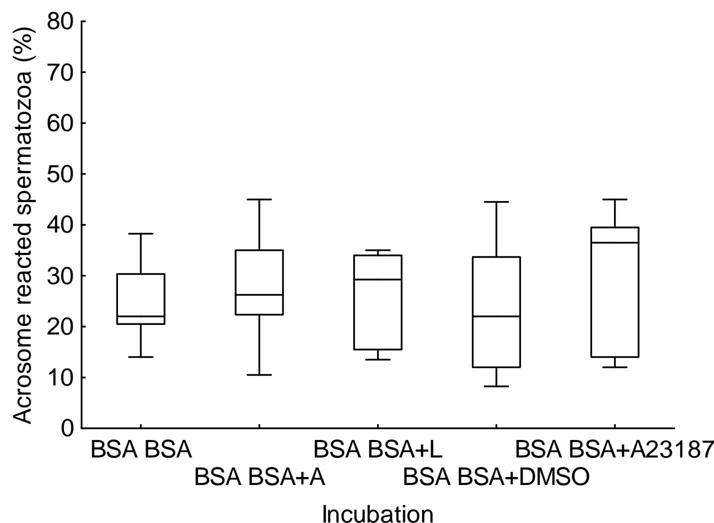


Figure 51 Acrosome reacted spermatozoa determined with *Pisum sativum* agglutinin after incubation with capacitating medium. Data were analysed using Mann-Whitney U-test (n=8). — Median ⎕ 25 %-75 % ⌶ Non-Outlier Range, BSA-capacitating medium, BSA+A-capacitating medium+100 ng/ml adiponectin, BSA+L-capacitating medium+100 ng/ml leptin, BSA+DMSO-capacitating medium+dimethyl sulfoxide, BSA+A23187-capacitating medium+10 µM acrosome reaction inductor, BSA x-incubation for 3 h and x is the following incubation for 30 min.

The number of acrosome reacted spermatozoa incubated with adiponectin for 3.5 h in capacitating medium (BSA+A BSA+A; 18.3 %, 9.67-41.7 %) did not differ significantly (P>0.05) from spermatozoa incubated with A23187 (BSA+A BSA+A23187; 27.0 %, 8.67-49.8 %, Figure 52 A).

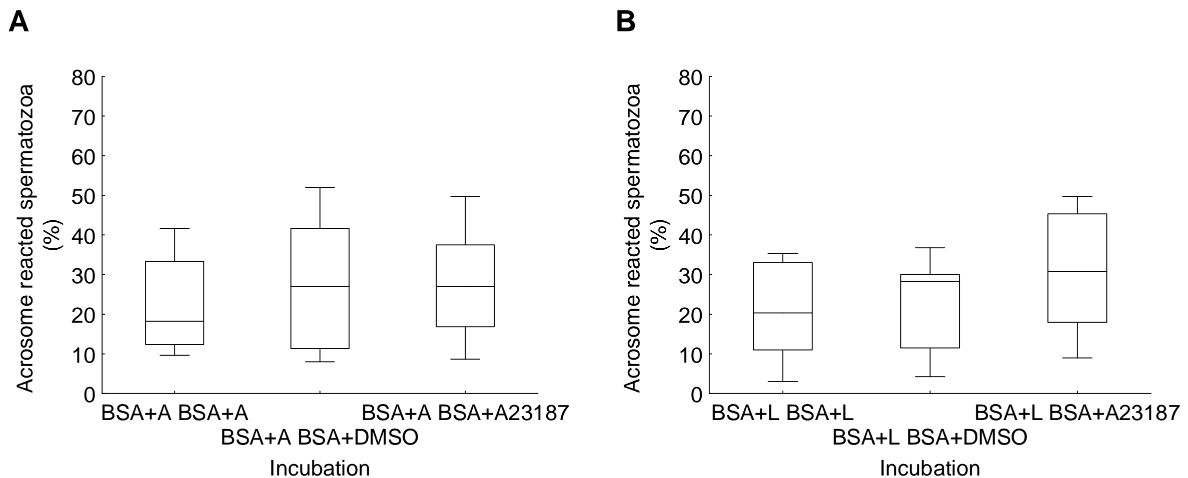


Figure 52 Acrosome reacted spermatozoa determined with *Pisum sativum* agglutinin after incubation with (A) capacitating medium+100 ng/ml adiponectin or (B) capacitating medium+100 ng/ml leptin. Data were analysed using Mann-Whitney U-test (n=8). — Median □ 25 %-75 % ⌶ Non-Outlier Range, BSA+A-capacitating medium+100 ng/ml adiponectin, BSA+DMSO-capacitating medium+dimethyl sulfoxide, BSA+A23187-capacitating medium+10 µM acrosome reaction inductor, BSA+A x-incubation for 3 h and x is the following incubation for 30 min, BSA+L-capacitating medium+100 ng/ml leptin, BSA+DMSO-capacitating medium+dimethyl sulfoxide, BSA+A23187-capacitating medium+10 µM acrosome reaction inductor, BSA+L x-incubation for 3 h and x is the following incubation for 30 min

Spermatozoa incubated with leptin in capacitating medium for 3.5 h (BSA+L BSA+L; 20.3 %, 3.00-35.3 %) did not reveal a significantly (P>0.05) different number of acrosome reacted spermatozoa compared with counterparts incubated with A23187 (BSA+L BSA+A23187; 30.8 %, 9.00-49.8 %, Figure 52 B).

4.3.4 Effects on apoptosis

Spermatozoa were incubated for 3 h with EBSS (non-capacitating medium) or EBSS+3 % BSA (capacitating medium) and additional aliquots of sperm solution were incubated with 1000 ng/ml adiponectin, 1000 ng/ml chemerin or 100 ng/ml leptin (n=3).

The incubation of spermatozoa with EBSS and adiponectin significantly (P=0.05) increased the percentages of spermatozoa with active caspase 3&7 compared with control (Table 13). Moreover, adiponectin incubation resulted in significantly (P=0.05) higher percentages of non-vital cells compared with control. Moreover, the incubation with chemerin significantly (P=0.05) increased the levels of caspase 3&7 positive and non-vital spermatozoa compared with control. Leptin incubation resulted in significantly (P=0.05) higher percentages of caspase3&7 positive spermatozoa compared with control.

In capacitated spermatozoa (EBSS+3 % BSA), the incubation with adiponectin, chemerin and leptin did not result in significantly different percentages of caspase 3&7 activity compared with control (P>0.05).

Capacitating spermatozoa had significantly (P=0.05) higher percentages of caspase 3&7 positive cells compared with non-capacitating spermatozoa.

Table 13 The influence of adipokine incubation on activity of caspase 3&7 in human spermatozoa. Data were analysed using Mann-Whitney U-test (n=3). Values are described as median (range). * P≤0.05 compared with EBSS; + P≤0.05 compared with the respective non-capacitating medium. EBSS-non-capcitating medium; EBSS+3 % BSA-capacitating medium

	Caspase negative, non-vital (%)	Caspase positive, non-vital (%)	Caspase negative, vital (%)	Caspase positive, vital (%)
EBSS	28.2 (28.2-29.5)	0.01 (0.00-0.01)	71.2 (70.3-71.2)	0.57 (0.10-0.57)
EBSS+1000 ng/ml adiponectin	32.1 * (30.6-32.1)	5.79 * (3.86-5.79)	51.4 * (51.4-60.2)	10.8 * (5.39-10.8)
EBSS+1000 ng/ml chemerin	34.9 * (33.4-34.9)	4.00 * (1.99-4.00)	52.4 * (52.4-60.6)	8.70 * (4.01-8.70)
EBSS+100 ng/ml leptin	27.8 * (25.9-27.8)	8.36 * (7.24-8.36)	48.9 * (48.9-54.2)	14.9 * (12.6-14.9)
EBSS+3 % BSA	30.1 + (29.7-33.4)	9.16 + (9.07-12.2)	49.3 + (42.2-50.4)	10.8 + (8.26-15.6)
EBSS+3 % BSA+1000 ng/ml adiponectin	34.0 + (32.3-35.8)	8.02 + (7.88-9.76)	50.0 (45.6-52.3)	7.52 (6.21-10.7)
EBSS+3 % BSA+1000 ng/ml chemerin	34.5 (32.9-37.3)	8.44 + (8.40-11.0)	48.1 + (44.7-51.1)	7.62 (6.14-9.76)
EBSS+3 % BSA+100 ng/ml leptin	32.3 + (31.8-35.7)	9.53 + (9.47-12.7)	47.0 + (38.8-48.1)	10.5 (7.78-16.2)

4.4 Inhibition of NAMPT enzyme activity

4.4.1 Effect of NAMPT inhibition on sperm motility

Spermatozoa were incubated with two different media: EBSS, a nutrient-deprived medium used for short-term incubation and HTF, a more nutrient-rich medium used for the long-term incubation. In a pilot study using spermatozoa of five donors, no significant impact on motility or vitality was detected after incubation with the NAMPT inhibitor FK866 in EBSS+3 % BSA after 3; 6; 12 or 24 h compared with controls (P>0.05, Table 14).

However, 24 h of incubation with 10 nM FK866 tended to result in 19-fold higher progressive (P=0.4) and 17-fold higher total motility (P=0.2) as well as an 1.4-fold increased number of viable cells (P=0.05) compared with controls. The motility of 100 nM FK866 incubated spermatozoa was 12.5-fold higher compared with controls (P=0.4), whereas the vitality was 1.4-fold higher (P=0.05). Overall, no dose-dependent effect was detectable for progressive motility (P=0.89), total motility (P=0.78), and vitality (P=0.23, data were analysed using Kruskal-Wallis test).

Table 14 The effect of the NAMPT inhibitor FK866 on semen parameters after short-term incubation of human spermatozoa with EBSS+3 % BSA. Data were analysed using Mann-Whitney U-test (n=5). Values are described as median (range). * P≤0.05 compared with 0 h; + P≤0.05 compared with 3 h.

FK866 conc.		0 nM	0.1 nM	1 nM	10 nM	100 nM
Progressive motility (%)	0 h	51.0 (40.0-63.0)	51.0 (40.0-63.0)	51.0 (40.0-63.0)	51.0 (40.0-63.0)	51.0 (40.0-63.0)
	3 h	54.0 (48.0-68.0)	50.0 (39.0-61.0)	49.0 (41.0-65.0)	48.0 (47.0-68.0)	50.5 (48.0-66.0)
	6 h	52.0 (42.0-64.5)	54.0 (43.0-58.0)	47.0 (41.0-60.0)	49.0 (35.0-62.0)	52.0 (45.0-60.0)
	12 h	46.0 (40.0-62.0)	48.0 (33.0-51.0)	49.0 (42.0-63.0)	42.5 (29.0-49.5)	43.0 (23.5-53.0)
	24 h	1.00 *+ (0.00-21.0)	6.00 *+ (0.00-25.0)	9.00 (0.00-59.0)	19.0 *+ (0.00-48.0)	12.5 *+ (0.00-38.0)
Total motility (%)	0 h	58.5 (45.0-73.0)	58.5 (45.0-73.0)	58.5 (45.0-73.0)	58.5 (45.0-73.0)	58.5 (45.0-73.0)
	3 h	65.0 (53.0-79.0)	58.5 (51.0-69.5)	63.0 (53.5-77.5)	61.5 (58.0-81.0)	57.5 (57.0-76.0)
	6 h	62.5 (47.0-74.0)	64.0 (54.0-78.0)	57.0 (51.0-73.0)	63.0 (49.5-75.0)	62.0 (49.0-70.0)
	12 h	57.0 (49.0-72.5)	57.0 (48.0-65.0)	58.5 (54.0-77.0)	47.5 (45.0-64.0)	48.0 (32.5-70.0)
	24 h	2.00 *+ (0.00-29.0)	18.0 *+ (0.00-29.0)	15.0 + (1.00-70.5)	34.0 *+ (0.00-61.0)	20.0 *+ (0.00-51.0)
Vitality (%)	0 h	70.0 (62.5-83.0)	70.0 (62.5-83.0)	70.0 (62.5-83.0)	70.0 (62.5-83.0)	70.0 (62.5-83.0)
	3 h	70.5 (59.5-82.0)	68.0 (60.0-86.0)	67.0 (61.0-81.0)	67.0 (56.5-82.0)	71.0 (56.5-79.0)
	6 h	66.5 (50.5-72.0)	64.0 (52.0-79.0)	64.0 (57.0-77.0)	68.0 (50.0-72.0)	65.0 (50.0-78.0)
	12 h	63.0 (51.0-73.0)	66.0 (53.0-72.0)	63.0 (55.0-81.0)	63.0 (53.0-70.5)	61.0 (53.0-74.0)
	24 h	42.5 *+ (2.00-53.5)	39.0 *+ (2.00-62.0)	63.0 (1.00-75.0)	60.0 * (40.5-69.0)	58.0 (39.5-73.0)

The incubation of human spermatozoa with FK866 in HTF+3 % BSA had no significant impact on motility or vitality over 24; 48 or 72 h compared with controls (P>0.05, Table 15). An incubation time of 72 h with 10 nM FK866 resulted in a tendency towards a 1.24-fold higher total motility (P=0.83) compared with controls. However, a dose-dependency was not detectable for progressive motility (P=0.60), total motility (P=0.72), vitality (P=0.91, data were analysed using Kruskal-Wallis test).

Table 15 The effect of the NAMPT inhibitor FK866 on semen parameters after long-term incubation of human spermatozoa with HTF+3 % BSA. Data were analysed using Mann-Whitney U-test (n=5). Values are described as median (range). * P≤0.05 compared with 0 h; + P≤0.05 compared with 24 h.

FK866 conc.		0 nM	0.1 nM	1 nM	10 nM	100 nM
Progressive motility (%)	0 h	43.0 (29.0-52.0)	43.0 (29.0-52.0)	43.0 (29.0-52.0)	43.0 (29.0-52.0)	43.0 (29.0-52.0)
	24 h	36.5 (0.00-66.0)	30.0 (0.00-52.5)	36.5 (1.00-50.5)	32.5 (0.50-54.5)	28.0 (12.0-54.0)
	48 h	10.0 * (0.00-37.7)	14.5 * (0.00-40.0)	8.00 * (0.00-28.5)	8.00 * (0.00-42.0)	11.5 * (0.00-30.0)
	72 h	12.0 * (0.00-28.5)	0.50 * (0.00-13.5)	4.00 *+ (0.00-14.5)	12.5 * (0.00-19.0)	3.00 *+ (0.00-14.0)
Total motility (%)	0 h	50.0 (36.5-59.5)	50.0 (36.5-59.5)	50.0 (36.5-59.5)	50.0 (36.5-59.5)	50.0 (36.5-59.5)
	24 h	38.5 (1.00-69.0)	33.0 (0.00-61.0)	38.5 (1.00-59.5)	35.0 (1.00-62.5)	33.0 (12.0-63.0)
	48 h	15.0 * (0.00-45.5)	27.5 * (0.00-49.0)	11.5 * (0.00-44.0)	15.5 * (0.00-50.0)	17.5 * (0.00-42.0)
	72 h	17.0 * (0.00-47.5)	6.50 * (0.00-27.0)	11.5 * (0.00-28.5)	21.0 * (0.00-38.5)	5.00 *+ (0.00-25.5)
Vitality (%)	0 h	65.0 (47.0-69.0)	65.0 (47.0-69.0)	65.0 (47.0-69.0)	65.0 (47.0-69.0)	65.0 (47.0-69.0)
	24 h	60.0 (23.5-71.5)	53.0 (20.5-69.0)	58.5 (32.5-74.0)	58.0 (31.0-69.0)	55.0 (23.0-65.0)
	48 h	60.5 (0.00-68.0)	50.0 (0.00-58.0)	56.0 (3.00-66.0)	54.0 (1.5-65.0)	50.5 (5.00-66.5)
	72 h	51.0 (1.00-63.0)	46.5 * (0.50-53.0)	45.0 * (1.00-61.0)	46.0 * (0.00-62.5)	46.0 * (2.30-58.5)

4.4.2 Effect of NAMPT inhibition on apoptosis signalling in spermatozoa

To investigate the apoptosis signalling cascade in the presence of the NAMPT inhibitor, a 24 h time course was chosen, because this period appeared to be appropriate due to the motility experiments. Spermatozoa were incubated with different FK866 concentrations for 24 h in EBSS+3 % BSA. No significant differences were found between FK866-incubated spermatozoa and controls with regard to motility values, mitochondrial membrane potential ($\Delta\Psi_m$), activity of caspase 3&7 and vitality (P>0.05, Table 16).

Due to the high variations in caspase activity and $\Delta\Psi_m$ between the donors, differences between FK866 and solvent control in spermatozoa from every single donor (Δ values) were determined. Incubation of spermatozoa with 1 nM FK866 resulted in significantly decreased Δ values of amplitude of lateral head displacement (ALH) compared with the control (Δ 1 nM FK866 -0.1 vs. Δ control 0, Figure 53 A). Furthermore, significantly more spermatozoa were viable after the incubation with 0.1 nM or 1 nM FK866 compared with the control (P<0.001; PI positive cells Δ 0.1 nM -5.98 or Δ 1 nM -3.05 vs. Δ control 0, Figure 53 B).

Table 16 Effect of the NAMPT inhibitor FK866 on caspase activity and mitochondrial membrane potential ($\Delta\Psi_m$) after incubation of human spermatozoa with EBSS+3 % BSA for 24 h. Data were analysed using Mann-Whitney U-test (n=11). Values are described as median (range).

FK866 concentration	0 nM	0.1 nM	1 nM	10 nM	100 nM
Vitality (Eosin %)	35.0 (0.00-45.5)	47.0 (3.00-79.0)	42.5 (2.50-85.0)	45.5 (0.00-42.5)	56.5 (0.50-82.0)
Progressive motility (%)	11.5 (0.00-50.5)	16.0 (0.00-36.5)	20.5 (0.00-47.0)	7.00 (0.00-50.5)	12.5 (0.00-39.0)
Total motility (%)	14.0 (0.00-86.5)	26.5 (0.00-44.0)	24.0 (0.00-52.5)	12.5 (0.00-79.0)	15.0 (0.00-50.5)
Progressive motility (CASA %)	3.00 (0.00-28.0)	16.0 (0.00-37.0)	9.00 (0.00-27.0)	6.00 (0.00-32.0)	7.00 (0.00-45.0)
Total motility (CASA %)	4.00 (0.00-38.0)	27.0 (0.00-47.0)	13.0 (0.00-34.0)	12.0 (0.00-46.0)	15.0 (0.00-5.00)
VAP (µm/sec)	35.2 (0.00-55.1)	39.6 (0.00-56.8)	27.8 (0.00-56.7)	32.6 (0.00-60.0)	27.2 (0.00-54.0)
VSL (µm/sec)	30.3 (0.00-50.2)	33.7 (0.00-51.9)	24.1 (0.00-52.8)	27.7 (0.00-54.5)	22.7 (0.00-49.2)
VCL (µm/sec)	64.9 (0.00-89.3)	67.3 (0.00-85.8)	45.4 (0.00-87.9)	56.6 (0.00-90.1)	62.7 (0.00-82.5)
ALH (µm)	3.10 (0.00-4.50)	3.10 (0.00-4.30)	2.40 (0.00-4.10)	3.30 (0.00-5.70)	3.20 (0.00-3.70)
BCF (Hz)	22.6 (0.00-30.1)	23.1 (0.00-28.5)	24.3 (0.00-29.2)	21.2 (0.00-29.2)	24.1 (0.00-29.0)
STR (%)	50.0 (0.00-89.0)	87.0 (0.00-95.0)	85.0 (0.00-91.0)	84.0 (0.00-90.0)	80.0 (0.00-89.0)
Lin (%)	55.5 (0.00-59.0)	55.0 (0.00-63.0)	55.0 (0.00-74.0)	48.0 (0.00-61.0)	44.0 (0.00-60.0)
Caspase 3&7 activity (%)	42.7 (14.5-94.5)	32.0 (10.1-95.2)	38.4 (21.8-95.6)	36.3 (22.0-93.6)	30.6 (14.5-94.0)
Vitality (PI %)	54.7 (27.3-78.3)	44.3 (25.7-72.7)	53.0 (26.2-73.1)	54.5 (27.9-73.5)	47.5 (28.5-75.2)
$\Delta\Psi_m$ (%)	40.0 (2.54-80.7)	41.6 (1.95-73.4)	45.9 (1.95-64.7)	41.7 (0.99-67.2)	34.1 (0.54-67.7)

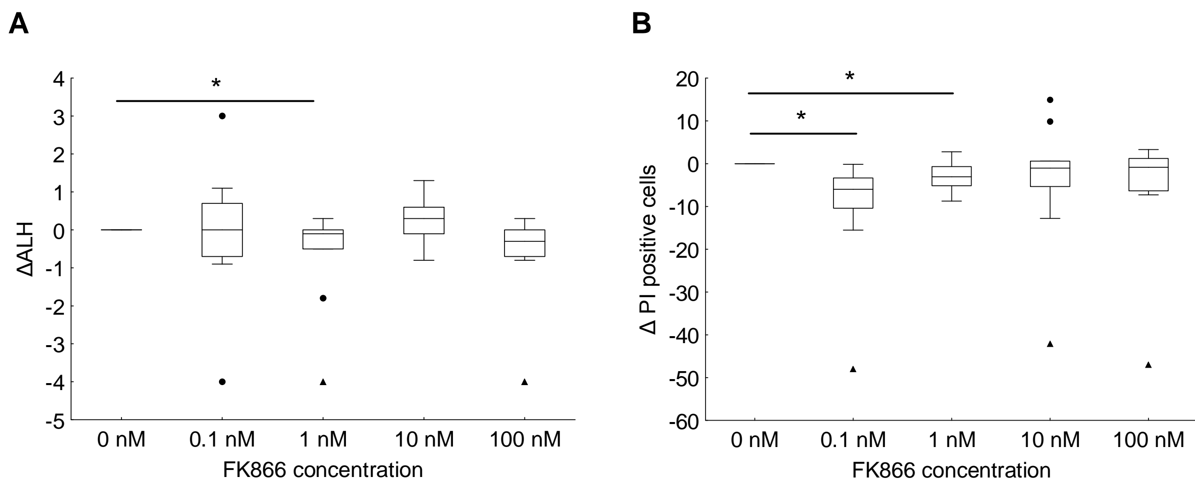


Figure 53 Δ values of amplitude of lateral head displacement (ALH) (A) and propidium iodide (PI) positive cells (B) after incubation of human spermatozoa with FK866 for a time period of 24 h with EBSS+3 % BSA. Data were analysed using Mann-Whitney U-test (n=11). * refers to significantly different values (P≤0.05) compared with the control (0 nM FK866 was set 0). — Median ☐ 25 %-75 % ⌶ Non-Outlier Range ● Outliers ▲ Extremes

4.4.3 Effect of NAMPT inhibition on capacitation

The effect of FK866 on the capacitation was determined by quantifying the tyrosine phosphorylation of sperm proteins after incubation in capacitating media for 24 h or 72 h using western blot analysis. GAPDH served as loading control. The immunoreactive bands had the following molecular weights: 24 kDa, 25/26 kDa, 35 kDa, 40 kDa, 48 kDa, 68 kDa, 90 kDa, and 105 kDa. The band densities that correlated with relative protein concentrations, diverged over a wide range between the individual specimens. This could be explained by the use of non-separated spermatozoa, representing a broad range of developmental stages. Therefore, no significant effect of FK866 on tyrosine phosphorylation after incubation for 24 h in EBSS+3 % BSA was detectable (n=11; Table 17, Figure 54; P>0.05).

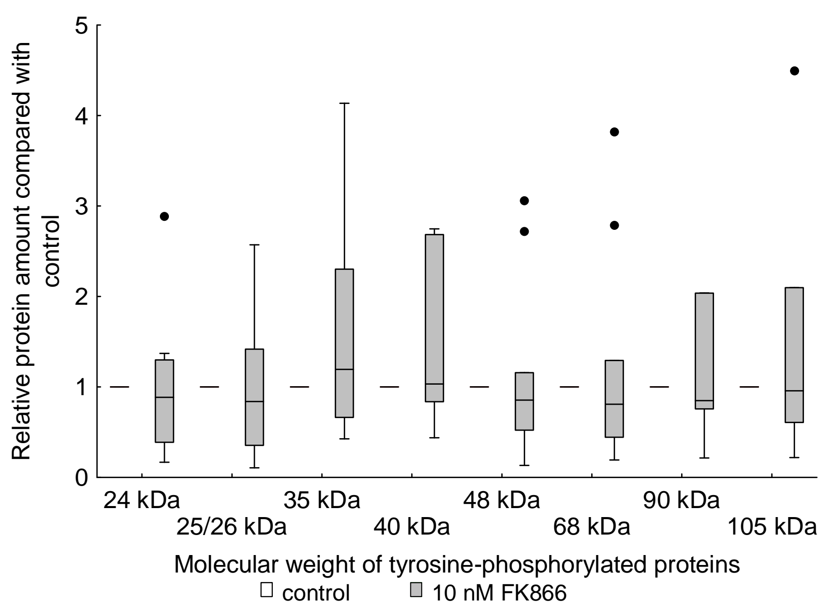


Figure 54 Effect of FK866 on the tyrosine phosphorylation status of capacitation involved proteins after incubation of human spermatozoa for 24 h. Data were analysed using Mann-Whitney U-test (n=11). GAPDH served as loading control. — Median □25 %-75 % ⌶ Non-Outlier Range ● Outliers

In addition, an incubation for 72 h using HTF+3 % BSA also showed no significant impact of FK866 on tyrosine phosphorylation (n=7; Table 17; P>0.05). Considering values from single donors, the tyrosine phosphorylation decreased in 62 % of the cases, only 12 % of the samples showed no change and 26 % showed an increase of their tyrosine phosphorylation over a time course of 24 h. The tyrosine phosphorylation revealed no differences after 72 h between 0 nM and 10 nM FK866 considering the single donor values.

Table 17 Effect of the NAMPT inhibitor FK866 on tyrosine phosphorylation status of capacitation involved proteins after incubation of human spermatozoa with EBSS+3 % BSA for 24 h and with HTF+3 % BSA for 72 h. Data were analysed using Mann-Whitney U-test (n=11 for 24 h, n=7 for 72 h). Values are described as median (range). Relative protein amounts of phosphorylated proteins are represented in arbitrary units (a.u.).

Capacitation involved proteins	24 h		72 h	
FK866 concentration	**0 nM**	**10 nM**	**0 nM**	**10 nM**
24 kDa (a.u.)	1.00 (1.00-1.00)	0.89 (0.17-9.90)	1.00 (1.00-1.00)	1.01 (0.54-1.67)
25/26 kDa (a.u.)	1.00 (1.00-1.00)	0.84 (0.11-5.77)	1.00 (1.00-1.00)	1.11 (0.59-2.00)
35 kDa (a.u.)	1.00 (1.00-1.00)	1.19 (0.43-4.14)	1.00 (1.00-1.00)	1.02 (0.49-1.77)
40 kDa (a.u.)	1.00 (1.00-1.00)	1.03 (0.44-2.75)	1.00 (1.00-1.00)	0.98 (0.44-1.68)
48 kDa (a.u.)	1.00 (1.00-1.00)	0.85 (0.13-3.06)	1.00 (1.00-1.00)	1.01 (0.44-1.99)
68 kDa (a.u.)	1.00 (1.00-1.00)	0.81 (0.19-3.82)	1.00 (1.00-1.00)	1.11 (0.21-3.24)
90 kDa (a.u.)	1.00 (1.00-1.00)	0.85 (0.22-2.04)	1.00 (1.00-1.00)	1.01 (0.44-1.78)
105 kDa (a.u.)	1.00 (1.00-1.00)	0.96 (0.22-4.49)	1.00 (1.00-1.00)	1.05 (0.61-3.12)

4.5 Difference of NAD^+ levels between spermatozoa subpopulations

As the detection kit was not validated for spermatozoa, a protocol and the appropriate sperm count were established. During the validation process, different spermatozoa counts were checked for their NAD^+ concentrations. Sperm counts ≤20x10^6 did not result in detectable NAD^+ concentrations, whereas NAD^+ was measurable in specimens with sperm counts >30-100x10^6 (data not shown). Hence, 30x10^6 spermatozoa were considered to be the appropriate sperm count to detect intracellular NAD^+, as higher sperm numbers are rarely achieved by patients. The validation was finalised by a precision experiment with three different donors (Table 18). The semen was divided into six aliquots for the precision measurement.

Table 18 Precision of NAD^+ level determination in human spermatozoa of 3 different donors. MV–mean value; SD–standard deviation; CV–coefficient of variation

	Donor 1		Donor 2		Donor 3	
	nM NAD/ mg protein	**nM NAD/ 10^6 sperm**	**nM NAD/ mg protein**	**nM NAD/ 10^6 sperm**	**nM NAD/ mg protein**	**nM NAD/ 10^6 sperm**
MV	0.73	37.8	0.67	34.6	1.06	53.1
SD	0.09	4.34	0.13	7.58	0.24	4.32
CV (%)	12.0	11.5	19.4	21.9	22.4	8.14

Two donors received a coefficient of variation (CV) below 15 % and were therefore acceptable for the validation for the NAD^+ levels in 10^6 sperm. The second donor did exceed

a CV of 20 %. This concludes that the precision measurement was only valid for two out of three donors.

Additionally, NAD^+ levels were determined in immature and mature spermatozoa (n=6). The NAD^+ concentrations were not significantly different between immature and mature spermatozoa after normalisation of NAD^+ levels to the protein amount (mean±SD; 1.28±0.45 nM NAD/mg protein vs.1.41±0.31 nM NAD/mg protein, P=0.42, Figure 55 A). NAD^+ levels were significantly higher in immature spermatozoa compared with mature counterparts, if NAD^+ levels were normalised to 10^6 spermatozoa (69.0±17.3 nM NAD/10^6 sperm vs. 50.1±8.94 nM NAD/10^6 sperm, P=0.04, Figure 55 B).

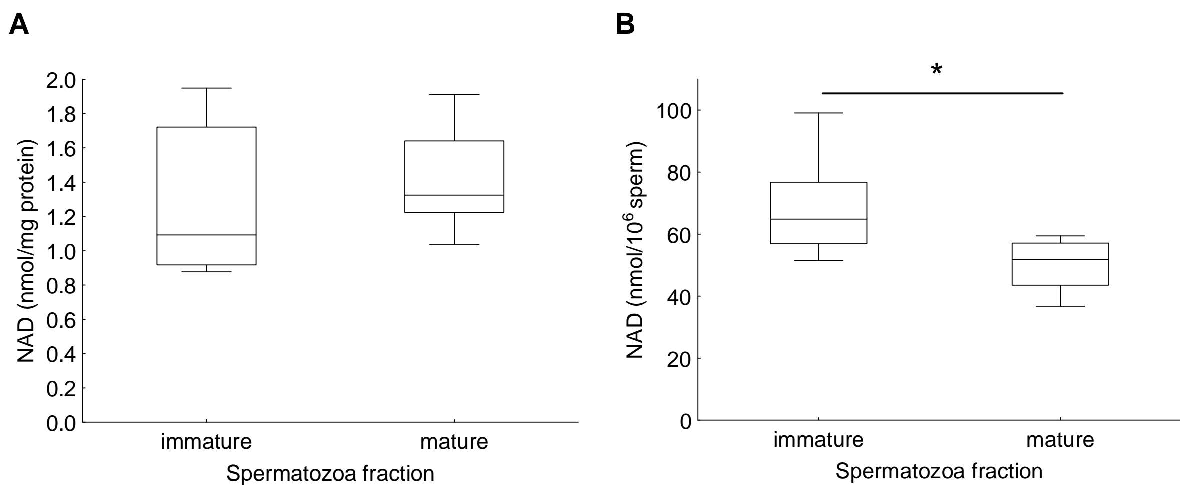


Figure 55 NAD^+ levels of mature and immature spermatozoa. Data were analysed using Mann-Whitney U-test (n=6). NAD^+ levels (nmol/mg protein) were not significantly different between these sperm fractions (A). NAD^+ levels (nmol/10^6 sperm) were significantly different between these sperm fractions (B). * P≤0.05 refers to significantly different values. — Median □ 25 %-75 % ⌶ Non-Outlier Range

Considering these results, protein concentrations/10^6 sperm were compared between these fractions. The protein concentrations/10^6 sperm tended to increase in immature spermatozoa compared with mature counterparts (n=6, median protein amount immature vs. mature spermatozoa; 6.16 µg protein/10^6 sperm vs. 3.56 µg protein/10^6 sperm, P=0.054).

4.6 Detection of autophagy in male reproductive tract

4.6.1 Detection of autophagy-related proteins in human spermatozoa and testis

The process of autophagy in human male reproductive tract was determined by the detection of LC3B and Beclin-1 protein by immunofluorescence and western blot analysis. A fluorescence signal against LC3B detected as typical spots or punctae was observed at the head of non-separated human ejaculated spermatozoa. The distribution and the strength of the punctae varied between the spermatozoa (Figure 56 A-F).

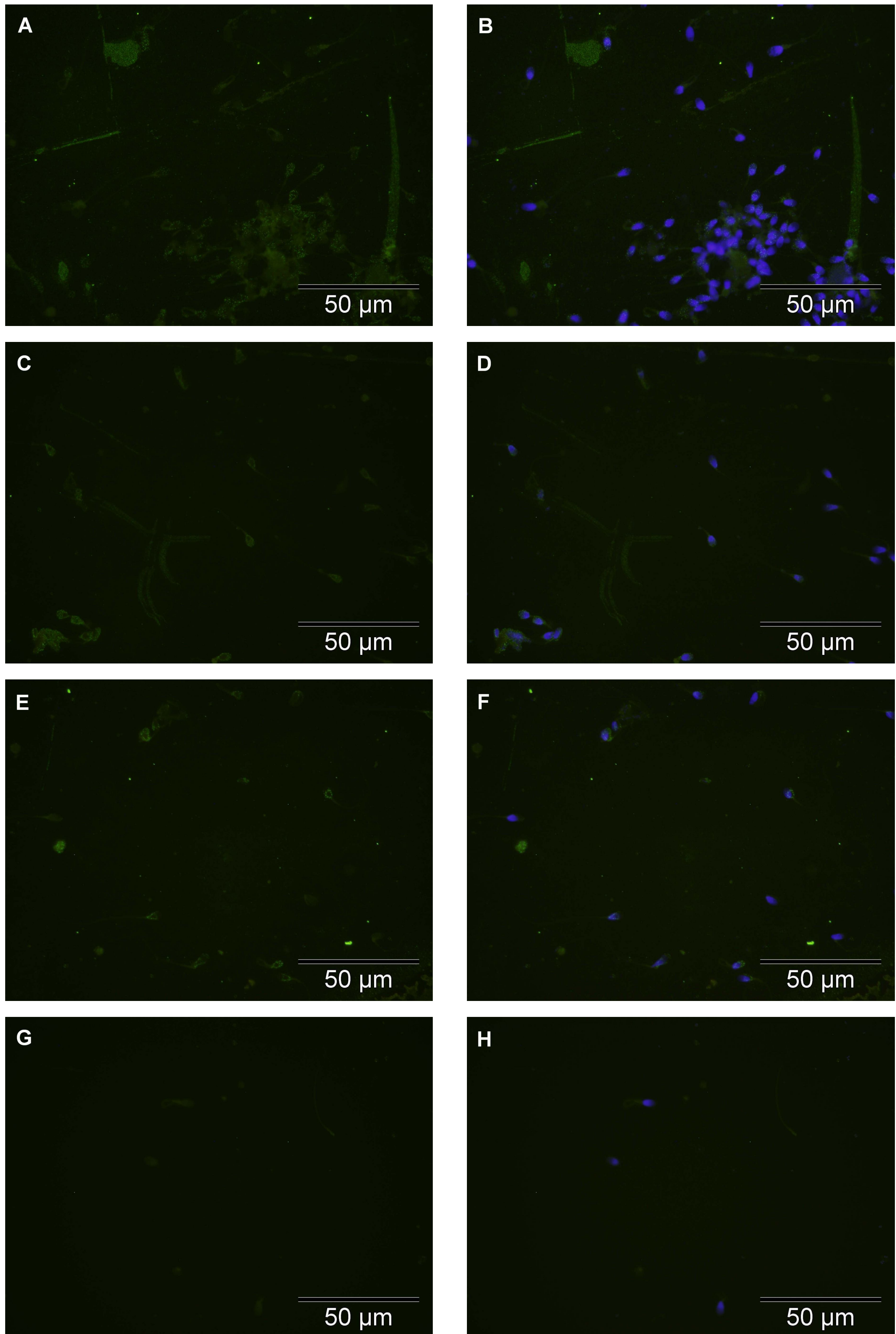


Figure 56 Detection of LC3B by immunofluorescence at human spermatozoa. Human spermatozoa were incubated with primary antibody against LC3B (A-F) and without LC3B antibody (G;H). Nucleus was counterstained with DAPI (B;D;F;H).

In several spermatozoa, LC3B was detected at the acrosomal region, in the whole head or only in the postacrosomal region of the head. Furthermore, no immunoreaction was detected at the midpiece or at the tail of the spermatozoa. In order to locate spermatozoa, the nucleus was counterstained with DAPI. As a control for unspecific binding of the secondary antibody, the spermatozoa were not incubated with the primary antibody against LC3B (Figure 56 G;H). LC3-I and LC3-II proteins can be discriminated by their difference in mobility on gel electrophoresis according to their molecular weight (immunoreactive bands at 14 kDa and 16 kDa). LC3B-I, LC3B-II and Beclin-1 protein (immunoreactive band at 60 kDa) were detected in non-separated spermatozoa extracts (n=4, Figure 57). HepG2 cells incubated with 0.25 mM palmitate for 4 h (kindly provided by M. Penke, Department of Women and Child Health, Hospital for Children and Adolescents, Center for Pediatric Research Leipzig, University Hospital Leipzig) served as positive control as already tested by Tan *et al.* (361). GAPDH served as loading control, determining differences in blotted protein amounts.

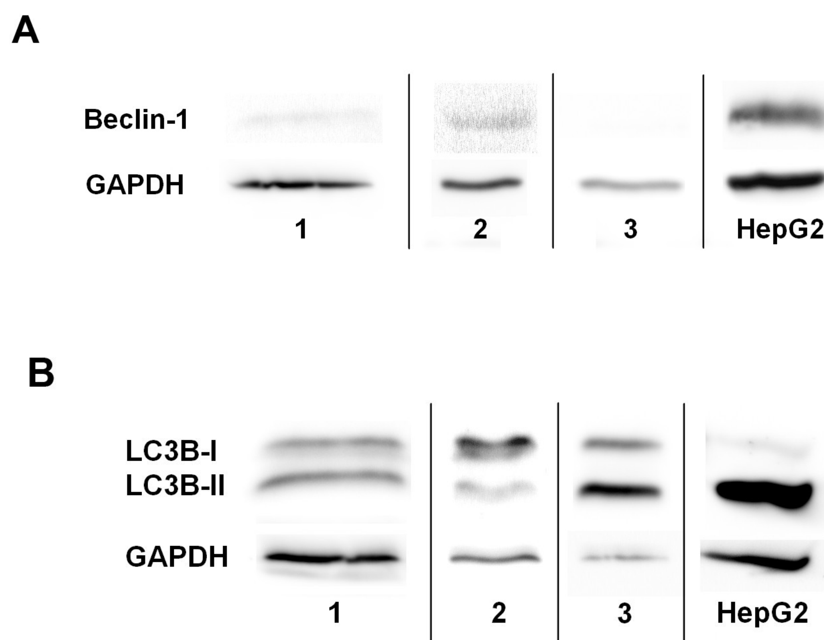


Figure 57 Representative western blots of non-separated human spermatozoa (n=4) against Beclin-1 (60 kDa; A), LC3B-I (16 kDa; B) and LC3B-II (14 kDa; B). GAPDH served as loading control and palmitate incubated HepG2 cells served as positive control. 1-3 represent three different donors within three different gels.

In addition, human testis (n=5) was tested for the presence of autophagy marker LC3B. It was detected in human testis of patients with a Johnsen score >8 to ensure, that no spermatogenetic arrest was occurring. LC3B was detected in the cytoplasm of Sertoli cells and spermatocytes, as well as in the nucleus of several spermatocytes and spermatids (Figure 58). The control tissue samples were incubated only with the secondary antibody and no unspecific binding was detectable (data not shown).

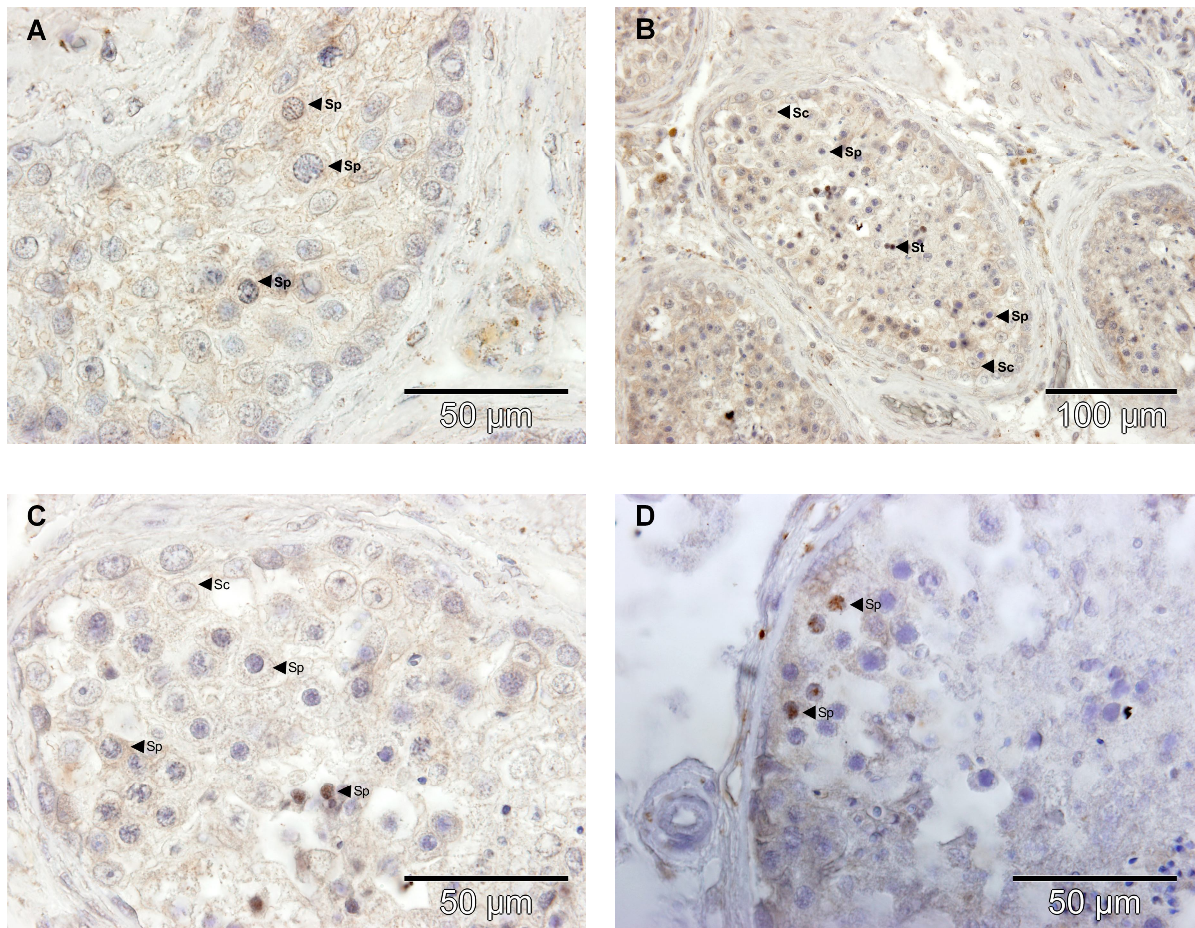


Figure 58 Immunohistochemical detection of LC3B in human testis. Specimens were treated with target retrieval solution (n=5). Testis sections were counterstained with hemalum (A-D). LC3B was expressed in the cytoplasm of spermatocytes (Sp, A;B;C), Sertoli cells (Sc, B;C) and in the nucleus of Sp (B;C;D) and spermatids (St, B).

4.6.2 LC3B is expressed in mature and immature spermatozoa

Native semen was separated into an immature and a mature fraction and the relative protein expression of LC3B-I and LC3B-II was investigated.

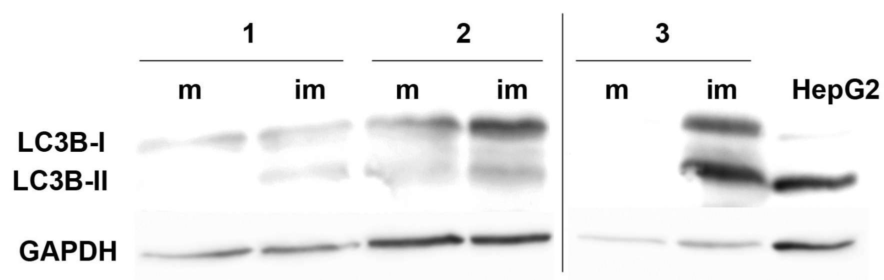


Figure 59 Representative western blots of mature (m) and immature (im) human spermatozoa against LC3B-I (16 kDa) and LC3B-II (14 kDa) protein (n=9). GAPDH served as loading control and palmitate incubated HepG2 cells served as positive control.

Considering the western blot data (n=9, Figure 59), mature spermatozoa had significantly lower protein levels of both LC3B forms than the immature counterparts (LC3B-I: 1±0 vs. 8.00±11.8 a.u., P=0.005; LC3B-II: 1±0 vs. 64.1±133 a.u., P=0.01, Figure 60).

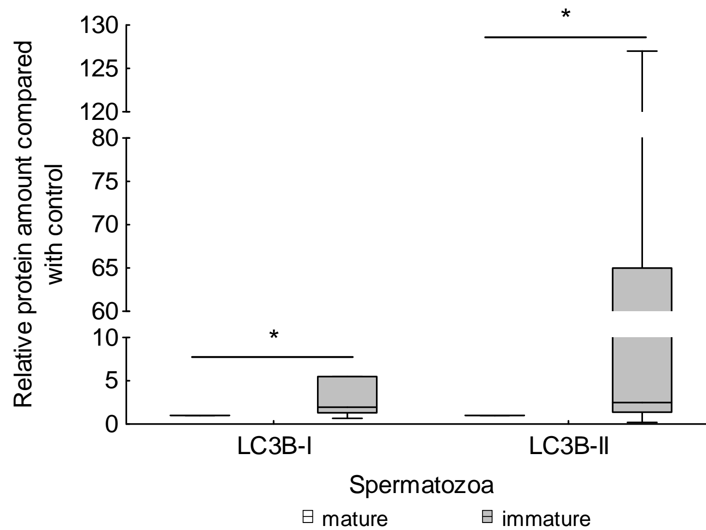
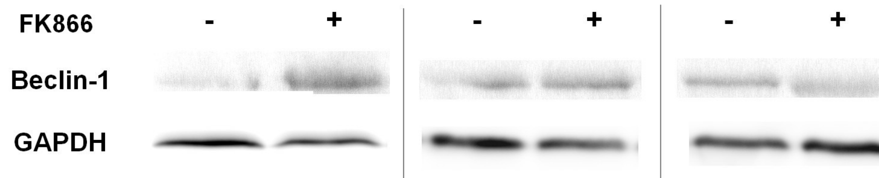


Figure 60 Relative LC3B protein expression of immature and mature spermatozoa. * P≤0.05 refers to significantly different LC3B protein expression using Mann-Whitney U-test (n=9). — Median □ 25 %-75 % ⌶ Non-Outlier Range

4.6.3 Impact of the NAMPT inhibitor FK866 on autophagy markers, motility pattern and vitality of human ejaculated spermatozoa

The bands for Beclin-1, LC3B-I, and LC3B-II were also detectable after incubation with FK866 for 24 h (n=16; Figure 61 A;B) and 72 h (n=16: Figure 62 A;B).

A



B

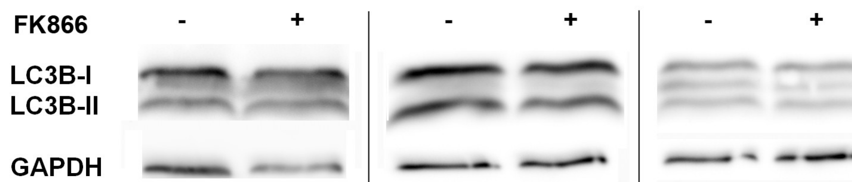


Figure 61 Representative western blots of non-separated human spermatozoa against Beclin-1 (A) and LC3B (B) after incubation with 10 nM FK866 for 24 h in EBSS+3 % BSA (n=16). GAPDH served as loading control.

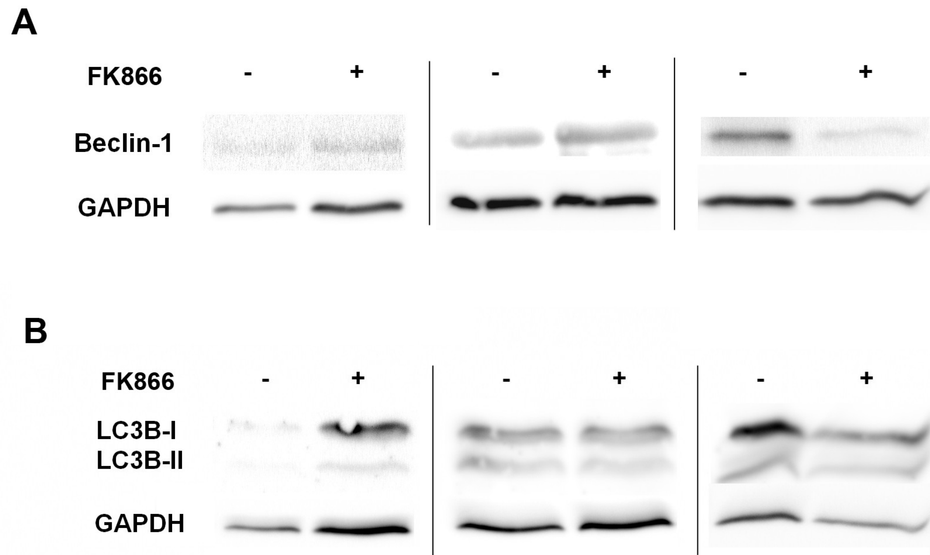


Figure 62 Representative western blots of non-separated human spermatozoa against Beclin-1 (A) and LC3B (B) after incubation with 10 nM FK866 for 72 h in HTF+3 % BSA (n=16). GAPDH served as loading control.

However, relative protein expression of Beclin-1, LC3B-I and LC3B-II did not differ significantly after 24 h of incubation with 10 nM FK866 compared with the control (n=16, P>0.05, Table 19). Additionally, no significant differences between the control and the FK866 incubated spermatozoa were detectable for vitality and motility patterns after 24 h (P>0.05). Due to the high variations of motility patterns, differences between FK866 and control were determined from every single donor (Δ values).

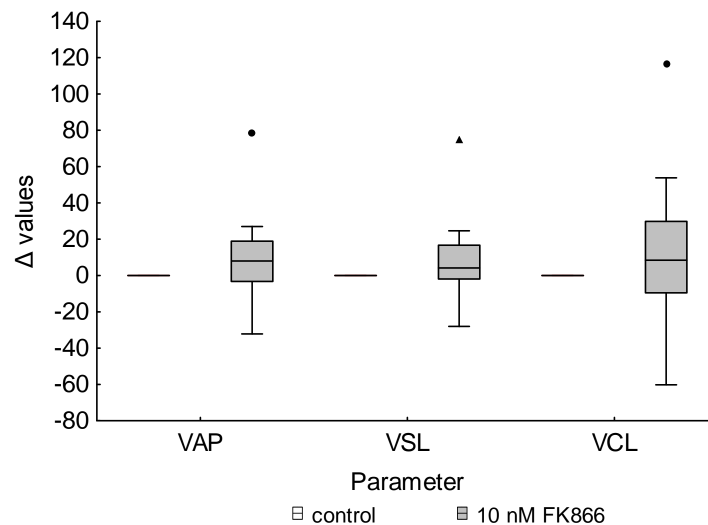


Figure 63 Δ VAP, Δ VSL and Δ VCL of human spermatozoa after an incubation period of 24 h with 10 nM FK866 in EBSS+3 % BSA compared to the control. The control was set 0. Data were analysed using Mann-Whitney U-test (n=16). — Median ☐ 25 %-75 % ⌶ Non-Outlier Range ● Outliers ▲ Extremes

Δ VAP (Δ 10 nM FK866 8.00 vs. Δ control 0), Δ VSL (Δ 10 nM FK866 4.15 vs. Δ control 0), Δ VCL (Δ 10 nM FK866 8.45 vs. Δ control 0) tended to be higher in 10 nM FK866 incubated spermatozoa compared to the control (P=0.05, Figure 63, Table 19).

Table 19 Effect of the NAMPT inhibitor FK866 on vitality, motility and Beclin-1 and LC3B protein expression of human spermatozoa after incubation with EBSS+3 % BSA for 24 h and HTF+3 % BSA for 72 h. Data were analysed using Mann-Whitney U-test (n=16 for 24 h, n=15 for 72 h). Values are described as median (range). Relative protein amounts of phosphorylated proteins are represented in arbitrary units (a.u.).

	24 h		72 h	
FK866 concentration	**0 nM**	**10 nM**	**0 nM**	**10 nM**
Vitality (Eosin %)	30.3 (3.00-81.5)	32.8 (3.50-74.5)	41.0 (3.00-67.0)	40.3 (8.00-58.0)
Progressive motility (%)	0.75 (0.00-59.5)	1.25 (0.00-69.0)	0.50 (0.00-9.50)	0.50 (0.00-9.00)
Total motility (%)	1.25 (0.00-67.5)	3.25 (0.00-71.5)	1.50 (0.00-23.0)	1.50 (0.00-21.0)
Progressive motility (CASA %)	1.00 (0.00-73.0)	1.50 (0.00-61.0)	9.00 (0.00-4.00)	0.00 (0.00-3.00)
Total motility (CASA %)	3.00 (0.00-79.0)	3.00 (0.00-70.0)	13.0 (0.00-6.00)	0.50 (0.00-5.00)
VAP (µm/sec)	26.2 (0.00-66.0)	30.4 (0.00-78.5)	27.8 (0.00-36.5)	0.00 (0.00-49.9)
VSL (µm/sec)	21.7 (0.00-61.5)	23.4 (0.00-75.2)	24.1 (0.00-28.0)	0.00 (0.00-36.8)
VCL (µm/sec)	47.7 (0.00-95.6)	56.5 (0.00-116)	45.4 (0.00-59.8)	0.00 (0.00-86.7)
ALH (µm)	2.25 (0.00-4.80)	3.65 (0.00-7.90)	2.40 (0.00-3.30)	0.00 (0.00-4.00)
BCF (Hz)	15.7 (0.00-29.6)	18.4 (0.00-30.8)	24.3 (0.00-36.0)	0.00 (0.00-35.7)
STR (%)	76.0 (0.00-99.0)	79.5 (0.00-96.0)	85.0 (0.00-98.0)	0.00 (0.00-95.0)
Lin (%)	43.0 (0.00-79.0)	45.5 (0.00-71.0)	55.0 (0.00-97.0)	0.00 (0.00-61.0)
Beclin-1 (a.u.)	1.00 (1.00-1.00)	1.14 (0.16-11.1)	1.00 (1.00-1.00)	1.53 (0.47-4.48)
LC3B-I (a.u.)	1.00 (1.00-1.00)	1.26 (0.50-88.7)	1.00 (1.00-1.00)	1.04 (0.19-307)
LC3B-II (a.u.)	1.00 (1.00-1.00)	1.00 (0.20-76.2)	1.00 (1.00-1.00)	1.32 (0.07-12.0)

Considering single specimens of different donors, Beclin-1, LC3B-I and LC3B-II protein expression increased in 40 % of the samples due to FK866 incubation for 24 h, whereas others demonstrated no change (25 %) or a decrease (35 %).

Additionally, non-separated spermatozoa were incubated for 72 h. However, after incubation of spermatozoa with 10 nM FK866 for 72 h the protein expression of Beclin-1, LC3B-I and LC3B-II, the vitality and motility patterns did not differ significantly compared with the control (n=16, P>0.05, Table 19). Furthermore, the Δ values for motility patterns were not significantly different (P>0.05). However, 56 % of the sperm samples of individual donors showed higher protein expression for Beclin-1, LC3B-I and LC3B-II after incubation for

72 h with 10 nM FK866 compared with the control, whereas in 11 % of the spermatozoa specimens no influence or in 33 % a decrease of the protein expression was detected.

4.6.4 The effect of chloroquine on the autophagic flux in human spermatozoa

Human ejaculated spermatozoa (n=8) were incubated with 10 µM chloroquine (CQ). To determine the toxic effect of CQ, spermatozoa were incubated for different time periods in capacitating medium. As sperm cells were incubated with EBSS+3 % BSA for 4 h and 24 h, no significant effect of CQ was detectable on motility or vitality (P>0.05, Table 20).

Table 20 Effect of chloroquine on vitality, motility and LC3B protein expression after incubation of human spermatozoa with EBSS+3 % BSA for 4 h and 24 h. Data were analysed using Mann-Whitney U-test (n=8). Values are described as median (range). Relative protein amounts of phosphorylated proteins are represented in arbitrary units (a.u.). ND-no data

	0 h	4 h		24 h	
Chloroquine concentration		**0 µM**	**10 µM**	**0 µM**	**10 µM**
Vitality (Eosin %)	74.5 (58.5-82.0)	71.5 (48.0-83.0)	67.5 (35.5-84.5)	62.5 (24.0-77.5)	57.3 (29.0-75.0)
Progressive motility (CASA %)	57.0 (25.0-74.0)	65.0 (22.0-82.0)	63.5 (21.0-79.0)	28.5 (6.00-59.0)	30.5 (7.00-49.0)
Total motility (CASA %)	88.5 (50.0-94.0)	77.5 (25.0-93.0)	80.0 (27.0-92.0)	37.0 (8.00-79.0)	39.0 (8.00-65.0)
VAP (µm/sec)	58.6 (52.5-75.1)	67.4 (60.8-88.2)	72.2 (61.8-88.5)	55.6 (34.9-66.0)	61.8 (39.5-70.2)
VSL (µm/sec)	53.9 (44.1-71.3)	63.1 (56.6-84.5)	67.5 (56.1-84.7)	50.7 (28.2-61.9)	55.9 (35.9-66.1)
VCL (µm/sec)	87.0 (78.1-119)	96.8 (84.1-115)	102 (85.0-125)	81.7 (58.8-95.7)	91.4 (61.2-109)
ALH (µm)	3.65 (3.00-5.10)	3.50 (2.80-4.30)	3.90 (3.00-4.80)	3.60 (2.80-4.20)	3.55 (2.80-4.70)
BCF (Hz)	28.8 (21.6-35.3)	31.5 (29.6-35.4)	31.6 (27.0-35.1)	28.6 (21.7-34.5)	28.5 (21.2-34.3)
STR (%)	88.0 (83.0-93.0)	91.0 (89.0-94.0)	90.5 (87.0-95.0)	89.0 (79.0-94.0)	88.5 (87.0-93.0)
Lin (%)	58.5 (51.0-72.0)	65.5 (61.0-75.0)	63.5 (59.0-75.0)	59.5 (48.0-69.0)	58.0 (56.0-70.0)
LC3B-I (a.u.)	ND	ND	ND	1.00 (1.00-1.00)	0.74 (0.17-4.32)
LC3B-II (a.u.)	ND	ND	ND	1.00 (1.00-1.00)	0.82 (0.03-3.80)

LC3B-I and LC3B-II protein expression did not differ significantly between the control and 10 µM CQ incubated spermatozoa, but a tendency of lower protein levels was detectable after CQ incubation for 24 h (P=0.18). If Δ values were compared between the control and intervention group, the Δ VCL (Δ 10 µM CQ 3.40 vs. Δ control 0) was significantly higher after 4 h in 10 µM CQ incubated spermatozoa compared with the control cells (P=0.007, Figure 64), whereas this effect was not detectable after 24 h.

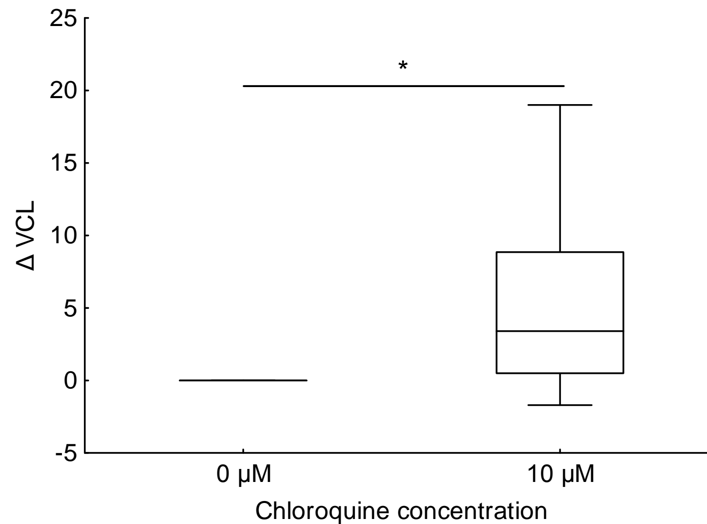


Figure 64 Δ VCL of non-separated human spermatozoa after incubation with chloroquine for a time period of 4 h (EBSS+3 % BSA, control was set to 0). Data were analysed using Mann-Whitney U-test (n=8). * P≤0.05 refers to significantly different values. — Median □ 25 %-75 % ⌶ Non-Outlier Range

Ejaculated spermatozoa were also incubated with HTF+3 % BSA for different time periods (4; 24; 48 and 72 h, Table 21). No significant differences in vitality and motility were found between the control and CQ incubated spermatozoa after 4; 24; 48 and 72 h. The LC3B-I and LC3B-II protein amounts did not differ significantly between the groups after a time period of 72 h (P>0.05).

The Δ values were determined according to the high variations of the vitality and motility patterns of each donor. After 4 h of incubation with 10 µM CQ, the Δ vitality was significantly lower compared with the control (P=0.04, Figure 65).

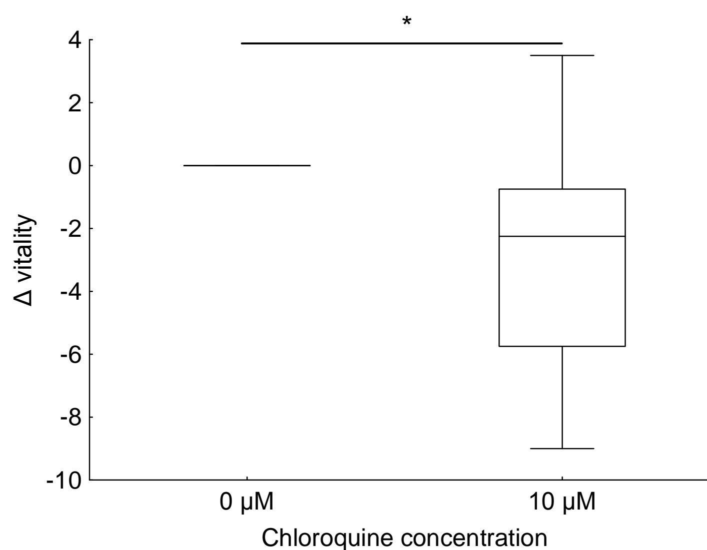


Figure 65 Δ Vitality of non-separated human spermatozoa incubated with chloroquine for 4 h in HTF+3 % BSA. * P≤0.05 refers to significantly different Δ vitality between control (set to 0) and CQ incubated spermatozoa using Mann-Whitney U-test (n=8). — Median □25 %-75 % ⌶Non-Outlier Range

Table 21 Effect of chloroquine on vitality, motility and LC3B protein expression after incubation of human spermatozoa with HTF+3 % BSA for 4; 24; 48 and 72 h. Data were analysed using Mann-Whitney U-test (n=8). Values are described as median (range). Relative protein amounts of phosphorylated proteins are represented in arbitrary units (a.u.). ND-no data

	0 h	4 h		24 h		48 h		72 h	
Chloroquine concentration		**0 µM**	**10 µM**	**0 µM**	**10 µM**	**0 µM**	**10 µM**	**0 µM**	**10 µM**
Vitality (Eosin %)	74.5 (58.5-82.0)	72.5 (51.0-82.5)	68.8 (48.5-82.5)	62.5 (24.0-77.5)	57.3 (29.0-75.0)	29.8 (5.50-66.0)	33.3 (9.50-62.0)	24.5 (3.50-53.5)	19.8 (3.50-55.0)
Progressive motility (CASA %)	57.0 (25.0-74.0)	65.0 (22.0-80.0)	62.5 (30.0-75.0)	28.5 (6.00-59.0)	30.5 (7.00-49.0)	3.00 (0.00-12.0)	2.00 (0.00-9.00)	0.00 (0.00-2.00)	0.00 (0.00-5.00)
Total motility (CASA %)	88.5 (50.0-94.0)	81.0 (27.0-94.0)	79.0 (41.0-94.0)	37.0 (8.00-79.0)	39.0 (8.00-65.0)	8.50 (0.00-25.0)	4.00 (0.00-14.0)	1.00 (0.00-7.00)	0.50 (0.00-11.0)
VAP (µm/sec)	58.6 (52.5-75.1)	74.2 (56.5-87.6)	73.0 (61.1-86.2)	55.6 (34.9-66.0)	61.8 (39.5-70.2)	26.1 (0.00-54.4)	24.5 (0.00-33.2)	7.30 (0.00-66.2)	9.25 (0.00-80.5)
VSL (µm/sec)	53.9 (44.1-71.3)	69.3 (50.9-81.0)	67.8 (57.4-83.5)	50.7 (28.2-61.9)	55.9 (35.9-66.1)	22.3 (0.00-50.9)	19.1 (0.00-27.8)	5.65 (0.00-66.2)	6.45 (0.00-79.2)
VCL (µm/sec)	87.0 (78.1-119)	102 (82.4-130)	104 (84.0-124)	81.7 (58.8-95.7)	91.4 (61.2-109)	45.4 (0.00-68.7)	43.6 (0.00-59.6)	14.0 (0.00-230)	14.8 (0.00-221)
ALH (µm)	3.65 (3.00-5.10)	3.45 (2.90-5.00)	3.75 (2.90-5.10)	3.60 (2.80-4.20)	3.55 (2.80-4.70)	2.50 (0.00-7.40)	2.70 (0.00-3.80)	0.00 (0.00-7.10)	0.00 (0.00-8.10)
BCF (Hz)	28.8 (21.6-35.3)	33.9 (28.7-37.3)	32.0 (27.2-36.5)	28.6 (21.7-34.5)	28.5 (21.2-34.3)	14.2 (0.00-29.5)	18.1 (0.00-31.3)	0.00 (0.00-60.0)	0.00 (0.00-17.3)
STR (%)	88.0 (83.0-93.0)	91.0 (87.0-96.0)	90.5 (87.0-95.0)	89.0 (79.0-94.0)	88.5 (87.0-93.0)	80.5 (0.00-91.0)	74.5 (0.00-85.0)	25.0 (0.00-100)	36.5 (0.00-98.0)
Lin (%)	58.5 (51.0-72.0)	64.5 (60.0-76.0)	64.5 (60.0-78.0)	59.5 (48.0-69.0)	58.0 (56.0-70.0)	48.5 (0.00-70.0)	43.5 (0.00-50.0)	13.5 (0.00-40.0)	18.0 (0.00-50.0)
LC3B-I (a.u.)	ND	ND	ND	ND	ND	ND	ND	1.00 (1.00-1.00)	0.71 (0.55-2.05)
LC3B-II (a.u.)	ND	ND	ND	ND	ND	ND	ND	1.00 (1.00-1.00)	1.06 (0.75-2.00)

However, the effect did not persist over 24; 48 or 72 h of incubation. As motility and vitality are not the only factors for the fertilisation capacity of spermatozoa, tyrosine phosphorylation as a hallmark for the capacitation was determined by western blot analysis after CQ incubation in capacitating media for 24 h and 72 h. The immunoreactive bands had the following molecular weights: 24 kDa, 25/26 kDa, 35 kDa, 40 kDa, 48 kDa, 68 kDa, 90 kDa, and 105 kDa. Probably by the use of non-separated spermatozoa, the relative protein amounts diverged over a wide range between the individual specimens. Therefore, no significant effect of CQ on tyrosine phosphorylation was detectable after incubation for 24 h in EBSS+3 % BSA (n=7; Table 22; P>0.05).

Table 22 Effect of chloroquine on tyrosine phosphorylation status of capacitation involved proteins after incubation of human spermatozoa with EBSS+3 % BSA for 24 h and with HTF+3 % BSA for 72 h. Data were analysed using Mann-Whitney U-test (n=7 for 24 h and 72 h). Values are described as median (range). Relative protein amounts of phosphorylated proteins are represented in arbitrary units (a.u.).

Capacitation involved proteins	**24 h**		**72 h**	
Chloroquine concentration	**0 µM**	**10 µM**	**0 µM**	**10 µM**
24 kDa (a.u.)	1.00 (1.00-1.00)	1.59 (0.64-2.55)	1.00 (1.00-1.00)	1.11 (0.44-1.30)
25/26 kDa (a.u.)	1.00 (1.00-1.00)	1.14 (0.62-2.43)	1.00 (1.00-1.00)	0.65 (0.50-1.57)
35 kDa (a.u.)	1.00 (1.00-1.00)	0.84 (0.79-1.53)	1.00 (1.00-1.00)	1.09 (0.79-1.32)
40 kDa (a.u.)	1.00 (1.00-1.00)	0.85 (0.73-0.98)	1.00 (1.00-1.00)	1.17 (0.74-1.41)
48 kDa (a.u.)	1.00 (1.00-1.00)	1.17 (0.79-1.85)	1.00 (1.00-1.00)	0.81 (0.41-2.17)
68 kDa (a.u.)	1.00 (1.00-1.00)	1.25 (0.48-2.90)	1.00 (1.00-1.00)	0.75 (0.34-1.34)
90 kDa (a.u.)	1.00 (1.00-1.00)	0.67 (0.60-0.82)	1.00 (1.00-1.00)	1.13 (0.67-1.30)
105 kDa (a.u.)	1.00 (1.00-1.00)	0.61 (0.46-0.93)	1.00 (1.00-1.00)	1.21 (0.77-1.42)

However, for the following molecular weights the tyrosine phosphorylation was significantly higher (P=0.03) in the control group compared with the CQ incubation for 24 h (n=7, Figure 66, Table 22; P=0.03).

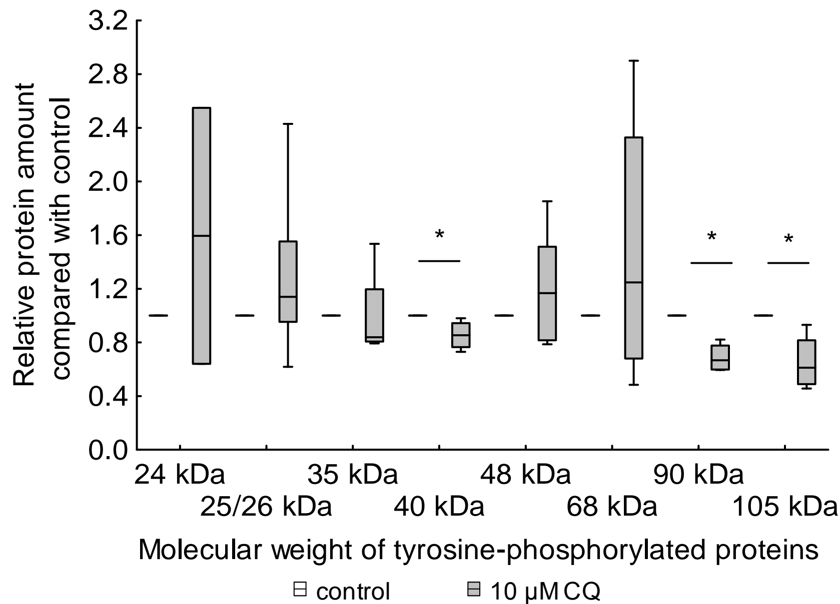


Figure 66 Effect of 10 µM chloroquine (CQ) on the capacitation status of non-separated human spermatozoa after a time period of 24 h with EBSS+3 % BSA. * P≤0.05 refers to significantly different tyrosine phosphorylation of control cells compared with CQ incubated spermatozoa using Mann-Whitney U-test (n=7). — Median □ 25 %-75 % ⌶ Non-Outlier Range

In addition, an incubation for 72 h using HTF+3 % BSA showed no significant impact of CQ on tyrosine phosphorylation (n=7; Figure 67, Table 22; P>0.05). Considering single values, the tyrosine phosphorylation decreased in 41 % of the cases, 19 % of the samples showed no change or in 40 % of the specimens an increase of the tyrosine phosphorylation was detected over a time course of 72 h due to CQ incubation.

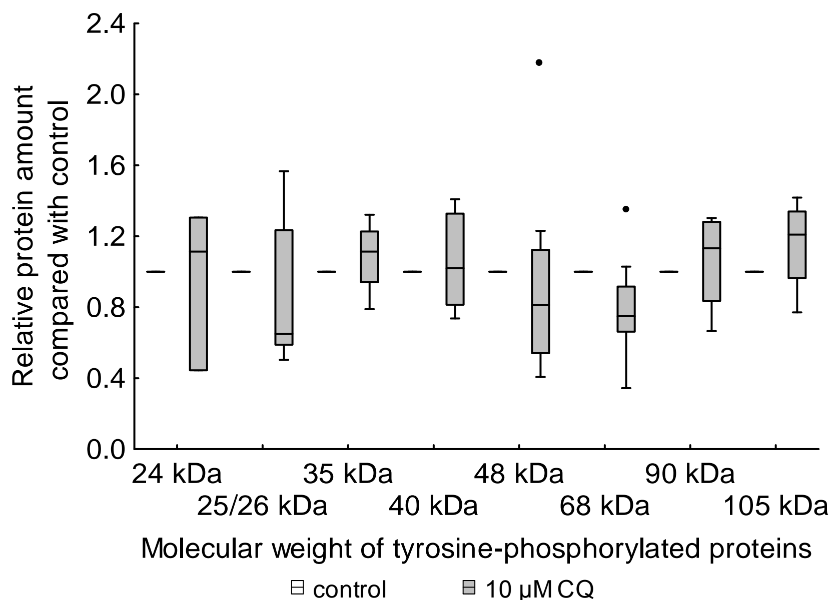


Figure 67 Effect of 10 µM chloroquine (CQ) on the capacitation status of non-separated human spermatozoa after a time period of 72 h with HTF+3 % BSA (n=8). Data were analysed using Mann-Whitney U-test (n=7). — Median □ 25 %-75 % ⌶ Non-Outlier Range ● Outliers

5 Discussion

5.1 Adipokines in seminal plasma depend on body weight

The following chapter 5.1 includes information of a published article[1].

"To our best knowledge, this is the first investigation describing the simultaneous presence of the adipokines adiponectin, chemerin, vaspin, leptin, resistin, progranulin and NAMPT in human seminal plasma. As obesity is associated with changes in the levels of adipokines in peripheral blood, these proteins could be the link between increased adipose tissue mass and impaired male fertility. A causal association between fat mass and sperm functionality was hypothesised by a number of recent publications (4-8). Our observation of a negative correlation between BMI and semen parameters in the whole group of donors supports this hypothesis. This is in agreement with findings that demonstrated a significantly negative correlation of BMI with normal-motile spermatozoa and a positive correlation with DFI (8, 362). Moreover, it was demonstrated that obesity is associated with a lower number of motile sperm, increased incidence for abnormal morphology (363, 364) and reduced mitochondrial activity (362). In agreement with these studies, we found lower numbers of normomorph spermatozoa for obese men, but the determined values were still considered as normal according to the reference values of the WHO (68). Moreover, nutritional and physical intervention in obese men lead to weight loss accompanied by a significant increase in the sperm count and normomorph spermatozoa (365). Contrary to these results, two studies could not find a detrimental effect of obesity on spermatozoa function (9, 366). Despite a body of evidence suggesting that obesity might affect semen quality, the pathogenesis of this putative interaction remains to be elucidated.

Interestingly, concentrations of vaspin, progranulin and NAMPT were significantly higher in SP than in serum, whereas adiponectin, chemerin, leptin and resistin concentrations were significantly lower. Only serum levels of adiponectin were moderately correlated to SP values. One explanation for the correlation between peripheral and SP adiponectin could be an exchange between these two compartments for adiponectin by a gap via the blood-testis barrier. Although the vascular barrier restricts the entry of serum proteins, adiponectin could be taken up into testis by leakage rather than saturable transport as described for leptin (367). Alternatively, adiponectin might be secreted by epididymal adipocytes presuming a paralleled secretion mechanism similar to that described for subcu-

[1] This article was published in Fertility and Sterility, Volume 99, Stephanie Thomas, Dorothea Kratzsch, Michael Schaab, Markus Scholz, Sonja Grunewald, Joachim Thiery, Uwe Paasch and Juergen Kratzsch, Seminal plasma adipokine levels are correlated with functional characteristics of spermatozoa, Pages 1256-1263.e3, Copyright Elsevier (2013).

taneous or visceral adipose tissue originated adiponectin. This hypothesis could be explained by the fact, that epididymal fat pad as well as the visceral adipose tissue increased in parallel with obesity in a mice model (368). However, it remains completely unclear why this mechanism may be specific only for adiponectin. The presence of paralleled adiponectin concentrations in serum and SP is still not proven for any secretion or crucial action of this adipokine on spermatozoa. Interestingly, adiponectin and its receptors AdipoR1 and AdipoR2 were detected in chicken testis recently (137). Accordingly, an adiponectin expression in human testis is likely. This fact is weakened by the presence of adiponectin in SP of vasectomised men, because vasa deferentia are severed and sealed. Subsequently, spermatozoa and secretes of testis are not longer part of the ejaculate. Therefore, testis may not be the only source for adiponectin in SP.
The lacking correlations for the other adipokines in both body fluids suggest a compartment-specific regulation of adipokines in reproductive tract and peripheral blood. This regulation appears to be largely independent of BMI and body fat as shown by missing correlations for adiponectin, leptin, chemerin, vaspin, and NAMPT in SP. In contrast, we found expected correlations of adiponectin, chemerin and leptin with BMI in serum.
The high levels of vaspin, progranulin and NAMPT in SP point to special physiological functions of these proteins in male reproductive tract although their source remains largely uncertain. Epithelial cells, accessory glands, testis, epididymal adipocytes or spermatozoa might contribute to the secretion of adipokines into the SP. Moreover, we found similar levels for all of the adipokines determined in SP of vasectomised compared with non-vasectomised donors. Thereby, chemerin and progranulin levels in SP were significantly lower in vasectomised men than in non-vasectomised men, whereas NAMPT concentrations tended to be higher. Accordingly, NAMPT did not appear to be mainly secreted by spermatozoa or testes related cells. Therebey, the presence of NAMPT in human and chicken testis has been demonstrated (315).
The putative presence of single adipokines in the seminal tract has been scarcely described so far. Chemerin might be produced by adipocytes from the epididymal fat pad according to investigations in 3T3-L1 adipocytes (159). Therefore, chemerin secretion by epididymal adipose tissue could increase with obesity and reduce semen quality. This hypothesis is supported by the fact, that chemerin levels in vasectomised men were significantly lower than in non-vasectomised men. Leptin was already found in human SP (24-26) and described to be secreted by human spermatozoa (369). It was also expressed in rat prostate, seminal vesicles (370) and human spermatocytes (371). Resistin was expressed in rat intestinal Leydig and Sertoli cells of seminiferous tubules (261) as well as in human SP (23) and human epididymal fat tissue (234). It is not clear if progranulin is secreted by human ejaculated spermatozoa and if this secretion could explain the amount of

protein found in seminal plasma. So far, progranulin was determined as an acrosomal protein of guinea pig spermatozoa (278). Additionally, progranulin mRNA was expressed in rat and mouse seminiferous tubule but rarely in mature spermatocytes (269). Whether this protein is also present in human acrosomes needs to be investigated.

For the first time, we could demonstrate that several adipokines in SP were associated with sperm function parameters. Our data shows that the regulation of these adipokines in SP was independent of BMI or waist circumference, which are both considered as surrogate markers of the visceral fat mass. In detail, SP adiponectin levels were significantly lower in overweight/obese men than in normal weight subjects, possibly due to the increased epididymal fat pad and visceral adipose tissue, which show parallels in a obese mice model (368). Nevertheless, adiponectin SP levels did not correlate to BMI or waist circumference. After adjusting for age, BMI, waist circumference, LH and FSH, SP chemerin correlated negatively with progressive motility. This implicates that the association of chemerin with spermatozoa function could be independent of body weight and sexual hormones.

Interestingly, leptin in SP correlated positively with sperm motility and concentration but not with BMI. Accordingly, leptin could have a direct effect on the spermatozoa itself mediated via its membrane receptor (25). Such a direct influence would mirror a BMI-independent leptin effect within the reproductive tract, which is in contrast to obesity-associated leptin resistance in peripheral blood (372). Thus, a direct effect of leptin on human spermatozoa was hypothesised as 10 nM leptin significantly increased progressive and total motility, acrosome reaction and NO production (373, 374). However, this effect could not be confirmed by Li *et al.* (374) in a comparable experiment. Both studies are based on leptin`s effect on already ejaculated spermatozoa but *in vivo*, leptin decreased epididymal sperm count and increased amount of abnormal sperm in rats (375). This suggests that leptin may have diverging effects on maturing and ejaculated spermatozoa.

Progranulin levels in SP were significantly lower in overweight/obese men than in normal weight subjects. Additionally, SP progranulin levels correlated significantly negative with BMI and positively with sperm motility, concentration, sperm count and normal morphology. After adjustment for age, BMI, waist circumference, LH and FSH, progranulin levels still correlated positively with progressive motility. This indicates that progranulin might be directly associated with semen quality rather than with obesity.

Our observational study has some restrictions: As age was a confounder for the comparison of BMI between the subgroups, its effect had to be excluded by a robust linear regression analysis. Beyond, we could not investigate putative associations of whole body fat mass with adipokine levels or spermatozoa function. However, BMI and waist circumference should enable to reflect such relationship at least by trend. We only demonstrated

statistical relationships but did not deliver any proof for pathophysiological effects of adipokines on sperm parameters. According to our presented data, we can only speculate the role of the detected adipokines in male reproduction.
In summary, we have shown that obesity is negatively correlated with some semen parameters. Secondly, adipokines are present in SP, whereby vaspin, progranulin and NAMPT levels were higher than in peripheral blood. Based on our correlation analysis, we speculate that SP adiponectin, leptin and progranulin could be positive surrogate markers of semen quality, whereas chemerin and vaspin might indicate a reduced quality." (437)

5.2 Presence and function of RNA and proteins in human spermatozoa

5.2.1 Consequences of the transcriptional arrest in spermatids

During spermatogenesis, somatic histones are initially replaced by transition proteins and later by protamines (376). This contributes to a high chromatin packaging, which refers to a transcriptional inactivity of human ejaculated spermatozoa (54), although mature mouse spermatozoal nuclei contained RNA polymerase and transcription factors (377). However, high levels of mRNA were found in round spermatids before the arrest of transcription starts during midspermiogenesis (378). The origin of the mRNA in spermatozoa is not revealed yet. It has been discussed that these mRNAs, full-length stable transcripts with inter-individual expression patterns, may be testicular origin and therefore remnants of the spermiogenesis and past events of the gene expression (57). The mRNA content is regulated in individual cases, as shown for the c-myc mRNA, which disappeared after capacitation (379). Long-term mRNA might be used to synthesise proteins in the female reproductive tract during storage in the sperm reservoir (380) and during capacitation. Moreover, sperm mRNA may play a crucial role in the early development of the zygote and the embryo (56) as during fertilisation the entire content of the spermatozoa is released into the cytoplasm of the oocyte (381). Subsequently, the oocyte degrades paternal mitochondria and sperm tail structures (382), whereas clusterin and protamine-2 spermatozoal transcripts retained in zygotes for 30 min and 3 h after fertilisation (56). To date, thousands of RNA transcripts were detected in ejaculates of individuals and pooled mature spermatozoa samples (383, 384). According to the RNA profile, half of the RNA populations are common in the ejaculates, whereas the other half is inter-individual.
Furthermore, the origin of the RNA might be mitochondrial (385) as these cell compartments include ribosomes. The 28S and 18S rRNAs are essential components of the 80S ribosome and therefore for the translational activity. Only the 18S rRNA was demonstrated to be present in spermatozoa (386). Electron-dense structures, corresponding to clearly identifiable ribosomes on the rough endoplasmic reticulum, were irregularly distributed in the cytoplasm of the neck to the beginning of the midpiece (386).

Although, the presence of mRNA in human spermatozoa is undeniable, the amount of mRNA diverges in high ranges. 10-60 ng RNA/10^6 spermatozoa were determined in the current study, which is comparable to the results of different research groups, who detected 7.07±1.05 ng/10^6 spermatozoa (54), median of 60.7 ng/10^6 spermatozoa (range 48.8-136.9 ng/10^6 spermatozoa) (387) and 29-400 ng/10^6 spermatozoa (388). This broad range of differences in RNA amounts may result from the presence or absence of somatic cells. Sperm specimens with higher RNA amounts might be contaminated with leucocytes or other round cells, because these cells contain much more RNA compared with spermatozoa. Accordingly, swim-up or density gradient centrifugation should be performed before RNA isolation. In the current study, we performed density gradient centrifugation and excluded leucocytes by using anti-CD45 magnetic beads. To ensure decontamination, we performed PCR with CD45 primers as recommended by Lambard *et al.* (379). If agarose gel electrophoresis showed a band at 206 bp corresponding to the leucocyte presence, samples were not included in further investigations. In addition, we performed a PCR with intron-overspanning primers against protamine 2 as a marker for contamination with genomic DNA. Once DNA was absent, bands were at 182 bp. After amplification of genomic DNA, PCR product was 345 bp long (389). Hence, DNA contaminated samples were excluded from the study.

5.2.2 Occurrence of translation in spermatozoa

Nuclear encoded transcripts are possibly translated by mitochondrial-like ribosomes, as cytoplasmic ribosomes do not seem to be functional in mature spermatozoa (390). The mammalian mitochondrial ribosome (55S) consists of both large (39S) and small (28S) subunits. The 39S subunits contain 16S and the 28S subunits contain 12S rRNAs. The 16S and 12S rRNAs are localised at the nucleus of mouse and human spermatozoa (391, 392). Translation occurred in the mitochondrial polysomes of spermatozoa (393) and the presence of 55S mitochondrial ribosomes and incorporation of labelled amino acids into polypeptides during sperm capacitation were verified (58). Moreover, this incorporation was blocked by mitochondrial translation inhibitors but not by a cytoplasmic translation inhibitor (58), which concludes that translation occurs in the mitochondria and not in the cytoplasm. The incorporation of labelled amino acids began within 2 min of incubation under capacitating conditions, indicating that sperm cells require newly synthesised proteins immediately at the beginning of capacitation (58). While the mitochondrial genome encodes only for 5 proteins, the protein amounts synthesised during capacitation are significantly higher, suggesting that there has to be another mechanism than the translation of the mitochondrial genome. The analysis of specific proteins synthesised during capacitation demonstrated, that nuclear-encoded proteins are indeed translated by mitochondrial-type ribosomes (58). Thus, sperm cells may use stable, long-term mRNA transcripts

to synthesise novel proteins (57, 393). Accordingly, the detection of proteins in spermatozoa is possible as well as the regulation of protein amounts by capacitation.

5.3 Expression of adipokines in male reproductive tract and their impact on sperm function parameters

5.3.1 Adiponectin

To date the expression of adiponectin and its receptors was only investigated in the reproductive tract of animal models. Adiponectin, AdipoR1 and AdipoR2 mRNA and protein have been found to be expressed in chicken testis (137). In detail, adiponectin protein was expressed in peritubular and interstitial cells, AdipoR1 in peritubular and interstitial cells and Leydig cells, and AdipoR2 was expressed in round and elongated spermatids and Sertoli cells (137). Furthermore, rat Leydig cells expressed adiponectin mRNA and protein and AdipoR1 mRNA was expressed in seminiferous tubules epithelium, whereas AdipoR2 was rather expressed in interstitium (136). Recently, mRNA and protein of adiponectin and its receptors were detected in bull spermatozoa (394).

In the recent study, we detected AdipoR1 protein in the cytoplasm of Sertoli and Leydig cells of human testis for the first time. Moreover, AdipoR1 mRNA and protein were present in human spermatozoa. In contrast to bull spermatozoa, AdipoR1 was localised at the connecting piece and tail of human spermatozoa but not at the equatorial region and acrosome (394).

Adiponectin might be secreted by spermatozoa as this adipokine is expressed in human and bull spermatozoa (394). According to the secretion experiments, adiponectin was not detectable in the supernatant of mature and immature spermatozoa after the incubation of 3 h and 24 h under capacitating conditions. This suggests that adiponectin is not secreted into the supernatant or the adiponectin concentrations were beneath the detection limit of the ELISA kit. Accordingly, more spermatozoa should be incubated with capacitating medium for different time periods to further investigate the secretion.

Beyond, in our experiments, the motility of swim up spermatozoa was influenced by recombinant adiponectin, whereas the effect was not dose-dependent. Thereby, adiponectin might affect the actin cytoskeleton of spermatozoa as shown for myocytes (395, 396). The filament structure is controlled by reversible polymerisation of globular (G)-actin, which forms filamentous (F)-actin. In human spermatozoa, actin is present in the acrosome, between plasma membrane and outer acrosome membrane, in postacrosomal area, neck and principal piece of the tail (397-401). The presence of actin at the tail suggests to play a role in sperm motility. This hypothesis is supported by a significant positive correlation of sperm motility and actin on the sperm surface (402) and high levels of F-actin in highly motile spermatozoa (403). Moreover, hyperactivated and total motility was significantly

reduced by the inhibition of actin polymerisation in human spermatozoa (404) and hyperactivated motility increased significantly by the induction of actin polymerisation (404). As adiponectin increased the F:G-actin ratio and subsequently the F-actin polymerisation in myocytes (396), it might be able to enhance the actin polymerisation in human spermatozoa and as a consequence increase the motility.
Besides, actin polymerisation might be crucial for capacitation and acrosome reaction (405, 406), as in uncapacitated spermatozoa the monomeric G-actin is predominately present, whereas actin polymerised into F-actin during capacitation (405, 407). Capacitation as a physiological process is also described to influence protein concentrations of adiponectin, AdipoR1 and AdipoR2 depending on the fertility status of bulls (394). By the induction of capacitation, the protein levels of this adipokine and its receptors decreased (394). Moreover, the authors suggest that adiponectin might influence the cholesterol efflux during the capacitation triggered by sperm-specific ATP-binding-cassette (ABC) transporter (394, 408), as adiponectin and its receptor increased the cholesterol efflux partially through ABCA1 (409). However, in the current study adiponectin's influence on capacitation and therefore the functionality of human AdipoR1 was investigated by incubation experiments. The tyrosine phosphorylation of proteins involved in capacitation increased significantly by incubation with adiponectin. Hence, adiponectin might not only influence the cholesterol efflux, but also the phosphorylation status of some proteins like IGF-IR β-subunit and the MAPK/ERK 1/2 in rat granulosa cells (410). In the current capacitation study a 43 kDa protein was detected. According to the molecular weight, phosphorylated MAPK (44 kDa), ERK 1 (42 kDa) or ERK 2 (44 kDa) might be present in spermatozoa. In previous studies, the presence and function of MAPK was revealed in human male reproductive system. In detail, the activation of MAPK is involved in spermatogenesis, germ cell apoptosis, motility development in the epididymis, capacitation and acrosome reaction (411, 412). According to the recent results, adiponectin might have a stimulating effect on capacitation status of human spermatozoa transduced by the AdipoR1.
The effects of adiponectin on the acrosome reaction were investigated by incubation experiments, whereas no influence on acrosome reaction in human spermatozoa was detectable. If actin is affected by adiponectin in human spermatozoa, it might cause the loss of actin during acrosome reaction (401). However, acrosome reaction was inhibited by the blockade of actin polymerisation (401) suggesting an important role of actin during acrosome reaction. This result might be inextricably linked to capacitation, where actin polymerisation occurs.
During acrosome reaction, a Ca^{2+} influx occurs and subsequently intracellular Ca^{2+} levels increase (413). In somatic cells, adiponectin is known to induce Ca^{2+} release from the endoplasmic reticulum by activating phospholipase C, thereby stimulating

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) mediated phosphorylation of AMPK (414). Adiponectin also stimulates extracellular Ca^{2+} influx and subsequently activates CaMKK and AMPK via binding to AdipoR1 in myocytes (415). In the end, the influence of adiponectin on MAPK, CaMKK, AMPK and Ca^{2+} release in spermatozoa should be investigated in future experiments.
The influence of adiponectin on apoptosis was also investigated. Under capacitating conditions, adiponectin did not influence caspase activity. Whereas under non-capacitating conditions, adiponectin increased significantly the activity of caspase 3&7 compared with the control. Nevertheless, the medium for spermatozoa incubation is crucial for the detection of caspase activity, as capacitation leads to a significant inactivation of apoptosis signalling (416). However, the number of donors was very low and therefore these experiments should be repeated with a higher number of donors. In somatic cells, adiponectin has also been reported to stimulate apoptosis (132, 417), but even promote cell survival (135, 418). Thereby, adiponectin induced apoptosis in cancer and endothelial cells due to its influence on Bcl-2 and caspases-3, -8 and -9 (419-421). In contrast, adiponectin protected pancreatic β-cells against apoptosis (422). These data demonstrate that adiponectin is either pro- or anti-apoptotic dependent on the cell type.
Overall, adiponectin is able to influence sperm parameters possibly triggered by a functional adiponectin receptor 1 and subsequently its effect on actin. This suggests an impact of adiponectin on the fertilisation of an oocyte.

5.3.2 Chemerin

So far, the presence of chemerin and its respective receptor has been detected in human testis (160), but not in spermatozoa. The presence of chemerin in human spermatozoa was not investigated directly in the current study. However, after incubation for 3 h and 24 h under capacitating conditions, no chemerin was detected in the supernatant of mature and immature spermatozoa. Suggesting that chemerin might not be present in spermatozoa or not secreted. Furthermore, the secreted chemerin concentrations might be below the detection limit of the ELISA kit. For the first time, we report the presence of ChemR23 protein at the connecting piece, midpiece and tail of human spermatozoa. The presence and functionality of ChemR23 is supported by the chemerin incubation experiments. Chemerin incubation concluded in a significant increase of the ALH, progressive and total motility. The mechanism of chemerin influencing sperm motility was not further investigated, but chemerin might be able to influence the MAPK signalling as shown for endothelial cells (423) or F-actin polymerisation as shown during phagocytosis (424). Whether chemerin is able to influence the capacitation or acrosome reaction was not investigated. However, chemerin stimulated intracellular Ca^{2+} release in CHO-K1 cells (139) and might be able to influence Ca^{2+} release and therefore acrosome reaction in human

spermatozoa. Moreover, chemerin induced caspase activity in human spermatozoa under non-capacitating conditions. This result is confirmed by chemerin triggering apoptosis in granulosa cells (425). In future experiments, the influence of chemerin on capacitation and acrosome reaction should be investigated.

5.3.3 Leptin

Leptin mRNA was present in boar epididymal and ejaculated spermatozoa (426). Leptin was also detected in human seminiferous tubules (24), especially in germ cells, mainly spermatocytes of fertile, varicocele and oligoasthenoteratozoospermic patients (371). Moreover, in the current study, leptin protein was present in the cytoplasm of Sertoli cells, spermatocytes and spermatogonia and in the nucleus of Sertoli cells and in ejaculated spermatozoa. The presence of leptin protein in human spermatozoa was also confirmed by Aquila *et al.* (369). However, in the recent study we were able to detect leptin protein by immunofluorescence but not by western blot analysis. Hence, leptin protein might be expressed in low levels in human spermatozoa and the blotted protein amount was to low to be detected. However, leptin is secreted by human spermatozoa (369), which was also confirmed by our results and underlines the expression data. Overall, not only leptin but also its receptor seems to be present in male reproductive tract. So far, leptin receptor mRNA has been expressed in Sertoli cells (427) and Leydig cells (428) of rats and in mice testis (429). In human spermatozoa, leptin receptor mRNA was absent (374). In contrast, leptin receptor mRNA was weakly expressed in 1 out of 10 fertile donors and absent in 20 out of 23 infertile patients leptin receptor expression (430). These results are consistent with our findings for mRNA expression of ObR in human spermatozoa. However, the protein of leptin receptor was detectable at the tail of human spermatozoa in the recent study and by Jope *et al.* (25). We validated the presence of the receptor by the use of a blocking peptide. Moreover, this receptor protein was detected in human testis, primarily in Leydig cells (371). The functionality of the leptin receptor was confirmed by the incubation of rat Leydig cells with leptin, which led to a significant and dose-dependent inhibition of human chorionic gonadotropin-stimulated testosterone production without a change in basal androgen release (428). Moreover, leptin influenced sperm motility and capacitation in human spermatozoa (373) underlining the presence of ObR in these cells, whereas we were not able to detect ObR by western blot analysis. In our study, we also detected an increase of sperm motility after incubation with human recombinant leptin, whereas another study group did not detect significant alterations in motility patterns under capacitating and non-capacitating conditions (374). Leptin might be able to alter the motility in spermatozoa due to its influence on the cytoskeleton as mentioned for adiponectin. In somatic cells, leptin is able to increase the F:G-actin ratio in rat portal veins, probably due to the inhibition of the F-actin depolymerisation (431). This increase is contributed by the signalling of

ObR (431). Leptin is able to depolymerise actin in a rat insulin cell line and the incubation with phalloidin binding to polymerised actin inhibits this depolymerisation (432). Moreover, leptin induced depolymerisation of F-actin of Ca^{2+}-activated K^{+} channels of hippocampal neurons (433). Overall, leptin is able to polymerise and depolymerise F-actin dependent on the cell line and tissue. How leptin influences the actin polymerisation in spermatozoa remains to be clarified. However, relative F-actin concentrations were significantly higher in immotile, immature caput epididymal guinea pig spermatozoa than in mature counterparts (434). Additionally, F-actin concentrations in capacitated guinea pig spermatozoa were significantly lower than in non-capacitating spermatozoa. In contrast, under capacitating conditions actin polymerisation occurred at the head of human spermatozoa particularly in the upper head region, whereas in non-capacitated sperm no F-actin was observed at the tail, but in the postacrosomal region (405). Factors that trigger protein tyrosine phosphorylation during capacitation stimulate actin polymerisation, whereas inhibitors of tyrosine phosphorylation block F-actin formation (405). Moreover, the inhibition of actin polymerisation by cytochalasin D in boar spermatozoa reduces the fertilisation rate (407). In the current study, increased tyrosine phosphorylation of the 85 kDa protein was detected after incubation with 100 ng/ml leptin for 3 h in HTF+3 % BSA. The effect of leptin on capacitation was contrasted by Li *et al.* (374). With regard to the current results, leptin might increase F-actin concentrations and therefore the motility and capacitation of human spermatozoa. After 3 h of capacitation, F-actin was maximally polymerised and the acrosome reaction started (405). Thus, it seems that G-actin is polymerised to F-actin during capacitation and depolymerised by enhancing intracellular Ca^{2+} concentrations, resulting in acrosomal exocytosis (405). Besides capacitation, actin polymerisation has an important role in the ZP-induced acrosome reaction of human sperm (401). F-actin disappeared rapidly after the Ca^{2+} ionophore A23187 induced acrosome reaction (405). A significant enhancement of acrosome reaction can be seen only after complete depolymerisation of F-actin (405). Phalloidin, which blocks F-actin depolymerisation to G-actin, inhibits Ca^{2+} ionophore induced acrosomal reaction (435, 436). Therefore, leptin might also affect acrosome reaction of human spermatozoa by the influence of cytoskeletal F-actin. So far, leptin increased spontaneous and progesterone induced acrosome reaction (373), whereas we and another study group were not able to detect an effect of leptin on acrosome reaction (374).

Overall, leptin had an impact on sperm motility and capacitation possibly due to its influence on actin.

5.3.4 NAMPT

In this study, we report for the first time the presence of NAMPT in human spermatozoa, with higher amounts detected in immature cells. Previously, NAMPT was detected in hu-

man seminal plasma (437) and in chicken testis biopsies (315). In testis of adult chicken, NAMPT was present in the cytoplasm of Sertoli cells, Leydig cells, primary and secondary spermatocytes, round and elongated spermatids. In comparison, NAMPT was expressed in the nucleus of prepubertal chicken testis cells. In our study, we were able to confirm these expression patterns. The nuclear and cellular localisation of NAMPT was determined in somatic cells before (281). Additionally, NAMPT expression was investigated in spermatozoa. Since we found higher NAMPT protein concentrations in immature than mature spermatozoa, immature spermatozoa may be able to release more NAMPT. The western blot and immunofluorescence data are supported by the secretion data revealing higher NAMPT levels in supernatant of immature spermatozoa compared with mature spermatozoa. The detection of NAMPT protein in the supernatant of human spermatozoa was demonstrated as described before for a number of cell types (284, 438). The demonstrated release of NAMPT into the supernatant seemed to be independent of sperm vitality, because the increase of NAMPT concentrations in supernatant was not associated with a decrease of vitality over time. The mechanism of NAMPT release is not known. In different cell types, NAMPT release was shown not to be inhibited by classical or ATP-binding cassette transporter-controlled secretion pathways (283, 284). However, the association between NAMPT expression and maturation status of human spermatozoa is not fully understood. We assume that immature sperm not only express more NAMPT protein but also have higher NAD^+ levels than mature spermatozoa. This may be either due to the higher energy requirements during development into mature spermatozoa or to NAD^+ consuming processes like reactions catalysed by sirtuins (439) and PARPs (440, 441). We were able to confirm the assumption in our study. Immature spermatozoa had significantly higher NAD^+ levels than their mature counterparts after normalisation to sperm count. Alternatively, NAMPT levels may be higher in immature sperm due to a higher level of intracellular ROS in the cytoplasmic droplet (442-444). NAMPT may confer resistance to oxidative stress (445) or may be produced in response to inflammatory signals (446). These facts suggest that in relation to a higher oxidative stress occurring in immature sperm, NAMPT is increased in compensation.

To investigate the effect of NAMPT on sperm function, the enzyme activity was inhibited by FK866 (358, 447), which binds in the active site (448). The motility tended to be increased by incubation with FK866 for 24 h. These experiments were performed with different culture media. Spermatozoa incubated with EBSS+3 % BSA were only viable up to 24 h, whereas sperms were viable up to 72 h in the HTF+3 % BSA medium. The first experiments were performed in the winter of 2011-2012. Further experiments of FK866 incubation were conducted in spring and summer of 2012. Interestingly, the significant differences in the motility patterns after FK866 incubations as seen in the winter experiments

were not repeatable in the summer. The impact of the season on sperm motility was demonstrated in fertile donors, who had significantly lower sperm count and motility in spring and summer season (449, 450). The seasonality was not only described for sperm motility, but also for mRNA profiles in swine spermatozoa (451). When men with idiopathic oligozoospermia were treated with tamoxifen citrate or testosterone undecanoate at different times of the year, patients showed higher response to the treatment in autumn and winter by sperm concentration, motility and morphology (452). The pregnancy incidence in autumn was also higher in control and treatment group (452). Therefore, we assume that NAMPT protein levels or activity could be altered depending on the season.

NAMPT inhibition was shown to induce apoptosis in cancer cells (453, 454), which is explained by the decrease of cellular NAD^+ levels (358), followed by ATP depletion (455) and subsequent apoptosis. In this study, we found no evidence that FK866 induced apoptosis, although human ejaculated spermatozoa were found to be capable of apoptosis (316, 317). Instead of causing apoptosis, FK866 was demonstrated to induce autophagy in a variety of cell types (358, 456).

In the current study, we investigated the effect of FK866 on capacitation. The duration of this process was recommended for 3 h (416). Knowing that FK866 is effective after 72 h (358), we performed incubations for a time period of 24 to 72 h. FK866 seemed to inhibit donor-dependently tyrosine phosphorylation and therefore capacitation of human spermatozoa after 24 h. The experiments were performed with non-separated spermatozoa, a mixture of immature and mature cells and resulted in a predominantly decrease of tyrosine phosphorylation in presence of FK866, which inhibits NAMPT activity. Hence, immature spermatozoa expressing higher NAMPT levels might be more susceptible to NAMPT inhibition. Overall, the decrease in tyrosine phosphorylation could be explained by the decrease of cellular NAD^+ levels (358), followed by ATP depletion (455) and therefore an impaired energy metabolism. As shown before, NADH is essential for capacitation, because NADH is able to trigger tyrosine phosphorylation in human spermatozoa (457). Moreover, NADH and NADPH were suggested as cofactors of the sperm oxidase responsible for oxygen production associated with sperm capacitation (458-460). Extracellular NAMPT was also shown to enhance the tyrosine phosphorylation of STAT3 in macrophages (461). Whether NAMPT has a direct impact on tyrosine phosphorylation and capacitation in spermatozoa needs to be clarified in further experiments.

Overall, we were able to show for the first time that NAMPT is present in human spermatozoa and in the conditioned supernatant in a maturation dependent manner. NAMPT inhibition seems to have a donor-dependent impact on the motility, vitality and the capacitation of human spermatozoa.

5.4 Autophagy in the male reproductive tract

Autophagy, as a process of degradation and a form of cell death, was detected in human ejaculated spermatozoa within the current study for the first time. Up to now this process has been revealed only in stallion ejaculated spermatozoa (343) and rat spermatocytes (462). Within the current study two different methods, immunofluorescence and western blotting, were performed, which are suitable for the determination of the autophagy process (463). In somatic cells, typical punctae/autophagic vesicles (358, 456) representing LC3-positive autophagsomes and autophagolysosomes (337, 340, 341) were detectable in the heads of human spermatozoa. The distribution and the number of these punctae were not identical throughout the ejaculate. This suggests that the spermatozoa accomplished different stages of autophagy. In addition to human spermatozoa, LC3B was detected in the human testis for the first time. In testicular sections, typical punctae were recognisable in the cytoplasm of spermatocytes.

According to the western blot results, LC3B-I, LC3B-II and Beclin-1 protein were detectable in non-separated spermatozoa, which were not incubated with the NAMPT inhibitor FK866 or an autophagy inducer. In contrast to rat spermatocytes (462), the protein levels of total LC3B and Beclin-1 were not significantly lower at the beginning of the experiment compared to 24 h or 72 h of incubation. Within the current study, the variations between the individual donors were very high. Consequently, the LC3B and Beclin-1 protein levels increased, decreased or did not alter within specimens of non-separated spermatozoa incubated with capacitating medium over a time period of 24 h or 72 h compared with the beginning. This result implicates that human capacitating spermatozoa are able to perform autophagy, whereas the regulation might be dependent on the maturation status.

Human semen can be separated into different fractions of spermatozoa with different maturation stages. The immature fraction is characterised by a high content of DNA damage, altered chromatin packaging, protamination status, and excessive production of ROS (442, 444, 464, 465). Other studies revealed the importance and maturation dependency of apoptosis in human spermatozoa. The immature subpopulation is suggested to be more reliable on apoptosis than the mature fraction, implicating that mature spermatozoa are more resistant to apoptosis (466). However, another study group demonstrated that immature spermatozoa have significantly less percentage of apoptotic cells than mature respectives (467).

Western blot analysis against LC3B-I and LC3B-II was performed with immature and mature spermatozoa to evaluate the autophagy process in different maturation stages. Immature spermatozoa had higher protein levels of LC3B-I and LC3B-II than their mature counterparts. This implicates that the autophagy process in immature spermatozoa might be more distinct than in mature ones, suggesting a dependency on maturation. Immature

spermatozoa might have less energy than mature cells and therefore might be in a state of nutrient deprivation, when autophagy is occurring at a higher rate (468). Therefore, they might perform this process to prolong lifespan or as a form of cell death (343, 469, 470). Furthermore, the increased rate of autophagic initiation or flux rate could be explained by higher levels of lysosomal enzymes, which degrade contents of autophagosomes (471-473). Mature spermatozoa might have less lysosomal enzymes due to an absent cytoplasmic droplet. Hence, the autophagy might be a remnant of the spermatogenesis and maturation. This result is contrasted by experiments within stallion spermatozoa, where a mature fraction of density gradient-centrifuged spermatozoa were more viable than native sperm and had also higher processed LC3B levels compared to their native respective cells (343). After storing native spermatozoa for five days, processed LC3B levels increased (343), which implicates that ejaculated spermatozoa are able to regulate the process of autophagy. In contrast, the mature fraction of these spermatozoa did not show an increase of LC3B-II levels for the same time span (343), which suggests that autophagy in mature spermatozoa might be blocked due to less lysosomal enzymes compared with immature spermatozoa. Hence, mature spermatozoa might not be able to induce or regulate the process of autophagy. In addition to human spermatozoa and rat spermatocytes (462), LC3B was detected in cytoplasm of human spermatocytes for the first time. Overall, autophagy might occur during spermatogenesis as mechanism of cell survival (462). However, the testis tissues showed only one stage of the autophagy process during spermatogenesis. Neither the regulation of the autophagy process in testis nor a potential mechanism of modulation has been revealed.

Spermatozoa were incubated with the NAMPT inhibitor FK866. This compound is known to induce autophagy in somatic cells after 72 h of incubation due to the decrease of intracellular NAD^+ levels (358, 456). In human spermatozoa, FK866 affected the protein levels of Beclin-1 and both forms of LC3B after 24 h and 72 h of incubation in capacitating medium. This implicates that autophagy could be induced by FK866 in ejaculated spermatozoa in a donor-dependent manner. The experiments were performed a mixture of immature and mature cells. Immature spermatozoa, expressing higher NAMPT and LC3B levels might be more susceptible to NAMPT inhibition. Hence, immature spermatozoa might be able to perform and regulate autophagy. The impact of FK866 on autophagy in immature and mature spermatozoa should be determined in future experiments.

All described experiments and references illustrated only a certain point in the process of autophagy. The autophagic flux has to be taken into account, as the increase of LC3B levels or the accumulation of autophagosomes can be caused by increased autophagic initiation or by blocking the autophagic flux. Currently it is not clarified how FK866 influences the autophagic flux or if ejaculated spermatozoa are able to react to blocking of the

autophagy by an inhibitor like chloroquine (CQ). This inhibitor interrupts the fusion between the autophagosome and the lysosome by an increase of the lysosomal pH (474) and leads to an accumulation of autophagosomes or LC3B-II in somatic cells (475, 476). Therefore, spermatozoa were incubated with 10 µM CQ (361, 476) to determine its effects on vitality, motility and LC3B protein expression. Overall, CQ had no toxic effect on human spermatozoa after different incubation times. In the current study, spermatozoa were incubated for 24 h and 72 h with CQ to reveal its effects on the LC3B protein levels. However, human spermatozoa did not show a significant increase of LC3B-II protein levels as mentioned for somatic cells after the incubation with CQ (359). After 24 h of incubation, CQ caused a decrease in the levels of LC3B-I and LC3B-II in a donor-dependent manner. According to these results, human spermatozoa might be influenced differently by CQ, autophagy might be a process which can predominately be influenced during spermatogenesis or the chosen time period was not appropriate. Although spermatozoa are free of lysosomes, they comprise lysosomal enzymes in the acrosome as well as in the cytoplasmic droplet of immature spermatozoa (477, 478). This suggests that the acrosome might be the modified form of a lysosome (29, 30). During maturation, the cytoplasmic droplet and lysosomal enzymes are reduced. The absence of lysosomes in spermatozoa might explain why spermatozoa do not react like somatic cells upon stimulation with CQ. We determined the effect of CQ on the capacitation by analysing tyrosine phosphorylation via western blot. The tyrosine phosphorylation of 4 proteins (molecular weight of 35; 40; 90 and 105 kDa) was decreased by the use CQ after an incubation period of 24 h. In contrast, in somatic cells CQ has been described to increase the tyrosine phosphorylation of the insulin receptor after insulin injection (479) and of the epidermal growth factor (EGF) receptor in EGF-stimulated cells (480).

Our study has certain limitations: (1) No transmission electron microscopy was performed to determine the number and maturation stage of autophagosomes (481) in human spermatozoa. (2) For western blot analysis, 40 µg protein were necessary. Since a sperm count of at least 100-120x10^6 sperms is required for the different incubation experiments, donor collective was restricted. To eliminate such recruitment bias, another method for the detection of autophagy should be used. (3) The incubation experiments with FK866 for 24 h and 72 h were not performed with one semen sample per donor, because sperm counts were not sufficient.

The recent study is a starting signal for further experiments within the male reproductive tract. Considering female reproduction, autophagy is occurring in fertilised oocytes and is essential for the preimplantation development (482). In this study, Atg5 null mice oocytes were fertilised either with Atg5 null sperm, which resulted in embryo lethality, or with wild-type sperm, which produced healthy pups. The authors suggest that the autophagy defi-

ciency is rescued by Atg5 derived from the zygote, but it might also be the sperm derived Atg5, which enables the oocyte to survive. This hypothesis supports our results of the presence of autophagy in spermatozoa.
The major finding of the present study was the detection of autophagy in human spermatozoa and testis. FK866 as an inhibitor of NAMPT was found to have an impact on LC3B expression. In immature spermatozoa, LC3B levels were higher compared with their mature counterparts. In mature spermatozoa, autophagy might not be induceable or functional as these cells are already differentiated. Autophagy as a process of surviving nutrient deprivation and prolonging lifespan might be carried out by immature spermatozoa due to the presence of more lysosomal enzymes.

6 Summary

Currently, the impact of obesity on fertility is extensively discussed. Several studies have shown the influence of increasing BMI on human male fertility. However, the investigation of the molecular pathogenesis of obesity-related subfertility was the major component of this study. We suggested that adipokines might be the potential link between obesity and male subfertility. The aim of this study was to evaluate the presence of adipokines in human male reproductive tract and their impact on sperm function parameters. In the first experiments, adipokine concentrations were determined in normal weight and overweight/obese men by ELISA. Adiponectin and progranulin concentrations in seminal plasma were significantly lower in overweight/obese men compared with normal weight men. Due to correlation analysis, the adipokines adiponectin and progranulin were suggested to function as positive markers for fertility prognosis, whereas chemerin tended to be a negative marker. Additionally, the comparison of sperm parameters in the overweight/obese group compared with the normal weight group resulted in decreased percentage of normal morphological spermatozoa and higher DFI values. These results support the already known detrimental effect of obesity on semen quality.

As the presence of adiponectin, chemerin, vaspin, leptin, resistin, progranulin and NAMPT in human seminal plasma was verified, we checked for the attendance of adipokines and their receptors in human spermatozoa and testis biopsies. Therefore, the presence of these proteins was investigated by immunofluorescence, immunohistochemistry and western blot analysis. Accordingly, we were able to detect adiponectin receptor 1, leptin and its receptor and NAMPT in spermatozoa and testis. The chemerin receptor was only detected in spermatozoa. The functionality of the adiponectin, chemerin and leptin receptors was investigated in spermatozoa by incubation experiments and subsequent analysis of sperm function parameters. Adiponectin, chemerin and leptin affected sperm motility. In addition, adiponectin and leptin also increased the tyrosine phosphorylation of proteins involved in capacitation. Adiponectin, chemerin and leptin increased activity of caspase 3&7 after incubation with non-capacitating medium, implicating the induction of apoptosis by these adipokines in human ejaculated spermatozoa.

In conclusion, this study provides evidence that adipokines are present in human seminal plasma and correlated with semen parameters. The link of adipokines to obesity-related subfertility was not finally resolved. Independent of BMI, progranulin might be a surrogate marker for male fertility. The results also indicate that human spermatozoa express functional receptors for the adipokines adiponectin, chemerin and leptin, which was proven by the impact of these adipokines on sperm motility, capacitation and apoptosis signalling cascade.

In the second part of this study, the source for NAMPT had to be elucidated in detail, as the concentrations of this adipokine in human seminal plasma were nearly 100-fold higher than in serum. It was hypothesised, that NAMPT might be expressed in and secreted by human ejaculated spermatozoa. Therefore we aimed to study the function of this adipokine. Accordingly, the NAMPT protein expression was investigated by immunofluorescence microscopy and western blot analysis. We discovered that NAMPT displayed a maturation dependent protein expression. Immature spermatozoa had significantly higher NAMPT protein and NAD^+ levels compared with mature counterparts, suggesting a special role in these sperm fractions. This result was confirmed by higher NAMPT concentrations in the supernatant of immature spermatozoa than in mature cells, implicating a secretion process. Moreover, the NAMPT activity was blocked by FK866 to investigate the effects on sperm function parameters. In primarily experiments, NAMPT inhibition was accompanied by increased motility and vitality, however this result could not be repeated in further experiments. Beyond, the inhibition of NAMPT activity did not influence the apoptosis signalling cascade, whereas capacitation was decreased donor-dependently.
Overall, the presence of NAMPT in human testis and spermatozoa was described for the first time, thereby NAMPT was suggested to be a maturation marker of human spermatozoa. NAMPT inhibition experiments should be repeated with immature and mature spermatozoa in order to verify NAMPT activity in spermatozoa. Consequently, NAD^+ levels as well as sperm function parameters should be determined in spermatozoa within different maturation stages after NAMPT inhibition.

In the third part of the study, the process of autophagy was investigated in human spermatozoa and testis. Autophagy is a life prolonging process and a form of cell death in somatic cells, which is induced by NAMPT inhibition. Recently, the presence of autophagy was detected in rat and stallion sperms. However, this process was not investigated before in human spermatozoa. We assume that human spermatozoa are able to perform autophagy due to NAMPT inhibition. In the current study, the expression of autophagy related proteins, LC3B and Beclin-1, was determined in human ejaculated spermatozoa by immunofluorescence and western blot analysis. The LC3B protein expression in testis was investigated by immunohistochemistry. Additionally, spermatozoa were incubated with the NAMPT inhibitor FK866 and the autophagic flux inhibitor chloroquine to determine the effect on LC3B protein expression. The experiments presented here, show that autophagy is detectable in human spermatozoa. In immature spermatozoa, the autophagy related proteins LC3B-I and LC3B-II were expressed in higher concentrations than in mature counterparts. However, the NAMPT inhibition resulted in a donor-dependent increase of LC3B and Beclin-1, suggesting an inter-individual regulation of autophagy in human

spermatozoa. In contrast to somatic cells, the inhibition of the autophagic flux by the incubation with chloroquine did not influence LC3B levels of spermatozoa. This implicates that either the autophagic flux in human spermatozoa is not susceptible by chloroquine or the autophagic flux can not be regulated once spermatozoa are differentiated.

In summary, the autophagy related proteins LC3B and Beclin-1 are present in human spermatozoa and testis. Immature spermatozoa might be more susceptible to autophagy than their mature counterparts. However, in the present study spermatozoa and testis samples were investigated at a specific moment in time. In future experiments, the process of autophagy should be studied at different points of time to investigate the function and regulation of this process in the male reproductive tract in detail.

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8 Supplement

8.1 Publications

Peer reviewed original research article

Thomas, S., Kratzsch, D., Schaab, M., Scholz, M., Grunewald, S., Thiery, J., Paasch, U., Kratzsch, J. (2013): Seminal plasma adipokine levels are correlated with functional characteristics of spermatozoa. Fertil. Steril. 99(5): 1256-1263.

Book chapter

Thomas, S., Grunewald, S., Paasch, U. (2012): Nahrungsergänzungsmittel, In: Harth,W, Brähler,E, Schuppe,H-C (Hrsg.). Praxishandbuch Männergesundheit. Berlin: MWV Medizinisch-Wissenschaftliche Verlagsgesellschaft, S.411-422

Oral presentation

Thomas, S., Kratzsch, D., Schaab, M., Grunewald, S., Kratzsch, J., Paasch, U. 2011 Adipokine Concentrations in Seminal Plasma are Correlated to Semen Quality in Normal-weight and Obese Men. Abstract CD 54. Symposium der Deutschen Gesellschaft für Endokrinologie 2011 OP-3-5

Poster presentations

Thomas, S., Grunewald, S., Paasch, U. Möglichkeiten und Grenzen der Beeinflussung der Spermienqualität durch Nahrungsergänzungsmittel im Rahmen der Adipositastherapie. 2010 J.Reproduktionsmed. Endokrinol. 7 (5): 438

Thomas, S., Kratzsch, J., Kratzsch, D., Schaab, M., Grunewald, S., Paasch, U. Adipokine concentrations in seminal plasma are correlated to semen quality in normal-weight and obese men. 2010 9th Leipzig Research Festival for Life Sciences 2010 p. 134

Kratzsch, D., Kratzsch, J., Thomas, S., Schaab, M., Grunewald, S., Paasch, U. Comparison of adipokine concentrations in serum and seminal plasma. 2010 9th Leipzig Research Festival for Life Sciences 2010 p. 135

Kratzsch, D., Thomas, S., Schaab, M., Grunewald, S., Paasch, U., Kratzsch, J. Comparison of Adipokine Concentrations in Serum and Seminal Plasma. Abstract CD 54. Symposion der Deutschen Gesellschaft für Endokrinologie 2011 PS1-06-6

Thomas, S., Kratzsch, D., Schaab, M., Grunewald, S., Paasch, U., Thiery, J., Kratzsch, J. 2011 Adipokines are Present in Seminal Plasma and Correlated to an Impaired Semen Quality in Obese Men. Clin Chem Lab Med 49:S443

Thomas, S., Schaab, M., Grunewald, S., Kratzsch, J., Paasch, U. Adipokine and adipokine receptor expression in human spermatozoa 2011 10th Leipzig Research Festival for Life Sciences 2011 p. 177

Thomas, S., Garten, A., Schaab, M., Grunewald, S., Kiess, W., Kratzsch, J., Paasch, U. NAMPT is Present in Human Semen. Abstract CD 55. Symposium der Deutschen Gesellschaft für Endokrinologie 2012 P-4-8

Thomas, S., Garten, A., Schaab, M., Grunewald, S., Kiess, W., Kratzsch, J., Paasch, U. NAMPT is Expressed and Released by Human Spermatozoa Depending on Maturation Stage 2012 J.Reproduktionsmed. Endokrinol. 9 (5): 396

Thomas, S., Garten, A., Schaab, M., Grunewald, S., Kiess, W., Kratzsch, J., Paasch, U Function of NAMPT in human spermatozoa. 2012 11th Leipzig Research Festival for Life Sciences 2012 p. 47

Thomas, S., Garten, A., Schaab, M., Grunewald, S., Kiess, W., Kratzsch, J., Paasch, U. NAMPT is expressed in human spermatozoa and testes. Abstract CD 56. Symposium der Deutschen Gesellschaft für Endokrinologie 2013 P86

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8.3 Chemicals

Product	Manufacturer	Catalogue number
0.2 M PBS/sodium azide pH 7.4	Dr. K. Hollborn & Söhne GmbH & Co KG, Leipzig, Germany	1013
4',6-Diamidino-2-Phenylindole (DAPI)	Sigma-Aldrich®, Germany	D9564
ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit	Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany	4303149
Acridine solution	was mixed and provided by the pharmacy	
Acrylamid/Bisacrylamid Rotiphorese Gel 30	Carl Roth GmbH + Co KG, Karlsruhe, Germany	3029.2
Adiponectin ELISA	Mediagnost, Reutlingen, Germany	E09
Adiponectin Receptor 1 antibody goat polyclonal	Novus Biologicals, Ltd., Cambridge, United Kingdom	NB-100-1518
Adiponectin, NATIVE, Human Serum	BioVendor GmbH, Heidelberg, Germany	RD162023050
AdipoR1 blocking peptide	Novus Biologicals, Ltd., Cambridge, United Kingdom	NB-100-1518PEP
Ammonium Persulfate (APS)	Carl Roth GmbH + Co KG, Karlsruhe, Germany	9592.2
Aniline blue solution	was mixed and provided by the pharmacy	
Annexin V Binding Buffer	Miltenyi Biotec, Bergisch Gladbach, Germany	130-092-820
anti-ChemR23 (R249) rabbit polyclonal	Bioworld Technology Co, Ltd., Nanjing, China	BS2199
Antigen target retrieval solution	Dako Deutschland GmbH, Hamburg, Germany	S1699
anti-NAMPT mAB (OMNI379)	Adipogen, Inc., Incheon, Korea	AG-20A-0034-C100
Anti-Phosphotyrosine, clone 4G10®	Upstate (Millipore), Billercia, USA	05-321
Aquatex	Merck Millipore, Merck KGaA, Darmstadt, Germany	1085620050
BCA Protein Assay Reagent (bicinchoninic acid)	Thermo Scientific Pierce Protein Biology Products, Bonn, Germany	23227
Beclin-1 (D40C5) Rabbit mAb	Cell Signaling Technology/New England Biolabs, GmbH, Frankfurt am Main, Germany	3495 S
Bovine anti-goat IgG (H+L)-HRPO, MinX Bo,Ck,Gp,Hs,Ho,Hu,Ms,Rb,Rt	Dianova GmbH, Hamburg, Germany	805-035-180
Bovine serum albumin	Sigma-Aldrich®, Germany	A9647
Bromophenolblue	Carl Roth GmbH + Co KG, Karlsruhe, Germany	A512.1
Buffered citric acid	Dr. K. Hollborn & Söhne GmbH & Co KG, Leipzig, Germany	8021745
Calcium Ionophore A23187	Sigma-Aldrich®, Germany	C7522-1MG
CD45 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany	130-045-801
Chemerin ELISA	Mediagnost, Reutlingen, Germany	E102

Chloroquine diphosphate salt	Sigma-Aldrich®, Germany	C6628-25G
Coomassie Blau R 250	Carl Roth GmbH + Co KG, Karlsruhe, Germany	3862.1
DePsipher Kit 100 Tests	Trevigen Inc., Gaithersburg, USA	TRE-6300-100-K
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich®, Germany	D8418
Dithiotreitol (DTT)	Sigma-Aldrich®, Germany	43815
DNA Molecular Weight Marker X (0.07-12.2 kbp)	Roche Diagnostics GmbH, Mann-heim, Germany	1 498 037
dNTPs (dATP, dCTP, dGTP, dTTP)	Promega, Madison, USA	U1511
Donkey anti-goat IgG (H+L) HRPO	Dianova GmbH, Hamburg, Ger-many	705-035-003
Donkey anti-goat IgG FITC	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	sc-2024
Dulbecco's PBS (10x)	PAA Laboratories GmbH, Cölbe, Germany	H15-011
Earle's Balanced Salt Solution	Life Technologies GmbH, Darm-stadt, Germany	24010-043
EnzyChrom NAD+/NADH As-say Kit	BioAssay Systems, Hayward, USA	E2ND-100
Ethidium bromide	SERVA Electrophoresis GmbH, Heidelberg, Germany	21238
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH + Co KG, Karlsruhe, Germany	CN06.1
FK-866	Adipogen, Inc., Incheon, Korea	AG-CR1-0011-M005
Formaldehyde 4 % solution	Otto Fischar GmbH & Co KG, Saarbrücken, Germany	27246
GAPDH antibody	Fitzgerald Industries International, Acton, USA	10R-G109a
GeneAmp® 10X PCR Buffer	Life Technologies GmbH, Darm-stadt, Germany	N8080129
Glacial acetic acid	J.T.Baker®, Avantor Performance Materials B.V	6052
Glutardialdehyde solution 25 %,	Merck Millipore, Merck KGaA, Darmstadt, Germany	104239
Glycerol solution, buffered	Dr. K. Hollborn & Söhne GmbH & Co KG, Leipzig, Germany	1111
Glycine	Carl Roth GmbH + Co KG, Karlsruhe, Germany	3908.1
Goat anti-mouse IgG-FITC	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	sc-2010
Goat anti-rabbit IgG-FITC	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	sc-2012
Green FLICA™ Caspase 3&7 Assay Kit (FAM-DEVD-FMK)	ImmunoChemistry Technologies, LLC, Bloomington, USA	94
HTF-Medium with Gentamicin á 100 ml	Irvine Scientific, Santa Ana, USA	90125
Immersol	Carl Zeiss AG, Feldbach, Schweiz	518 N
ISolate® Sperm Separation Medium 2x50ml	Irvine Scientific, Santa Ana, USA	99264
LC3B (D11) XP® Rabbit mAb	Cell Signaling Technology/New England Biolabs, GmbH, Frank-furt am Main, Germany	3868 S
Lectin from *Pisum sativum*	Sigma-Aldrich®, Germany	L0770

Leptin RIA	Mediagnost, Reutlingen, Germany	R40
LiChrosolv®	Merck Millipore, Merck KGaA, Darmstadt, Germany	115333
Liquid DAB+ Substrate Chromogen System	Dako Deutschland GmbH, Hamburg, Germany	K346711
Magnesium chloride solution	Sigma-Aldrich®, Germany	M8787
Mercaptoethanol	Carl Roth GmbH + Co KG, Karlsruhe, Germany	4227.1
Methanol	J.T.Baker®, Avantor Performance Materials B.V	8045
Mounting Medium	Sigma-Aldrich®, Germany	M1289-10ML
NAMPT (Visfatin/PBEF) (human) ELISA Kit	Adipogen, Inc., Incheon, Korea	AG-45A-0006
New Fuchsin Substrate System	Dako Deutschland GmbH, Hamburg, Germany	K0698
NP-40 (Igepal)	Sigma-Aldrich®, Germany	74385
Ob (A-20)	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	sc-842
Ob (A-20) P	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	sc-842 P
ObR (C-20)	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	sc-1832
ObR (C-20) P	Santa Cruz Biotechnology, Inc., Heidelberg, Germany	sc-1832 P
PBS pH 7.2, 0.5 % BSA und 2 mM EDTA	was mixed and provided by the pharmacy	
Percoll	GE Healthcare Europe GmbH, Freiburg, Germany	17-0891-02
Peroxidase conjugated Affini Pure Donkey Anti-Goat IgG (H+L)	Dianova GmbH, Hamburg, Germany	705-035-003
Peroxidase conjugated goat anti-rabbit IgG	Dianova GmbH, Hamburg, Germany	111-035-003
Peroxidase Horse Anti-Mouse IgG Antibody	Vector Laboratories Inc., Burlingame, USA	PI-2000
Phenylmethylsulfonyl fluoride (PMSF)	Carl Roth GmbH + Co KG, Karlsruhe, Germany	6367
Phosphate buffered saline (PBS)	PAA Laboratories GmbH, Cölbe, Germany	H15-011
Picrin acid (aqueous)	Dr. K. Hollborn & Söhne GmbH & Co KG, Leipzig, Germany	
Progranulin ELISA	Mediagnost, Reutlingen, Germany	E103
Proteinase K (Ready-to-use)	Dako Deutschland GmbH, Hamburg, Germany	S3020
Recombinant human chemerin	provided by S. Schultz/A.G. Beck-Sickinger, Institute of Biochemistry, University of Leipzig	
Recombinant Human Leptin	PeproTech, Hamburg, Germany	300-27
Resistin ELISA	Mediagnost, Reutlingen, Germany	E50
SignalStain® Boost IHC Detection Reagent (HRP, Mouse)	Cell Signaling Technology/New England Biolabs, GmbH, Frankfurt am Main, Germany	8125 P
SignalStain® Boost IHC Detection Reagent (HRP, Rabbit)	Cell Signaling Technology/New England Biolabs, GmbH, Frank-	8114

	furt am Main, Germany	
Sodium chloride (NaCl)	Carl Roth GmbH + Co KG, Karlsruhe, Germany	HN00.2
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH + Co KG, Karlsruhe, Germany	2326.1
Sodium hydrogen carbonate	Carl Roth GmbH + Co KG, Karlsruhe, Germany	8551.1
Sodium orthovanadate	Sigma-Aldrich®, Germany	S6508
Sodium-Deoxycholat	Sigma-Aldrich®, Germany	D6750
Spectra™ Multicolor Broad Range Protein Ladder	Fermentas, Fisher Scientific GmbH, Schwerte, Germany	SM1841
SuperScript® II Reverse Transcriptase	Life Technologies GmbH, Darm-stadt, Germany	18080044
SuperSensitive™ Link-Label IHC Detection System	BioGenex, Fremont, USA	QA900-9L
SuperSignal West Femto Chemiluminescent Substrate	Thermo Scientific Pierce Protein Biology Products, Bonn, Germany	34095
Tetramethylethylenediamine (TEMED)	Carl Roth GmbH + Co KG, Karlsruhe, Germany	2367.3
Tris	Carl Roth GmbH + Co KG, Karlsruhe, Germany	5429.1
Triton X-100	Carl Roth GmbH + Co KG, Karlsruhe, Germany	3051.3
TRIzol® Reagent	Life Technologies GmbH, Darm-stadt, Germany	15596-026
Tween-20	Merck Millipore, Merck KGaA, Darmstadt, Germany	655204
UltraPure™ Agarose	Life Technologies GmbH, Darm-stadt, Germany	16500100
Vaspin ELISA	Adipogen, Inc., Incheon, Korea	AG-45A-0017

8.4 Material and equipment

Material	**Manufacturer**
50 ml Tubes	Sarstedt AG & Co, Nümbrecht, Germany
BD FACSCalibur	BD Biosciences, Heidelberg, Germany
Berthold-Gammacounter LB 2111	Berthold Technologies, Bad Wildbad, Ger-many
Biofuge 28 RS	Heraeus Sepatech, Osterode, Germany
Biomedical freezer, -40°C	Sanyo, Leicestershire, UK
BioRad Mini Protean 3	Bio-Rad Laboratories GmbH, Munich, Ger-many
Centrifuge 5415 C	Eppendorf GmbH, Engelsdorf, Germany
Centrifuge 5430	Eppendorf GmbH, Engelsdorf, Germany
Centrifuge Universal 30-G	Hettich Zentrifugen, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany
Compartment dryer Incuce II	MMM Medcenter, Planegg, Germany
Computer-aided sperm analysis (CASA)	IVOS, Hamilton-Thorne Biosciences, Inc., Beverly, USA
Cover slip 22x22 mm	Menzel-Gläser, Gerhard Menzel GmbH, Braunschweig, Germany
Cover slip 24x50 mm	Menzel-Gläser, Gerhard Menzel GmbH, Braunschweig, Germany

DakoCytomationPen	Dako Deutschland GmbH, Hamburg, Germany
Eppendorf Safe Lock 1.5 ml	Eppendorf AG, Hamburg, Germany
Eppendorf Safe Lock 2 ml	Eppendorf AG, Hamburg, Germany
Eppendorf-pipette	Eppendorf AG, Hamburg, Germany
Freezer -80°C	Heraeus Thermo, Langenselbold, Germany
Freezer, -20 C	Liebherr, Bulle, Switzerland
G:Box, GeneSnap from Syngene	Syngene, A Division of Synoptics Ltd., Cambridge, UK
Gel Blotting Paper GB 005	Whatman™, Part of GE Healthcare Life Sciences, Munich, Germany
Handy Aspirator WP-25	Yamato, Scientific Co., LTD, Japan
Imager G:Box EF	VWR, Darmstadt, Germany
Incubator C16	Labotect GmbH, Göttingen, Germany
Leja Standard Count 2 Chamber Slide, 20 micron	Leja, Nieuw-Vennep, Netherlands
MACS and Octo MACS	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS Separation Columns MS Columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnetic stirrer Color Squid	IKA®-Werke GmbH & Co.KG, Staufen, Germany
Microplate Reader BioTek Synergy2	BioTek, Bad Friedrichshall, Germany
Microscope Olympus CX 21	Olympus Deutschland GmbH, Hamburg, Germany
Microscope Olympus BX 41	Olympus Deutschland GmbH, Hamburg, Germany
Mini Trans-Blot Cell	Bio-Rad Laboratories GmbH, Munich, Germany
Mini-PROTEAN Electrophoresis System	Bio-Rad Laboratories GmbH, Munich, Germany
Modular System	Roche, Mannheim, Germany
Multichannel-pipette	Eppendorf AG, Hamburg, Germany
NanoDrop	Thermo Fisher Scientific Inc., Wilmington, USA
Neubauer chamber improved	Marienfeld Laboratory Glassware, Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Pipette tips (10, 200, 1000 µl)	Sarstedt AG & Co, Nümbrecht, Germany
Plate shaker KS 125 basic	IKA®-Werke GmbH & Co.KG, Staufen, Germany
Plate shaker, Rotamax 120	Heidolph Instruments, Schwabach, Germany
Power supply LNG 350-0.6	Phase GmbH, Lübeck, Germany
Protran BA 83, Nitrocellulose 0.2 µm	GE Healthcare, Life Sciences, Whatman™
Rack	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Rocking Shaker Edmund Bühler TL-10	Edmund Bühler GmbH, Hechingen, Germany
Rotilabo ®-PCR Racks	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Rotilabo ®-Racks	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Semi Dry Blotter Pegasus	Phase GmbH, Lübeck, Germany

Slide with dull edge	Menzel-Gläser, Gerhard Menzel GmbH, Braunschweig, Germany
Slide without dull edge	Menzel-Gläser, Gerhard Menzel GmbH, Braunschweig, Germany
Sonotrode MS1	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Superfrost® Plus slide	Thermo Scientific, Menzel-Gläser, Menzel GmbH & Co KG, Braunschweig, Germany
Threaded bottle 100 ml	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Tube 4.5 ml, konisch	Sarstedt AG & Co, Nümbrecht, Germany
Tube Thermostate	Gebr. Liebisch GmbH & Co. KG Labortechnik, Bielefeld, Germany
Ultrasonic desintegrator UP100H	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Varifuge 3.OR	Heraeus Sepatech, Osterode, Germany
Vortex-Genie 2	Scientific Industries, Bohemia, N.Y., USA

8.5 Western blot buffers

Supplemental Table 1 Composition and storage of buffers used for western blotting.

Upper buffer	0.5 M Tris pH 6.8	Storage at fridge
Lower buffer	1.5 M Tris 0.4 % SDS pH 8.8	Storage at fridge
4x Laemmli buffer	2.4 ml 1 M Tris pH 6.8 0.8 g SDS 4 ml glycerol 1 ml β-mercaptoethanol 0.01 % bromophenolblue 2.8 ml aqua dest	Storage at room temperature or -20 °C as aliquots
Loading buffer	25 mM Tris 192 mM glycine 0.1 % SDS pH 8.3	Storage at fridge
Transfer buffer	25 mM Tris 192 mM glycine 20 % methanol pH 8.3	Storage at fridge
Washing buffer	0.3 % Tween.20 0.05 % Triton X-100 TBS (20 mM Tris, 150 mM NaCl, pH 7.4)	Storage at fridge
Blocking buffer	3 % BSA 0.1 % Tween 20 20 mM Tris 150 mM NaCl	Storage at fridge
Antibody buffer	3 % BSA 0.1 % Tween-20 20 mM Tris 150 mM NaCl	Storage at fridge

8.6 Antibody incubation conditions for western blotting, immunofluorescence and immunohistology

Supplemental Table 2 Antibody incubation conditions for western blotting. *minimal cross-reaction to bovine, chicken, guinea pig, syrian hamster, horse, human, mouse, rabbit, rat serum proteins

	Antibody	Final dilution	Incubation
Primary antibody	Anti-Phosphotyrosine, clone 4G10, mouse anti-human, monoclonal	1:1000	1 h at RT
	GAPDH, mouse anti-human, monoclonal	1:15000	1 h at RT or over night at 4 °C
	Leptin (A-20) rabbit anti-human, polyclonal	1:200	Over night at 4 °C
	Leptin receptor (ObR, C-20) goat anti-human, polyclonal	1:400	Over night at 4 °C
	Adiponectin Receptor 1 goat anti-human, polyclonal	1:1000	Over night at 4 °C
	Beclin-1 (D40C5) rabbit anti-human, monoclonal	1:1000	Over night at 4 °C
	LC3B (D11) XP® rabbit anti-human, monoclonal	1:1000	Over night at 4 °C
	NAMPT (OMNI379) mouse anti-human, monoclonal	1:2000	Over night at 4 °C
Secondary antibody	Peroxidase Horse Anti-mouse IgG (H+L)	1:10000	1 h at RT
	Peroxidase conjugated AffiniPure Goat Anti-Rabbit IgG (H+L)	1:10000-20000	1 h at RT
	Peroxidase conjugated AffiniPure Bovine anti-goat IgG (H+L)*	1:40000	1 h at RT
	Goat anti-rabbit IgG (H&L), HRP linked Antibody	1:2000 for Beclin-1 and LC3B	1 h at RT

Supplemental Table 3 Antibody incubation conditions for immunofluorescence.

	Antibody	Final dilution	Incubation
Primary antibody	Leptin (A-20) rabbit anti-human, polyclonal	1:10	Over night at 4 °C
	Leptin receptor (ObR, C-20) goat anti-human, polyclonal	1:10	Over night at 4 °C
	ChemR23(R249) rabbit, anti-human, polyclonal	1:50 1:100	Over night at 4 °C
	Adiponectin Receptor 1 goat anti-human, polyclonal	1:20	Over night at 4 °C
	NAMPT (OMNI379) Mouse anti-human, monoclonal	1:50	Over night at 4 °C
	LC3B (D11) XP® rabbit anti-human, monoclonal	1:20	Over night at 4 °C
Secondary antibody	Goat anti-rabbit IgG-FITC	1:100	1 h at RT
	Donkey anti-goat IgG FITC	1:100	1 h at RT
	Goat anti-mouse IgG-FITC	1:100	1 h at RT
Blocking peptide	Leptin	2 µg/50 µl	Over night at 4 °C
	ObR	2 µg/50 µl	Over night at 4 °C
	AdipoR1	4 µg/50 µl	Over night at 4 °C

Supplemental Table 4 Antibody incubation conditions for immunohistochemistry.

Primary antibody	Final dilution	Incubation	Secondary antibody
Leptin (A-20) rabbit anti-human, polyclonal	1:100 1:200	Over night at 4 °C	Super Sensitive Link-Label IHC Detection System
Leptin receptor (ObR, C-20) goat anti-human, polyclonal	1:50 1:100	Over night at 4 °C	Peroxidase conjugated Affini Pure Donkey Anti-Goat IgG (H+L)
Adiponectin Receptor 1 goat anti-human, polyclonal	1:50 1:100	Over night at 4 °C	Peroxidase conjugated AffiniPure Bovine anti-goat IgG (H+L)
NAMPT (OMNI379) Mouse anti-human, monoclonal	1:50	Over night at 4 °C	SignalStain Boost IHC Detection Reagent (HRP, Mouse)
LC3B (D11) XP® rabbit anti-human, monoclonal	1:25 1:50	Over night at 4 °C	SignalStain Boost IHC Detection Reagent (HRP, Rabbit)

Curriculum Vitae

Personal data

Name	Stephanie Juliane Thomas
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Education and career

1992-1996	Franz-Mehring Grundschule, Leipzig
1996-2004	Neue Nikolaischule (Gymnasium), Leipzig
2004-2009	Degree in Nutritional Science Diploma thesis: Der Einfluss von Leptin in Interaktion mit Insulin auf den Lipidstoffwechel in Rattenhepatomzellen
12/2009-03/2013	Research associate at the Medical Faculty of the University of Leipzig, Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics and Department of Dermatology, Venerology and Allergology, European Training Center of Andrology Doctoral thesis: The impact of adipokines on human male fertility

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Date Signature

Acknowledgement

First of all, I want to thank Prof. Dr. rer. nat. habil. Jürgen Kratzsch from the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University of Leipzig, Prof. Dr. med. habil. Uwe Paasch, and PD Dr. med. habil. Sonja Grunewald from the Department of Dermatology, Venerology and Allergology, European Training Center of Andrology, University of Leipzig for the chance to perform this dissertation under their guidance. Moreover, I thank Prof. Dr. troph. habil. Gabriele Stangl from the Faculty of Natural Sciences III, Institute for Agricultural and Nutritional Sciences, Martin-Luther-University Halle-Wittenberg for supervising this dissertation.

Additionally, I appreciate the technical support of the co-workers from the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics and the Department of Dermatology, Venerology and Allergology, European Training Center of Andrology, University of Leipzig. Special thanks to Dr. med. Dorothea Kratzsch for the support. Thanks to Dr. rer. nat. Michael Schaab, who gave me an introduction of molecular genetic methods. I thank Dr. rer. nat. Antje Garten, Susanne Schuster, Melanie Penke and Theresa Gorki for the discussions about NAMPT. Futhermore, I want to thank Susanne Pyttel for illuminating and critical discussions and the great support especially during the pregnancy and the parental leave. Thanks to Dr. rer. nat. Kristina Sass from the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University of Leipzig.

I want to thank S. Schultz and A.G. Beck-Sickinger from the Institute of Biochemistry, University of Leipzig, who kindly provided human recombinant chemerin and A. Paradowska from the Urology, Children Urology, and Andrology, University Hospital Giessen and Marburg, Giessen, Germany for providing human spermatozoa DNA.

Additionally, I thank Jurij Riammer, who supported me in every situation, encouraged me to continue till the end and for his comprehension for long hours at work.

Special thanks go to my parents for the support throughout my whole life and especially during the parental leave. They encouranged me during my study and dissertation with great enthusiasm and helped through difficult times, when I doubted myself. They never questioned my decisions in an open way, but got me to understand the consequences of my doings in a hidden way. Furthermore, my mother-in-law helped me through hard times during parental leave and took care of my daughter as I wrote my dissertation.

In the end, I want to thank Undine Jakob and Anita Arslanow, who are not only fellow students of mine but also great friends. They supported me both in difficult times, handed out advices and encouraged me to go on and to never doubt myself.

Last but not least, thanks to my daughter for her patience during the last months. Her cheerfulness gave me the strength to go on with this work.

Declaration under Oath

I declare under penalty of perjury that this thesis “The impact of adipokines on human male fertility” is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

I declare that the thesis has not been used previously at this or any other university for reasons of graduation.

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Date Signature