

ASSOCIATIONS OF ADIPOSE TISSUE CHARACTERISTICS IN
NON-ALCOHOLIC FATTY LIVER DISEASE AND DISTURBED
SEX STEROID PROFILE IN OBESE MALE PATIENTS

Marlies Bekaert

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LIST OF ABBREVIATIONS

ACC	acetyl coenzyme A carboxylase
ADA	American Diabetes Association
ADT	androgen deprivation therapy
AFABP	adipocyte-fatty-acid-binding protein
ALT	alanine aminotransferase
AMPK	AMP-activated protein kinase
aPKC	atypical protein kinase C
AR	androgen receptor
ARKO	androgen receptor knockout
ArKO	aromatase knockout
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AU	arbitrary units
BAT	brown adipose tissue
BMI	body mass index
BP	blood pressure
CMKLR	chemokine-like receptor
CT	computed tomography
CTGF	connective tissue growth factor
CV	coefficients of variation
CVD	cardiovascular diseases
CYP	cytochrome P450
DAG	diacylglycerol
DHT	dihydrotestosterone
DXA	Dual-energy X-ray absorptiometry
E ₂	Estradiol
EASD	European Association for the Study of Diabetes
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
FA	fatty acids
FAS	fatty acid synthase
FFA	free fatty acids
FOXO1	forkhead box protein O1
FSH	follicle stimulating hormone
FT	free testosterone
G6P	glucose-6-phosphatase
GGT	γ-glutamyltransferase
GLP-1	glucagon-like peptide-1
GnRH	gonadotropin-releasing hormone
GSK3β	glycogen synthase kinase 3β
HCC	hepatocellular carcinoma
HDL	high-density lipoprotein
HOMA-IR	homeostasis model of assessment for insulin resistance

HPG	hypothalamic-pituitary-gonadal
HSC	hepatic stellate cells
hs-CRP	high-sensitivity C-reactive protein
HSD	hydroxysteroid dehydrogenase
HSL	hormone-sensitive lipase
IKK	I κ B kinase
IL	interleukin
IR	insulin resistance
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL	low-density lipoprotein
LH	luteinizing hormone
LOQ	limit of quantification
LPL	lipoprotein lipase
MAPK	Ras/mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MetS	metabolic syndrome
MO	morbidly obese
MRI	magnetic resonance imaging
mTorc1	mammalian target of rapamycin complex 1
NAFLD	nonalcoholic fatty liver disease
NAS	NAFLD activity score
NASH	nonalcoholic steatohepatitis
NCEP-ATPIII	National Cholesterol and Education Program - Adult Treatment Panel III
NHANES	National Health and Nutrition Examination Survey
NEFA	non-esterified fatty acid
NF κ B	nuclear factor- κ B
PCR	polymerase chain reaction
PDK 1	phosphoinositide-dependent protein kinase 1
PEPCK	phosphoenolpyruvate carboxykinase
PGC1 α	peroxisome proliferator-activated receptor- γ co-activator 1 α
PI3K	phosphatidylinositol 3-kinase
PIP ₂	phosphatidylinositol (4,5)-bisphosphate
PIP ₃	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B
PKC	protein kinase C
PPAR γ	peroxisome proliferator-activated receptor gamma
RBP4	retinol-binding protein 4
RCT	randomized controlled trials
ROS	reactive oxygen species
SAT	subcutaneous adipose tissue
SFRP4	secreted frizzled-related protein 4
SHBG	sex-hormone-binding globulin
SREBP-1c	sterol regulatory element-binding protein-1c

SS	simple steatosis
T	testosterone
T2D	type 2 diabetes
TF	transcription factors
TG	triglycerides
TLR4	toll-like receptor 4
TNF- α	tumor necrosis factor- α
TZD	thiazolidinediones
VAT	visceral adipose tissue
VLDL	very-low-density lipoproteins
WAT	white adipose tissue
WC	waist circumference
WHO	World Health Organization

SAMENVATTING

Onze huidige samenleving wordt uitgedaagd door een steeds toenemende prevalentie van obesitas. Dit wordt veroorzaakt door een overvloed aan voeding en een toenemende sedentaire levensstijl en is geassocieerd met belangrijke metabole complicaties zoals type 2 diabetes, *non-alcoholic fatty liver disease* (NAFLD) en hart- en vaatlijden. Hoewel obesitas gedefinieerd wordt als een abnormale en overmatige opstapeling van vetweefsel doorheen het lichaam met negatieve impact op de gezondheid, blijft de exacte pathofysiologie die leidt tot de gevolgen ervan nog steeds onvolledig gekend. Op heden wordt het steeds duidelijker dat een aantal signaalwegen kunnen interageren en resulteren in de ontwikkeling van metabole ziekten. Als gevolg van de overmaat aan energie die opgenomen wordt zullen de vetweefsel depots zich gaan uitzetten, wat leidt tot ontwikkeling van grotere vetcellen. Uiteindelijk zal dit resulteren in disfunctie van het vetweefsel, dat gekarakteriseerd wordt door een aangetast lipide metabolisme met verhoogde vrijstelling van vetzuren, lokale inflammatie en een verstoorde vrijstelling van adipokines (signaalstoffen afkomstig uit de vetcellen). Deze lekkage van lipiden vanuit de klassieke vetweefsel depots geeft aanleiding tot ectopische vetaccumulatie in organen en weefsels zoals de lever, pancreas, spier en hart, wat bijdraagt tot de ontwikkeling van metabole complicaties. Bovendien vermoedt men dat inflammatoire factoren en het verstoord adipokine secretieprofiel ook bijdraagt tot deze gevolgen.

NAFLD wordt vaak aangeduid als de hepatische manifestatie van obesitas en omvat een breed spectrum aan leverziekten, gaande van eenvoudige leververvetting (*simple fatty liver disease of steatose*) tot *non-alcoholic steatohepatitis* (NASH) en zelfs eindstadium levercarcinoom. Hoewel het nog steeds onvolledig gekend is hoe obesitas leidt tot NAFLD, wordt verondersteld dat insulineresistentie (IR) en disfunctioneel vetweefsel centrale componenten vormen in de pathogenese van NAFLD. Het wordt steeds meer erkend dat een verstoord secretiepatroon van adipokines een centrale rol zou kunnen spelen bij de ontwikkeling van metabole ziekten, inclusief NAFLD en de progressie naar NASH. Ter illustratie, serumspiegels van adiponectine, één van de bekendste adipokines, zijn verlaagd bij obesitas en worden geassocieerd met zowel IR als de ernst van NAFLD. Echter, recent werden nog veel meer adipokines beschreven (> 700), waarvan hun rol in de ontwikkeling van obesitas-gerelateerde aandoeningen zoals NAFLD nog onthuld moet worden.

Nog een gekende bijwerking van obesitas is een verstoord metabolisme van de geslachtshormonen. Bij obese mannen is het voornaamste geslachtshormoon testosteron (T) vaak verlaagd. Hepatische IR die leidt tot een laag *sex hormone binding globulin* (SHBG) gehalte vormt een belangrijke oorzaak voor dit

laag T gehalte. Echter, bij ernstige obesitas ziet men eveneens een onderdrukking van de hypothalame-hypofysaire-gonadale as. Ook hiervan is de pathofysiologie nog steeds onduidelijk en er bestaan verschillende hypothesen. Een verstoorde hypothalame signalering van leptine bijvoorbeeld, werd verondersteld hierin een mogelijk mechanisme te zijn. Een andere verklaring zou een verhoogde enzymatische aromatase activiteit in de vergrote adipocyten kunnen zijn, welke leiden tot een grotere omzetting van T naar estradiol. Dit zou dan op zich kunnen leiden tot een grotere suppressie ter hoogte van de hypothalame-hypofysaire-gonadale as. Gezien T normaal belangrijke anabole acties vertoont en de lichaamssamenstelling gunstig zou kunnen beïnvloeden, is meer inzicht in de pathofysiologie van deze lage androgeen gehalten van groot belang alsook in de gevolgen ervan bij obese mannen.

Om meer inzicht te krijgen in deze obesitas-gerelateerde complicaties, was het doel van dit proefschrift om het disfunctioneel vetweefsel bij obese patiënten te onderzoeken alsook het verband met biopsie-geverifieerd NAFLD en het verstoord gehalte in geslachtshormonen bij mannen te bestuderen. In het bijzonder werd getracht om de mogelijke rol van adipokines in NAFLD te analyseren en een beter zicht te krijgen op de complexe relatie tussen de lichaamssamenstelling, geslachtshormonen en metabole parameters bij obese mannen.

Hoofdstuk I bevat een algemene achtergrond omtrent de pathofysiologie van obesitas en de gerelateerde metabole complicaties op basis van de huidig beschikbare literatuur. Daarnaast worden de onderzoeksdoeleinden in dit hoofdstuk beschreven. De studiepopulaties en methodologie die doorheen dit proefschrift gebruikt werden worden vermeld in **hoofdstuk II**.

In **hoofdstuk III** werden associaties bestudeerd tussen niet-klassieke adipokines en de histopathologische ernst in patiënten met biopsie-geverifieerd NAFLD. Vooreerst werd een systematische review uitgevoerd omtrent de huidige kennis over recent beschreven adipokines en hun relatie met lever histologie in patiënten met NAFLD (**hoofdstuk 3.1**). Hoewel chemerine, *adipocyte-fatty-acid-binding protein* (AFABP) en resistine onafhankelijk geassocieerd werden met leverschade en steatose in verschillende studies, vertoonden deze onderzoeken vaak te veel verschillen om hun mogelijke bijdrage in NAFLD voldoende te bewijzen. In het algemeen blijft de beschikbare data over de impact van deze recente adipokines op de progressie van NAFLD beperkt, met een grote heterogeniteit tussen de studieresultaten en deze werden vaak gebaseerd op kleine patiëntengroepen. Deze review benadrukt de dringende noodzaak aan longitudinale studies met grotere en meer homogene patiëntengroepen en met het gebruik van gestandaardiseerde meetmethoden om de concentraties van adipokines te bepalen.

In **hoofdstuk 3.2.** werd een selectie gemaakt van nieuwere adipokines, waarvan een bijdrage in NAFLD verwacht werd op basis van *in vitro* data. De histologische parameters in leverbiopten en zowel het serumgehalte als de expressie in visceraal vetweefsel van deze adipokines werden onderzocht in een cross-sectionele studie met obese patiënten van middelbare leeftijd met NAFLD. Het doel van deze studie was te onderzoeken of deze adipokines onafhankelijke associaties vertoonden met de graad van steatose, inflammatie, ballooning in de levercellen en/of fibrose in leverbiopten, om zo hun mogelijke bijdrage in de ontwikkeling van NAFLD en/of de progressie ervan te verduidelijken. Gezien van de adipokines afkomstig van visceraal vetweefsel verondersteld wordt dat zij direct aan de lever geleverd worden via de portale vene, werd zowel het serumgehalte als de viscerale expressie gemeten van adiponectine, omentine, chemerine, *monocyte chemoattractant protein-1* (MCP-1) en *secreted frizzled-related protein 4* (SFRP4). Hierbij werd bevestigd dat de serumspiegels van adiponectine een negatieve associatie vertonen met de NAFLD *activity score* (NAS), hoewel deze associatie verdween na correctie met IR. Deze bevinding suggereert dat lage circulerende adiponectinespiegels kunnen bijdragen tot IR, wat op zijn beurt NAFLD kan beïnvloeden. Vervolgens was de chemerine expressie in visceraal vetweefsel verlaagd en negatief geassocieerd met NAS en hepatische steatose, onafhankelijk van de leeftijd, BMI en IR. Dit doet vermoeden dat een lagere expressie van chemerine in visceraal vetweefsel van obese patiënten betrokken kan zijn in de pathofysiologie van NAFLD, hoewel prospectieve studies op grotere schaal noodzakelijk zijn om onze bevindingen te bevestigen.

De link tussen disfunctioneel vetweefsel en geslachtshormonen werd onderzocht in ernstig obese mannen in vergelijking met gezonde controle mannen (**hoofdstuk IV**). Het doel was om mogelijke determinanten van totaal en vrij T (TT en FT) te bepalen, met de nadruk op disfunctioneel vetweefsel, metabole parameters en de expressie van het aromatase enzym in vetweefsel. In deze studie konden geen verschillen in de *in situ* aromatase expressie, zowel in subcutaan als visceraal vetweefsel, aangetoond worden tussen obese mannen (met of zonder type 2 diabetes) en mannen met een normaal gewicht. Deze bevinding werkt de hypothese rond verhoogde aromatase activiteit tegen. Echter, de vetcelgrootte in subcutaan vetweefsel was omgekeerd geassocieerd met zowel TT als FT spiegels, na multivariate regressieanalyse gecorrigeerd voor leeftijd, groep (controle versus obesitas/diabetes), IR en triglycerides. Deze resultaten suggereren een direct negatieve impact van de vetcelgrootte, maar niet IR, op de TT en FT gehalten bij obese mannen, wat de mogelijke rol van disfunctioneel vetweefsel in het verstoord profiel van geslachtshormonen bij obese mannen benadrukt.

Finaal worden de belangrijkste bevindingen van onze studies samengevat en besproken in **hoofdstuk V**, met de nadruk op: de kenmerken van NAFLD bij obesitas, de associaties tussen adipokines en leverhistologie bij patiënten met NAFLD met een bijzondere belangstelling voor chemerine, en de mogelijke rol van disfunctioneel vetweefsel in lage T waarden bij obese mannen. Bovendien worden in dit hoofdstuk ook de klinische relevantie van deze bevindingen en perspectieven op toekomstig onderzoek besproken.

Tot slot vormen de bevindingen van dit proefschrift aanvullend bewijs over de impact van disfunctioneel vetweefsel bij obesitas, met een mogelijke rol in de ontwikkeling van NAFLD en het verstoord profiel van geslachtshormonen bij obese mannen. Hoewel prospectieve studies op grotere schaal noodzakelijk zijn om deze bevindingen te bevestigen en uit te breiden, suggereren ze dat het herstellen van de normale functie van vetweefsel en/of het voorkomen van disfunctie zou kunnen helpen om de obesitas-gerelateerde bijwerkingen tegen te werken. Bovendien zou de farmacologische manipulatie van adipokines in de toekomst een mogelijk alternatieve therapeutische optie kunnen bieden.

SUMMARY

Our society is challenged to combat with a continuously growing prevalence of obesity. This is due to ample food supplies and more sedentary lifestyles and is associated with important metabolic comorbidities such as type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD). While obesity has been defined as abnormal and excessive accumulation of adipose tissue throughout the human body that interferes with health, the exact pathophysiology causing its consequences remains to be fully elucidated. Nowadays, it is becoming clear that a number of pathways may interact, resulting in the development of metabolic disease. In response to an excessive energy supply, adipose tissue depots will expand with larger adipocyte cell sizes. At a certain point, this leads to adipose tissue dysfunction, characterized by a disturbed lipid metabolism with elevated release of fatty acids, local tissue inflammation and an impaired release of adipokines. This overspill of lipids outside the 'classical' adipose tissue depots leads to ectopic lipid deposition in organs and tissues such as the liver, pancreas, muscle and heart, contributing to the development of metabolic comorbidities. In addition, a contribution of inflammatory factors and the impaired adipokine profile to these consequences is suspected.

NAFLD is often referred to as the hepatic manifestation of obesity, and encompasses a wide spectrum of liver diseases ranging from simple fatty liver disease (steatosis) to non-alcoholic steatohepatitis (NASH) and even end-stage liver disease. Although it is still incompletely understood how obesity leads to NAFLD, insulin resistance (IR) and adipose tissue dysfunction are suggested to be central pathogenic components of overall NAFLD. It is increasingly recognized that an impaired pattern in adipokine secretion could play a pivotal role in the development of metabolic diseases, including NAFLD and the progression to NASH. For instance, serum adiponectin levels are lowered in obesity and associated with both IR and NAFLD severity. However, many more adipokines (> 700) have been described recently, and their possible role in the development of obesity-related disorders such as NAFLD remains to be explored.

Another well-known manifestation of obesity is disturbance of the normal sex steroid metabolism. Obese men often present with low levels of the principal male sex steroid testosterone (T). A major factor for these decreased T levels relies on lower levels of the sex hormone binding globulin (SHBG), probably resulting from hepatic IR. In morbid obesity, however, a true downregulation of the hypothalamic-pituitary-gonadal axis can be observed. Pathophysiology thereof is still unclear and several hypotheses still stand. For instance, disturbed hypothalamic signaling of another adipokine,

leptin, has been proposed as a possible mechanism. Another possibility might be increased aromatase enzyme activity at the enlarged adipose tissue depot leading to an increased conversion of T to estradiol, which then would lead to enhanced suppression at hypothalamic-pituitary level. As T is considered to display important anabolic actions and might favorably influence body composition, insights in the pathophysiology of these low androgen levels and its consequences in obese men is of interest.

To gain more insight into these obesity-related phenomena, this thesis aimed to examine adipose tissue dysfunction in obese subjects and to investigate its link with biopsy-proven NAFLD and disturbed sex steroid levels in men. In particular, we aimed to investigate the potential role of adipokines in NAFLD and to gain a better view on the complex relationship between body composition, sex steroid levels and metabolic parameters in obese men.

In **chapter I**, a general background with a review of current available literature regarding the pathophysiology of obesity and its metabolic comorbidities is given together with our research aims. The study populations and methodology used throughout this thesis are reported in **chapter II**.

In **chapter III**, we investigated the associations between non-classical adipokines and histopathological severity in biopsy-proven NAFLD patients. Firstly, a systematic review was performed to recapitulate current knowledge on recently described adipokines and their relation with liver histology in NAFLD patients (**chapter 3.1**). Although chemerin, adipocyte-fatty-acid-binding protein (AFABP) and resistin were independently associated with hepatic injury and steatosis in several reports, these studies were often too divergent to provide significant evidence on their potential involvement in NAFLD. Overall, data on the impact of these recently described adipokines on disease progression remain scarce with a major heterogeneity across study results and often confined to small patient numbers. The review highlights the urgent need for longitudinal studies on larger and homogenous patient groups with the use of standardized assays to measure adipokine levels.

In **chapter 3.2**, a selection of more novel adipokines was made, of which a contribution to NAFLD was suspected based on *in vitro* data. Their serum levels and visceral adipose tissue (VAT) expression was associated with hepatic histological parameters in a cross-sectional cohort of obese middle-aged NAFLD patients. We aimed to investigate whether these adipokines were independently associated with steatosis grade, inflammation, hepatocyte ballooning and/or fibrosis grade of liver biopsies, in order to clarify their potential contribution to NAFLD development and/or progression. Because adipokines derived from VAT are considered to be delivered directly to the liver via its portal vein, we measured

both circulating levels and VAT expression of adiponectin, omentin, chemerin, monocyte chemoattractant protein-1 (MCP-1) and secreted frizzled-related protein 4 (SFRP4). We confirmed that serum adiponectin levels were negatively associated with NAFLD activity score (NAS), however this was lost after correction for IR. This finding suggests that low circulating levels of adiponectin may contribute to IR, which in turn may influence NAFLD. Further, chemerin VAT expression in male NAFLD patients was reduced and inversely associated with overall NAS and hepatic steatosis independent of age, BMI and IR. This suggests that lower VAT expression of chemerin in obese patients may be involved in the pathophysiology of NAFLD, although larger-scaled prospective studies are needed to confirm our findings.

The relation of dysfunctional adipose tissue with sex steroid profile was investigated in a cohort of morbidly obese and healthy control men (**chapter IV**). We aimed to explore potential determinants of total and free T (TT and FT) levels, focusing on adipose tissue dysfunction, metabolic parameters and adipose tissue aromatase expression. In this study, we could not find differences in *in situ* aromatase expression, both in subcutaneous adipose tissue (SAT) and VAT, between obese men (with or without type 2 diabetes) and normal-weight men, thus counteracting the increased aromatase activity hypothesis. SAT cell size, however, was independently inversely associated with both TT and FT levels after multivariate regression analysis corrected for age, group (control versus obesity/diabetes), IR, and triglyceride levels. These results suggest a direct negative impact of adipocyte size, but not IR, on TT and FT levels in obese men, highlighting a potential role of adipose tissue dysfunction in the disturbed sex steroid profile of obese male subjects.

Finally, the main findings of our studies are summarized and discussed in **chapter V**, focusing on the characteristics of NAFLD in obesity, the associations between adipokines and liver histology in patients with NAFLD with a particular interest in chemerin, and the potential role of adipose tissue dysfunction in low T levels of obese men. In addition, clinical relevance of these findings is discussed as well as perspectives on future research.

In conclusion, the findings in this thesis have provided additional evidence on the impact of adipose tissue dysfunction in case of obesity, with a potential role in the development of NAFLD and the impaired sex steroid profile of obese men. Although larger-scaled and prospective studies are needed to confirm and expand these findings, it suggests that restoring normal adipose tissue function and/or preventing dysfunction might help to counteract obesity-related consequences. Alternatively, the pharmacologic manipulation of adipokines might represent a future therapeutic option.

I. GENERAL INTRODUCTION

1. Background

Worldwide epidemic rates of overweight and obesity, due to ample food supplies and more sedentary lifestyles, result in a persistently increasing burden for modern society. Obesity is a major risk factor for the development of metabolic comorbidities such as type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases (CVD) [1]. According to the World Health Organization (WHO), overweight and obesity are defined as abnormal or excessive fat accumulation that represents a risk to health [2]. The term ‘abnormal’ in this definition most likely represents the importance of adipose tissue dysfunction, rather than solely excess fat *per se*, in the pathogenesis of obesity-related metabolic complications.

Adipose tissue dysfunction can develop when a nutritional overload leads to failure in storage of lipids, which might result in overspill of lipids outside the ‘classical’ adipose tissue depots and ectopic deposition into organs and tissues such as liver and muscle. This then causes local tissue dysfunction, called lipotoxicity [1]. Although the exact mechanism on how adipose tissue becomes dysfunctional is incompletely understood, several pathological mechanisms are considered to be implicated including mitochondrial damage, increased adipose cell size, decreased insulin sensitivity of fat cells and failure of their storage function with consequent peripheral lipotoxicity, but also adipose tissue macrophage infiltration, inflammation and changes in adipokine secretion [3]. In light of the epidemiologic burden of obesity and its metabolic consequences arising worldwide, a thorough understanding of the etiology and underlying mechanisms is necessary.

NAFLD is often referred to as the hepatic manifestation of obesity, as its prevalence is simultaneously rising with the combined epidemic of obesity and type 2 diabetes. Although the exact pathophysiology of NAFLD remains to be fully elucidated, insulin resistance (IR) and adipose tissue dysfunction are suggested to play a central role in this. In addition, the potential impact of impaired adipokine release is currently a major point of interest in clarifying the development of metabolic diseases, including NAFLD and the progression to NASH. However, the range of discovered adipokines is currently enormously high (> 700), whereas knowledge of their function remains mainly unclear.

A well-known association exists between body composition and sex steroids, the steroid hormones involved in the development of secondary sexual characteristics and reproductive functions, and which are at least partly responsible for the sexual dimorphism in body composition between men and women

[4]. In light of the increasing prevalence of obesity worldwide, the interest in sex steroids as potential determinants of body composition is similarly increasing. Obese men, who are often characterized by low levels of the principal male sex steroid testosterone (T), have increased levels of visceral and subcutaneous fat that accumulates preferentially in the abdominal region. Visceral fat in particular, is associated with increased metabolic risk [5, 6]. Consequently, there is a high need to understand and unravel the potential causal relationship between obesity and disturbed sex steroid levels.

In this general introduction, an overview of current knowledge regarding the pathophysiology of obesity and its metabolic comorbidities is described in the first chapter. Secondly, the link between obesity and NAFLD, more specifically the contribution of IR and adipokines to the pathogenesis of NAFLD, is discussed in the following chapter. Finally, the regulation and synthesis of sex steroids in men as well as their interaction with adipose tissue and the disturbed sex steroid pattern in case of obesity is described in the last chapter.

2. Body fat: friend or foe?

Obesity is characterized by excess body fat or adipose tissue and associated with a high risk of developing metabolic comorbidities such as IR, type 2 diabetes and CVD. However, patients with congenital lipodystrophy that are characterized with an almost complete absence of adipose tissue, have been described as having a similar metabolic profile with IR, type 2 diabetes, dyslipidemia and CVD [7]. This suggests that adipose tissue *per se* is not as harmful as it may seem but that its function is determining metabolic complications in obesity. Thus, more knowledge on the pathological consequences of excess adipose tissue accumulation is necessary in order to better understand, prevent and treat obesity-related complications. Humans mainly possess white adipose tissue (WAT), but semi-recently the presence of brown adipose tissue (BAT) has been demonstrated. Both are involved in energy metabolism, but differ largely with respect to fuel utilisation. While WAT is excessively present throughout the whole body and stores surplus lipids as energy for times of metabolic need, BAT is only to a very limited extent and variably present in the human body and uses lipids as a source of heat production through thermogenesis [8]. Besides thermogenesis, the role of BAT is rather unclear and will not be the scope of this thesis as we will focus on WAT.

2.1. Adipose tissue anatomy and histology

The main biological function of adipose tissue is to manage lipid storage and availability, providing both long- and short-term regulation and delivery of stored lipids in response to the body's energy need. These lipids are stored in the adipocytes within one large lipid droplet that occupies the majority of intracellular space, compressing the cytoplasm and nucleus into a thin border. Adipocytes are organized into adipose tissue together with fibroblasts, macrophages, small blood vessels, nerve tissue and adipocyte precursor cells (preadipocytes) [8]. The volume of an adipocyte is determined by a balance of 3 processes occurring within the adipocyte: fatty acid (FA) uptake, lipogenesis and lipolysis, which will be described in the section of lipid handling and storage (section 2.2.1.).

There are two major anatomic subdivisions of adipose tissue: intra-abdominal or visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). While SAT is located beneath the skin, VAT is found within the abdominal cavity. VAT is further divided into intraperitoneal and retroperitoneal depots, although the borderline between them is not completely clear. The majority of VAT is composed of intraperitoneal fat, which comprises omental and mesenteric adipose tissue. In addition to the main SAT

and VAT compartments, several organs such as the heart and kidneys are surrounded by small amounts of adipose tissue [8, 9]. SAT can also be subdivided by region into abdominal or upper-body and gluteal/femoral or lower-body SAT, which in turn differ in fat uptake and release, and hormone production. Indeed, free fatty acid (FFA) release from upper-body SAT was higher than from lower-body SAT, both in men and women [10-12]. Intra-abdominal adipose tissue or VAT is characterized with more capillaries and efferent sympathetic axons per unit volume than SAT and unlike SAT, it drains directly into the hepatic portal vein. Excess VAT results in substantially higher metabolic risk for any amount of total body fat, at least because it is more resistant to insulin and insulin-mediated suppression of lipolysis, but perhaps also due to differences in its secretory factors. However, an important question remains whether VAT is a causal factor or simply a marker of a dysmetabolic profile [13-16]. In contrast, SAT, in particular lower-body SAT, is less associated with metabolic risk due to its enhanced ability to expand, allowing more storage of excess dietary FA, and due to reduced hypoxia and fibrosis [17, 18]. Although body mass index (BMI) - body weight divided by the square of the body height (kg/m^2) - is associated with obesity-related health risks, it is not the most accurate marker of metabolic risk as it does not differentiate between muscle mass and fat mass and there is no discrimination between SAT and VAT despite the differential associated metabolic risk [19]. Waist circumference (WC) measured at the smallest circumference of the natural waist, appears to be a better estimate of abdominal obesity. However, caution is advised when interpreting these results because this measure of abdominal fat still does not differentiate between SAT and VAT [20]. In clinical practice, regional fat distribution can be estimated with skinfold measurements, ultrasound and Dual-energy X-ray absorptiometry (DXA), although the added clinical value seems limited. The differentiation between intra-abdominal fat (VAT) and abdominal SAT requires advanced imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI), which are less easily used in clinical practice [21, 22]. The distribution of fat and particularly of VAT, may be influenced by aging, gender, smoking, sedentary lifestyle and nutritional status [23]. The relation between body composition, including fat distribution, and gender will be described more in detail in section 4.2.

2.2. General physiology of adipose tissue

2.2.1. Lipid handling and storage

As mentioned before, the major function of adipose tissue is lipid storage. After a meal, dietary lipids or FA are absorbed from the intestine and released into general circulation as chylomicrons at the thoracic

duct. In the circulation, lipids are transported as triglycerides (TG) into specific transporting proteins such as chylomicrons and very-low-density lipoproteins (VLDL; synthesized by the liver). Once lipoproteins arrive at the target cell, lipoprotein lipase (LPL), an enzyme that resides on the endothelial cells of blood vessels, is activated and enables the adipocytes or other metabolically active cells to take up FA [24]. Once entered in the adipocytes, lipids are stored in the form of TG to provide a reservoir of energy. Synthesis of TG within the adipocyte consists of the esterification of 3 molecules of FA to one molecule glycerol - a process called lipogenesis. TG are hydrolyzed back into FFA and glycerol in case of fasting - a process called lipolysis. The FA released in the circulation after lipolysis, called non-esterified fatty acids (NEFA), serve as a fuel for metabolically active tissues where they are oxidized by mitochondria into energy in the form of adenosine triphosphate (ATP). Lipogenesis can also include *de novo* synthesis of FA and subsequently TG, a process that is catalyzed by acetyl coenzyme A carboxylase (ACC) and involves metabolized substrates of glucose. Each of these processes is strictly regulated by extracellular signals such as insulin, catecholamines, FFA and cytokines [8, 25] (Figure 1).

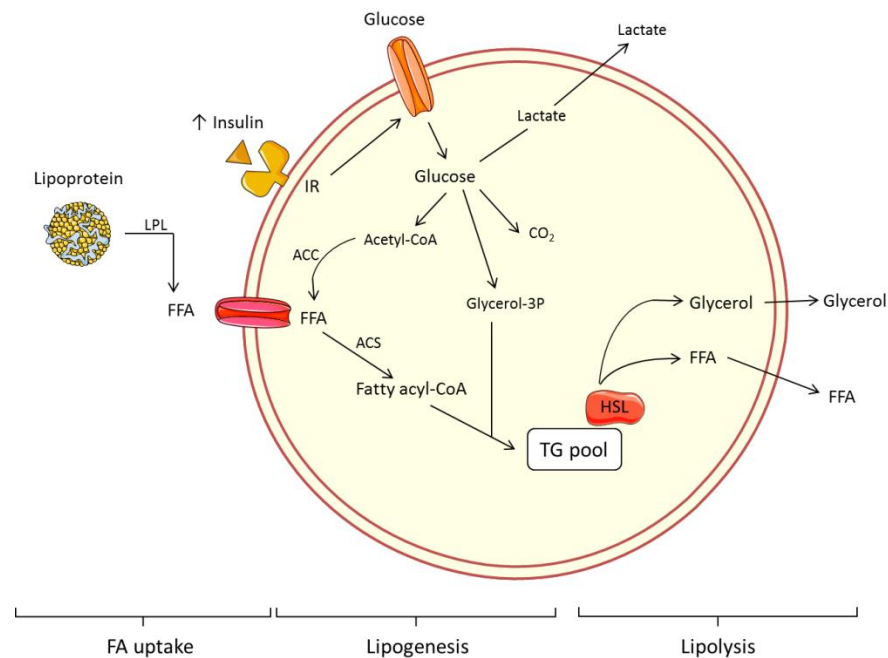


Figure 1 Schematic presentation of the processes of FA uptake, lipogenesis and lipolysis within one adipocyte. Dietary lipids are transported in the circulation as lipoproteins, from which they are released as free fatty acids (FFA) by lipoprotein lipase (LPL). Once entered the adipocyte, FFA are transformed into triglycerides (TG) by acetyl coenzyme A synthetase (ACS) and binding with glycerol-3P, a substrate that is derived from glucose. Insulin normally enhances glucose uptake, providing storage of glucose in the form of glycogen and lipids. Indeed, more glucose results in higher amounts of substrates for both *de novo* lipogenesis as well as the conversion of entered FFA into TG. Consequently, the higher rates of lipogenesis increase the TG pool within the adipocyte, together with

synthesized glycogen providing long-term storage of energy. ACC: acetyl coenzyme A carboxylase; HSL: hormone-sensitive lipase; IR: insulin receptor. Adapted from [8].

2.2.2. Regulatory factors of the metabolic system

Metabolic homeostasis is normally strictly controlled, as illustrated for example by the link between alterations in the function of crucial transcription factors (TF) and the occurrence of major metabolic diseases. Organisms are able to adapt their metabolic activity to environmental (nutritional) changes by modifying the transcriptional control of gene expression of key enzymes. These modifications particularly occur in response to hormonal impulses and take place during the alternation between fasting and feeding to achieve energy homeostasis. Disruption of this transcriptional and hormonal control leads to the development of metabolic disease, reflecting the importance of a finely tuned glucose and lipid metabolism [26]. Blood glucose levels for instance, are strictly kept in a narrow range of 4 to 7 mM despite the strong differential supply between fasting and feeding. Indeed, a strict regulation of glucose availability is crucial for the correct functioning of different cell types (avoidance of hypoglycemia for e.g. erythrocytes which are exclusively dependent on glucose as a fuel), but also to efficiently store glucose for later use. After feeding, the pancreas senses glucose and insulin secretion is stimulated by increased glucose levels, inducing the quick removal of glucose by uptake in peripheral tissues. Simultaneously, insulin promotes energy storage by glycogen synthesis in liver and muscle as well as FA synthesis in liver and adipose tissue. In times of fasting or physical activity, insulin levels remain low and the contraregulatory hormone glucagon stimulates the hepatic production of glucose (gluconeogenesis) and its release from glycogen (glycogenolysis). While the liver functions as a “buffer” that provides glucose when energy is needed and stores energy when glucose is abundant, muscle tissue mainly consumes glucose as an energy source and the intramyocellular glycogen is in fact only available for local use [27].

Insulin and glucagon are thus considered to be major regulatory hormones involved in metabolic homeostasis. Firstly, pro-insulin is synthesized by β -cells of the pancreas, which is cleaved into insulin and peptide C by proconvertases. Insulin is then stored in secretory vesicles, of which secretion is directly linked to the sensing of glucose levels [28]. Released insulin proteins circulate to insulin-responsive tissues such as adipose tissue, liver and muscle, where they interact with the membrane insulin receptor, a tyrosine kinase receptor. Receptor activation results in the phosphorylation of insulin receptor substrate 1 and 2 (IRS1 and IRS2), followed by several sequential phosphorylation steps that

subsequently activate the Ras/mitogen-activated protein kinase (MAPK) pathway. This pathway seems to be mostly involved in cellular growth and proliferation rather than metabolic homeostasis. Simultaneously however, the activated IRS proteins induce activation of phosphatidylinositol 3-kinase (PI3K), which converts phosphatidylinositol (4,5)-bisphosphate (PIP₂) into phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). This results in a phosphorylation cascade that leads to the activation of phosphoinositide-dependent kinase 1 (PDK1) and Akt/protein kinase B (PKB). In metabolically active tissues such as the liver and skeletal muscle, PDK1 and Akt/PKB regulate glucose and lipid metabolism by increasing glucose uptake and the synthesis of glycogen and lipids and by decreasing hepatic gluconeogenesis [27].

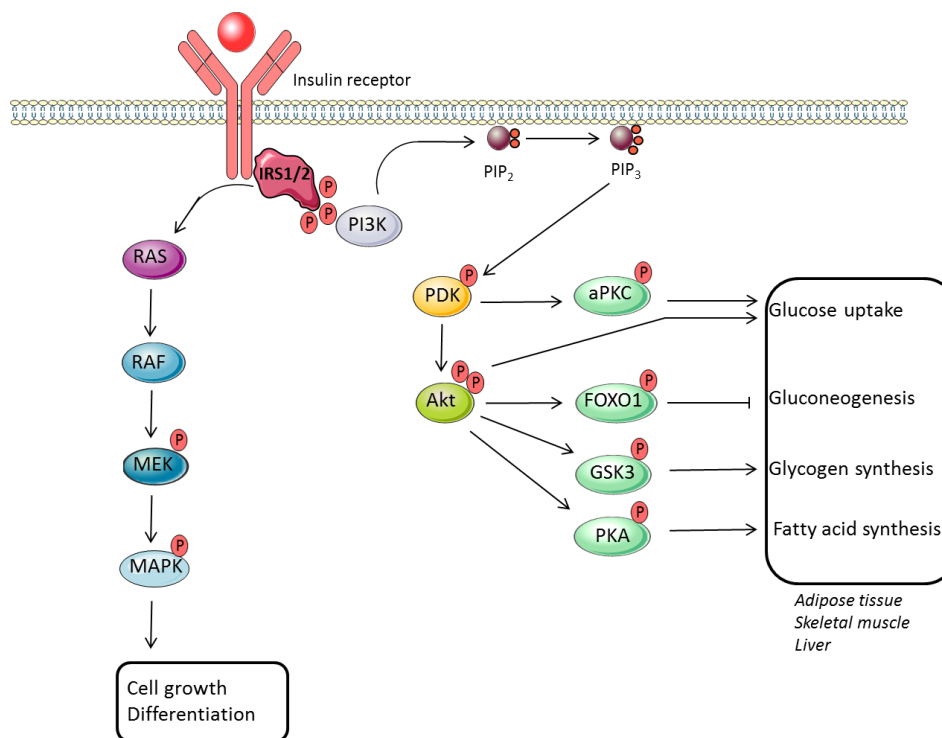


Figure 2 Insulin signaling in healthy individuals. Insulin-mediated activation of the insulin receptor results in the phosphorylation of insulin receptor substrate 1 and 2 (IRS1 and IRS2), followed by the initiation of a phosphorylation cascade that ultimately activates the mitogen-activated protein kinase (MAPK) pathway and the Akt/protein kinase B (PKB) pathway. While the MAPK pathway regulates cell growth and differentiation, phosphoinositide-dependent kinase (PDK) and Akt/PKB activation leads to an increased glucose uptake, glycogen and lipid synthesis as well as a decreased gluconeogenesis in metabolically active tissues such as the liver, skeletal muscle and adipose tissue. aPKC, atypical protein kinase C; FOXO1, forkhead box protein O1; GSK3, glycogen synthase kinase 3; PI3K, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol (4,5)-bisphosphate; PIP₃, phosphatidylinositol (3,4,5)-trisphosphate; PKA, protein kinase A. Adapted from [29]

2.2.3. Adipose tissue as an endocrine organ

Besides the storage and mobilization of lipids, adipose tissue operates as an endocrine organ expressing steroid metabolizing enzymes and secreting numerous regulatory products, named adipokines, which exert local, peripheral and central effects. In literature, numerous adipokines have been described already and their number is still rising. The discovery of more than 700 different proteins have been indicated as adipokines in literature and the research group of Lehr *et al.* has recently reported another 44 novel adipokines as secretary products in medium of differentiated primary adipocytes derived from healthy individuals [30]. Adipokines are polypeptides that are mainly, but not exclusively, produced by adipocytes. Some inflammatory cytokines, such as tumor necrosis factor (TNF)- α and interleukin (IL)-6, are additionally secreted by immune cells infiltrating the adipose tissue [31]. Although the function of many adipokines remains unknown, certain adipokines have been described to have a role in regulating whole body energy homeostasis and metabolism and/or inflammatory responses. Therefore, adipokines are generally subdivided into pro-inflammatory and anti-inflammatory adipokines, of which the balance in their secretion pattern is strictly regulated in normal physiological conditions [32, 33]. Important differences in the adipokine secretion pattern exist among the distinct regional adipose tissue depots. Leptin for instance, has been described as an important regulator of the energy balance and food intake. Higher leptin levels are more closely correlated with SAT expression than with VAT expression [34, 35]. While adiponectin, a known anti-inflammatory adipokine, is predominantly produced in SAT, You T *et al.* reported that its expression was higher in abdominal compared to gluteal SAT of abdominally obese women and weight loss preferentially increased this abdominal SAT expression and release [11]. Additionally, adipose tissue is also an important site of steroid hormone metabolism, as several steroid metabolizing enzymes are expressed. For example, adipose tissue expresses both aromatase enzyme, which converts T into estradiol (E_2), and 11 β -Hydroxysteroid dehydrogenase (11 β -HSD) enzyme, which catalyzes the conversion of cortisone to cortisol [36]. This relation between adipose tissue and sex steroid metabolism will be described more in detail in chapter III.

2.3. Obesity and metabolic consequences

As mentioned, obesity is defined as a medical condition in which there is surplus accumulation of fat in the body, which is linked to metabolic disturbances resulting in increased illness, disability, and even mortality. The WHO classified obesity according to BMI categories into class I (30-34.9 kg/m²; mild), class II (35-39.9 kg/m²; moderate) and class III (≥ 40 kg/m²; severe). In 2013, approximately 50% of the adult

population in developed countries was either overweight (BMI > 25 kg/m²) or obese (BMI > 30 kg/m²) [37]. According to the WHO, the global prevalence has doubled between 1980 and 2014, with 20-30% of the European adults being obese [38]. This prevalence is still rising worldwide, increasing health care costs and leading to a huge burden for modern society. Metabolic derangements associated with obesity include hypertension, dyslipidemia and IR, often collectively termed as the metabolic syndrome (MetS). The MetS thus comprises a cluster of cardiometabolic risk factors, with IR and excess body fat as central features. MetS is considered a practical tool to describe this cluster of factors that regardless of cause, identifies individuals at risk of CVD and type 2 diabetes. Following criteria for MetS have been identified by the National Cholesterol and Education Program (NCEP) - the Adult Treatment Panel III (ATPIII) criteria: central obesity (indicated by WC > 40 inches for men, > 35 inches for women), dyslipidemia with high TG (≥ 150 mg/dl) and/or low high-density lipoprotein (HDL) cholesterol (< 40 mg/dl for men, < 50 mg/dl for women), hypertension (blood pressure $\geq 130/85$ mmHg) and impaired fasting glucose (≥ 110 mg/dl). The presence of at least 3 of these features is considered sufficient to diagnose MetS [39, 40]. Based on the National Health and Nutrition Examination Survey (NHANES), approximately 35% of all US adults and 1 out of 10 US adolescents currently have MetS [41, 42]. Furthermore, >40% of adults over the age of 50 have MetS [43]. It is generally known that the presence of MetS generates an increased risk of all-cause mortality and CVD, and results in a 7-fold increased risk of developing type 2 diabetes [44, 45]. Although obesity is a well-known risk factor for IR, type 2 diabetes and CVD, not every obese patient is insulin resistant or at high risk of diabetes and CVD. This explains why obesity is not an obligatory component in the definition of MetS and thus not necessarily present in patients with MetS. Indeed, both metabolically healthy obese phenotypes as well as metabolically obese but normal-weight phenotypes have been identified, depending on their fat distribution and insulin sensitivity, which are presented as the main factors that define phenotypes within the same BMI range. Metabolically healthy obese patients may represent an adaptation to reestablish a new homeostatic state by expanding adipose tissue and maintain a normal glucose and lipid metabolism in case of high availability of caloric-rich food [46]. However, major inconsistency still exists on this topic. Besides its role in energy storage, adipose tissue has impact on whole-body metabolism at least in part by secreting adipokines and influencing immune cell functions, as we will discuss in following sections.

2.3.1. Adipose tissue dysfunction

In obesity, the excessive energy supply initially results in adipose tissue expansion both at SAT and VAT depots. Adipose tissue is highly plastic and can respond rapidly to changes in energy balance through adipocyte enlargement (hypertrophy) or increasing the number of adipocytes (hyperplasia), depending on the fat depot and initial adipocyte size. Although the number of abdominal adipocytes is often suggested to remain constant during adulthood, recent reports suggest that mainly lower-body SAT may also expand through hyperplasia. However, in upper-body or central obesity this adipose tissue expansion is mainly characterized by larger adipocyte cell sizes [18, 47, 48]. If not counterbalanced, this adipocyte enlargement can lead to adipose tissue dysfunction, potentially due to an insufficient blood supply and a higher rate of macrophage infiltration. Adipose tissue dysfunction is characterized by a disturbed lipid metabolism with an elevated release of NEFA, inflammation and an impaired release of adipokines. As these processes can cause IR and local inflammation in other organs, it is also called lipotoxicity [49].

2.3.1.1. Non-esterified fatty acids (NEFA) and lipid metabolites

NEFA are released into the circulation after lipolysis within the adipocytes. In case of obesity, increased lipolysis as a result of resistance to the anti-lipolytic effect of insulin, may lead to elevated NEFA levels in serum. Elevated NEFA have been indicated as important mediators of adverse metabolic effects including IR and a disturbed glucose and lipid metabolism, by playing a key role in the process of fat overflow to ectopic tissue sites. NEFA entering skeletal muscle cells – intramyocellular lipids – may impair insulin sensitivity and glucose uptake. In the liver, excess NEFA are converted into TG (lipogenesis) and glucose (gluconeogenesis), contributing to the development of hepatic steatosis, IR and hyperglycemia [16, 50]. Although a higher proportion of hepatic NEFA delivery is expected as VAT increases - because of the direct delivery via the hepatic portal vein, the importance of VAT-derived NEFA delivery to the liver is still controversial. On the contrary, it has been reported that the proportion of portal NEFA derived from VAT was lower than the relative amount derived from lipolysis in SAT. Both fat depots are thus suggested to be important suppliers of NEFA to the liver and other organs, and other factors than VAT-derived lipid deposition might explain the worsened metabolic profile of viscerally obese patients [51]. Moreover, in some obese individuals, the release of NEFA per kilogram of adipose tissue was downregulated instead of increased, suggesting that obesity-related hepatic and systemic IR can also occur without the elevation of NEFA concentrations [52].

In addition to the elevated NEFA release, the oxidative capacity of adipocytes fails to sufficiently compete with excess supply of lipids. Consequently, this impaired lipid oxidation results in the production of lipid metabolites such as fatty acyl-CoA, diacylglycerol (DAG) and ceramides. These metabolites and DAG in particular, are suggested to interfere with insulin signaling by activating protein kinase C (PKC) locally as well as in muscle and liver. Activated PKC phosphorylates IRS1 and IRS2 at serine residues, thereby inhibiting insulin signal transduction and glucose uptake [53].

2.3.1.2. Inflammation

Accumulating evidence suggests that an inflammatory response in the adipose tissue may play a critical role in the development of obesity-related metabolic comorbidities [54, 55]. FA for instance, may induce intracellular inflammation by stimulating nuclear factor- κ B (NF κ B), a known inflammatory TF that induces the transcription of inflammatory cytokines, and by activating c-Jun N-terminal kinase (JNK), which interferes with insulin signaling and also activates NF κ B. Extracellularly, FA can also serve as ligands for the toll-like receptor 4 (TLR4) and stimulate cytokine production of macrophages, which additionally contributes to systemic inflammation [56]. Furthermore, the increased mitochondrial oxidation of FA in adipocytes can result in reactive oxygen species (ROS) production leading to oxidative stress, which together with endoplasmic reticulum (ER) stress stimulates inflammatory pathways and may induce IR [57]. These results suggest that inflammation is closely linked with mechanisms of IR. The expanded adipose tissue of obese subjects is indeed characterized by more and different infiltrating immune cells, contributing to an increased local and systemic inflammatory response [58].

2.3.1.3. Adipokines

As described earlier, adipose tissue produces numerous adipokines, of which the secretion may influence metabolic homeostasis. It is generally accepted that the adipose tissue of obese subjects has a different secretion pattern of adipokines as compared to normal-weight subjects, leading to the release of more pro-inflammatory and less anti-inflammatory adipokines. Adipose tissue dysfunction thus results in dysregulation of the adipokine secretion pattern, which in turn might contribute to the development of metabolic dysfunction by altering glucose and lipid homeostasis as well as the inflammatory response [32, 59]. Although a substantial amount of discrepancy still exists throughout literature, several adipokines have gained particular interest for their suspected role in the pathophysiology of obesity and its metabolic comorbidities. Adiponectin for instance, is a well-known adipose tissue-specific adipokine with insulin-sensitizing and anti-inflammatory effects. Its adipose tissue expression and serum levels are lower in obese and insulin resistant patients and upregulation of

adiponectin secretion has increased insulin sensitivity in animal models [60]. Leptin is another adipokine, specifically expressed by adipocytes, that normally inhibits appetite and food intake and stimulates energy expenditure [61]. In obesity however, higher expression and serum levels were reported and the existence of (partial) leptin resistance was hypothesized, as some beneficial effects of leptin were lost while pro-inflammatory effects were induced in these patients [62, 63].

In addition, adipocytes and immune cells, particularly adipose tissue-infiltrating macrophages, are the primary sources of inflammatory molecules such as cytokines and chemokines [64]. Cytokines are known to induce an inflammatory response and to interfere with insulin signaling. A well-known cytokine is TNF- α , of which higher expression and serum levels were found in obese patients and a positive association was found with IR [65]. However, it has been suggested that the effects of TNF- α on IR are attributable to a complex inflammation network that is capable to initiate different cytokine cascades and synergistically interfere with insulin signaling [66]. Chemokines, for example monocyte chemoattractant protein-1 (MCP-1), play a major role in selectively recruiting immune cells to the site of inflammation (chemotaxis) and chemokine receptors are highly expressed in adipose tissue of obese subjects [67]. Table 1 gives an overview on functions of other adipokines that have been suggested to be implicated in obesity.

Overall, it is accepted that adipokines play an important role in the crosstalk between adipose tissue and other tissues including skeletal muscle, liver and even the brain. However, considering the wide spectrum of both anti- and pro-inflammatory adipokines, it is likely that a crosstalk of several adipokines in turn may be involved in metabolic dysfunction [68]. Further studies are needed to elucidate important crosstalk pathways.

Table 1 Functions of adipokines that have been implicated in obesity

Adipokine	Serum and/or expression in obesity	Target tissue/cells	Main effects of adipokines in target tissue	References
Chemerin	↑ [69]	Adipose tissue	Increased adipogenesis, glucose uptake, FA synthesis and secretion of adiponectin and leptin; increased or decreased insulin signaling; decreased lipolysis and IL-6 secretion	[70, 71]
		Skeletal muscle	Decreased insulin signaling and glucose uptake; increased activation of NFκB with an initiated inflammatory response	[69]
		Macrophages and dendritic cells	Increased chemotaxis	[72, 73]
Omentin	↓ [74]	Cardiomyocytes	Increased insulin sensitivity and cardiac function	[75]
		Adipose tissue	Increased insulin sensitivity and glucose uptake	[76]
		Endothelial cells	Decreased inflammatory response	[77, 78]
MCP-1	↑ [79]	Adipose tissue	Lowered insulin signaling and activated chemotaxis	[80, 81]
IL-6	↑ [83]	Liver	Promoted hepatic fibrosis	[82]
		Adipose tissue	Lowered insulin signaling and glucose uptake and higher circulating fasting glucose levels	[83-85]
		Skeletal muscle	Higher glucose uptake and fatty acid oxidation	[86]
Resistin	↑ [88]	Liver	Lowered insulin signaling and glycogen synthesis	[87]
		Adipose tissue	Lowered insulin signaling and activated FA and cytokine release	[89-91]
		Liver	Lowered insulin signaling, glycogen synthesis and glucose uptake; increased glucose production, circulating fasting glucose levels and inflammatory response	[92-97]
Visfatin	↑ [99] or ↓ [100]	Macrophages	Increased pro-inflammatory response	[98]
		Adipose tissue	Stimulated insulin signaling and glucose uptake; lowered circulating glucose levels	[101, 102]
		Hypothalamus	Increased insulin signaling and glucose uptake	[103]
RBP4	↑ [105]	Monocytes	Increased cytokine release and pro-inflammatory response	[104]
		Skeletal muscle	Lowered insulin signaling	[106]
Vaspin	↑ [107]	Liver	Increased glucose production	[106]
		Adipose tissue	Increased insulin signaling and normalized cytokine expression and serum glucose levels	[108]
AFABP	↑ [110]	Hypothalamus	Decreased food intake and blood glucose levels	[109]
		Adipose tissue	Lowered insulin signaling and adiponectin secretion; increased lipolysis	[111, 112]
		Macrophages	Increased pro-inflammatory response	[112]

FA, fatty acids; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin-6; RBP4, retinol-binding protein 4; AFABP, adipocyte-fatty-acid-binding protein.

2.3.2. Insulin resistance (IR)

In general, IR is defined as the decreased ability of tissues to respond to insulin action and is known to be a major predictor of the development of type 2 diabetes. In adipose tissue, insulin normally stimulates storage of TG, uptake of glucose and FFA (derived from lipoproteins), lipogenesis and inhibits lipolysis, all of which are dysregulated in obesity. In case of IR in myocytes, insulin-mediated glucose uptake is impaired with subsequent reductions in glycogen synthesis. In hepatocytes, IR is manifested by defects in the ability of insulin to suppress glucose production and promote glycogenesis [113]. As described in the preceding sections, several factors such as FFA and impaired adipokine release from adipose tissue have been directly or indirectly involved in the development of aberrant insulin signaling. Accumulating evidence suggests that these factors may synergistically contribute to IR. For instance, saturated FA, but not unsaturated FA, as well as TNF- α both can induce the synthesis of ceramide, a well-known metabolite that interferes with insulin signaling [114, 115]. Except for the impaired FA and adipokine release, obesity-associated systemic inflammation is characterized by the activation of several inflammatory kinases and TF such as JNK and NF κ B, which in turn interfere with insulin action in adipocytes and hepatocytes [116]. Although VAT is more metabolically active and often more closely associated with IR compared to SAT, evidence proved that dysfunctional SAT remains a key player in the development of IR and inflammation by contributing to the circulating levels of FFA and adipokines [117, 118]. In response to the insulin resistant state, pancreatic β -cells initially secrete more insulin, attempting to overcome the hyperglycemia, resulting in hyperinsulinaemia (high blood insulin levels). Although hyperinsulinaemia may in part compensate for some IR, it may contribute to other complications, i.e. hypertension and hepatic steatosis [119]. Furthermore, this compensatory effort will ultimately fail in most obese patients, leading to development of glucose intolerance and diabetes.

2.3.3. Ectopic lipid deposition

Intracellular lipid content of organs and tissues that constitute lean body mass (skeletal muscle, liver, heart, kidneys) is normally tightly regulated. A small reservoir of FA is needed in all cells, because of their essential role to form phospholipid bilayers of cell membranes and the phospholipid messengers that transmit intracellular signals. Any lipid overload however, may lead to cell dysfunction by lipotoxicity and lipid-induced programmed cell death (lipoapoptosis) [120]. Because these tissues have a limited oxidative capacity, any FA surplus must enter non-oxidative pathways with the production of toxic lipid metabolites such as ceramide as a consequence. Together with FA-induced inhibition of anti-

apoptotic factors, ceramide induces, for instance through activation of NF κ B and increased nitric oxide production, lipoapoptosis in β -cells, cardiomyocytes and hepatocytes [121].

2.4. Therapeutic approaches counteracting obesity

2.4.1. Lifestyle interventions: diet and physical activity

The primary recommended interventions towards obesity consist of lifestyle modifications that result in weight loss such as diet adjustments, exercise and behavioral changes. Weight loss is associated with an improvement of metabolic and cardiovascular risk. Over a period of one to three years, lifestyle interventions have contributed to an improvement of IR, diabetes, hypertension and dyslipidemia [122-125]. Furthermore, at a pre-clinical level weight loss has been shown to decrease macrophage infiltration and pro-inflammatory gene expression in adipose tissue of obese subjects [126]. The weight loss goal is considered to be at least 10 % of initial body weight in order to improve health and prevent or ameliorate comorbidities [127]. Recently, a meta-analysis reported that lifestyle interventions with more physical activity in (morbidly) obese patients (BMI ≥ 35 kg/m²; WHO class II or III), result in significant improved effects on weight loss, fat mass, WC, blood pressure, total cholesterol, low-density lipoprotein (LDL) cholesterol, TG and fasting insulin [128]. However, a major disadvantage of lifestyle modifications (with diet and exercise) is that subjects often fail to achieve long-term weight loss [129]. Once the weight goal is achieved, weight maintenance remains a lifetime challenge for these patients and requires long-term support to sustain diet, physical activity and behavioral changes. The important role of physical activity and behavioral changes herein should not be underestimated. Even when patients achieved weight loss through pharmacological or surgical interventions, lifestyle modifications remain important to achieve treatment goals and maintain their favorable body weight [130]. For example, a randomized controlled trial (RCT) compared obese patients that received an intensive and guided lifestyle intervention program, including dietary changes and increased physical activity, with obese patients on metformin or placebo who only received information on diet and exercise. After three years, the lifestyle intervention group showed greater weight loss (-5.6 kg) compared to those on metformin (-2.1 kg) or placebo (-0.1 kg). The incidence of type 2 diabetes and MetS was reduced by, 58 % and 41 %, respectively, in comparison with the placebo group [123]. Weight loss followed by weight maintenance with lifestyle modification is, although challenging, possible but often requires intensive and long-term support.

2.4.2. Pharmacological interventions

Up till now, there are no truly effective pharmacological agents to fully treat morbid obesity. Pharmacological therapy is often directed at correcting metabolic comorbidities such as insulin-sensitizing and lipid-lowering drugs, but a detailed description thereof is beyond the scope of this thesis. Some insulin-sensitizing drugs however, are frequently used in pharmacological treatment of overweight and obesity. The most familiar drugs in this category for treating patients with obesity are metformin and glucagon-like peptide-1 (GLP-1) receptor agonists [131]. The mechanism of metformin action predominantly involves reducing hepatic glucose production and improving peripheral insulin sensitivity [132, 133]. Metformin provides a low risk of hypoglycemia but gastrointestinal adverse events such as nausea and diarrhea are common [134, 135]. GLP-1 receptor agonists such as liraglutide and exenatide are incretin mimetic drugs that cause slower stomach emptying, stimulate glucose-dependent insulin secretion, reduce glucagon secretion and reduce glucose production in the liver. As a result, they lead to lowering of hyperglycemia and are associated with weight loss. Common adverse events of GLP-1 receptor agonists however, again include nausea and vomiting. Furthermore, these drugs need to be administered in parenteral manner [136, 137].

Orlistat is one of the most familiar drugs to induce weight loss. It acts as a gastrointestinal lipase inhibitor and thus leads to dietary fat malabsorption. The use of orlistat promotes significant weight loss and has been shown to minimize weight regain after a period of intensive lifestyle intervention. Orlistat in addition to lifestyle changes resulted in a lower incidence of type 2 diabetes and lowered both total and LDL cholesterol, independent of weight loss, in patients with obesity. However, orlistat has adverse effects on gastrointestinal tract and is expensive, limiting its current use [138-140]. Currently, there are some additional weight loss-inducing drugs available that act centrally, such as lorcaserin, phentermine/topiramate and naltrexone/bupropion. These drugs are mainly involved in the regulation of food intake and/or energy expenditure but several adverse effects, such as dizziness, anxiety, depression, vomiting and more, were reported. Therefore, long-term studies investigating safety, benefits and the better understanding of the mechanisms of action of these drugs are needed [141]. Importantly, clinical trials have reported that the combination of pharmacotherapy and lifestyle interventions promotes greater and longer sustained weight loss in comparison with lifestyle interventions only [142].

2.4.3. Bariatric surgery

Bariatric surgery is a worldwide recognized invasive approach to achieve significant weight loss in class II or III obese patients. Weight loss is induced by restricting the amount of food a stomach can hold (gastric restriction), creating malabsorption or a combination of both. Three commonly performed bariatric procedures are gastric banding, sleeve gastrectomy and Roux-en-Y gastric bypass, all of which are carried out laparoscopically. In Belgium, patients can only qualify for bariatric surgery reimbursement if they fulfill current eligibility criteria, which require candidates to have (i) BMI ≥ 40 kg/m² or BMI ≥ 35 kg/m² in conjunction with one or more obesity-induced comorbidity (diabetes, sleep apnea, therapy resistant hypertension,..), (ii) acceptable surgical risks, (iii) an ability to participate in long-term follow-up and (iv) an understanding of the surgical procedure and the lifestyle changes that they will need to make [143]. Surgically induced weight loss is associated with improvement or even resolution of obesity-related comorbidities in 75-100% of patients and results in less overall mortality compared to medically treated patients [144, 145]. Although bariatric surgery generally leads to successful weight loss and recovery of metabolic risk factors on short-term, limited data are available about the long-term outcomes per surgery method [146]. Moreover, adverse outcomes of bariatric surgery depend upon the surgical procedure and may vary up to 20% in high-risk patients. Based on current available data, peri-operative mortality rates are very low, with 0.1% for gastric banding and 0.5% for gastric bypass [144]. However, only a few studies reported the amount of reoperations (due to insufficient results) that were needed after bariatric surgery, which ranged from 2% to 40% of the participants depending on the procedure [147]. Bariatric surgery also contains potential procedure risks such as bleeding, internal hernia, anastomosis ulceration and stenosis. A multidisciplinary team is thus needed to evaluate the patients preoperatively and to provide postoperative care if necessary. Altogether, long-term follow-up is highly required for patients after surgery to assess weight loss, resolution of comorbidities, long-term complications and to provide continuing education and nutritional support [143].

3. Non-alcoholic fatty liver disease (NAFLD) as a hepatic manifestation of obesity

NAFLD is currently the most common chronic liver disease in developed countries and it is often referred to as the hepatic manifestation of obesity, due to its well-established association with obesity-related metabolic abnormalities [148-151]. In Western countries, NAFLD is estimated to affect 20-30% of the general adult population. The prevalence of NAFLD might even rise up to 70% in obese and diabetic subjects, who represent a high-risk population [152]. NAFLD is generally asymptomatic and as an entity encompasses a broad spectrum of pathologic mechanisms and disease states, with FA infiltration as the initial insult. Pure fatty liver or simple steatosis (SS) is considered to be the first phase of NAFLD and is characterized by the presence of lipid droplets in at least 5% of hepatocytes. This initial phase of hepatic steatosis is considered as relatively benign with a low likelihood of disease progression. In the more advanced form of NAFLD however, named non-alcoholic steatohepatitis (NASH), there is additional development of inflammation and hepatocellular damage, with or without fibrosis [153, 154]. Disease progression to NASH (with or without fibrosis) occurs only in a fraction of patients, with approximately 10-25% of patients with SS evolving to NASH and a prevalence of 3-5% in the general population [152]. Once NASH has developed, disease progression can proceed potentially leading to cirrhosis in 15-30% of the cases and even hepatocellular carcinoma in 5-13% of the patient with cirrhosis [153, 155]. As mentioned, the progression of NAFLD occurs mainly asymptomatic and only when cirrhosis has developed, clinical manifestations and liver failure may develop as a result of portal hypertension; e.g. esophageal and gastric varices or bleedings, edemas, splenomegaly, jaundice, weakness, weight loss ... [156]. An overview of the different stages in NAFLD is displayed in Figure 3, together with the major characteristics per stage. In addition to the liver-related disease morbidity and mortality, presence of NAFLD has been associated with an increased cardiovascular risk [157]. Although the pathophysiology of NAFLD is incompletely understood, age [158, 159], race, gender [159] and components of the metabolic syndrome have been independently associated with advanced disease [150, 158-162]. For example, a large study with 26 527 Asian subjects showed that the prevalence of NAFLD was 31% in men and 16% in women [163]. Indeed, there is a higher risk to develop NAFLD if subjects are older and male, with a potential link between NAFLD and sex steroids [164]. Most important features in NAFLD pathophysiology are described in detail in following sections 3.1. and 3.2. As the prevalence of NAFLD is rising along with the epidemic of obesity and type 2 diabetes, identifying the patients that are more likely to progress to advanced stages of disease is becoming increasingly important [165, 166].

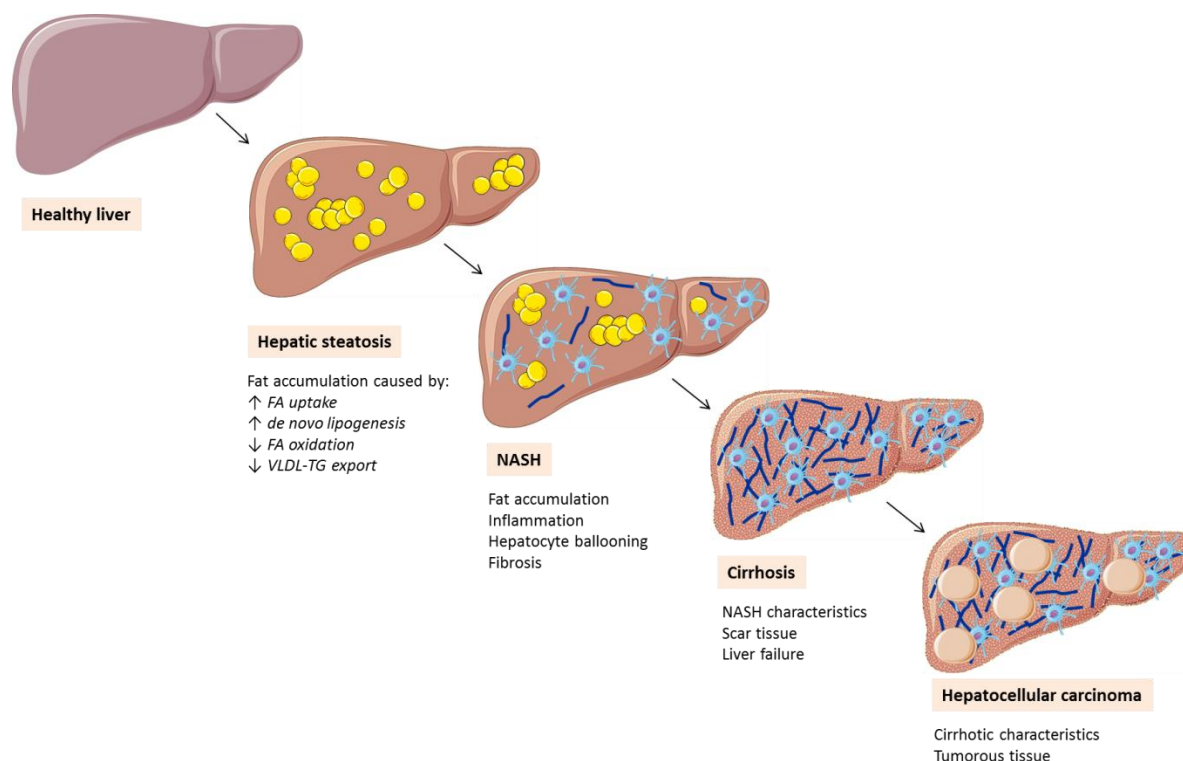


Figure 3 Stages of non-alcoholic fatty liver disease (NAFLD). The first stage, termed hepatic steatosis, merely involves fat accumulation caused by an imbalance between lipid storage and lipid removal. When additionally hepatic inflammation, hepatocyte ballooning and/or fibrosis are present, the liver damage is collectively termed as non-alcoholic steatohepatitis (NASH). NASH can further evolve to cirrhosis with concomitant liver failure or even result in hepatocellular carcinoma (HCC).

3.1. Fatty liver and insulin resistance

NAFLD is independently associated with IR, which in turn is often positively associated with the severity of fatty infiltration and liver damage in those patients with NAFLD [167, 168]. This suggests that IR may play a role in the pathogenesis and/or progression of liver disease, or that both features have a common pathogenic mechanism. Since fat accumulation is the common histological feature among all forms of NAFLD, it is of high importance to understand the critical step when this surplus of lipids results in hepatocellular damage. Fat accumulation in the liver is dependent on the intrahepatic pool of lipids that are stored as TG, which results from following sources: i) FA release by the adipose tissue, due to lipolysis; ii) dietary fat; iii) *de novo* lipogenesis [169].

In normal physiological conditions, the intrahepatic TG levels are kept at low steady-state concentrations by a tightly regulated balance between FA/TG influx and efflux. In addition, the liver functions as a “buffer system” which provides glucose when nutrients are scarce (via gluconeogenesis)

and stores glucose as glycogen when food is abundant. Once the hepatic glycogen storage is full, glucose is converted into lipids for long-term energy storage as fat by *de novo* lipogenesis [26]. Insulin normally regulates these processes by inhibiting gluconeogenesis and inducing *de novo* lipogenesis by stimulating sterol regulatory element-binding protein 1c (SREBP-1c), an important lipogenic TF. In case of IR, however, the expression of genes involved in glucose metabolism is altered, i.e. genes involved in glycogen synthesis are downregulated and those of gluconeogenesis (e.g. phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P)) are upregulated, promoting hyperglycemia. As discussed the pancreatic betacells try to compensate for these phenomena by increasing the production of insulin and in order to maintain normal glucose levels, this leads to hyperinsulinemia. This subsequently results in an overstimulation of SREBP-1c, promoting *de novo* lipogenesis [57, 170]. These findings suggest that *de novo* lipogenesis and glucose metabolism are regulated through divergent signaling pathways and that IR is mainly affecting the pathway(s) involved in glucose metabolism, whereas the resulting hyperinsulinemia can continue to stimulate the lipogenesis [171].

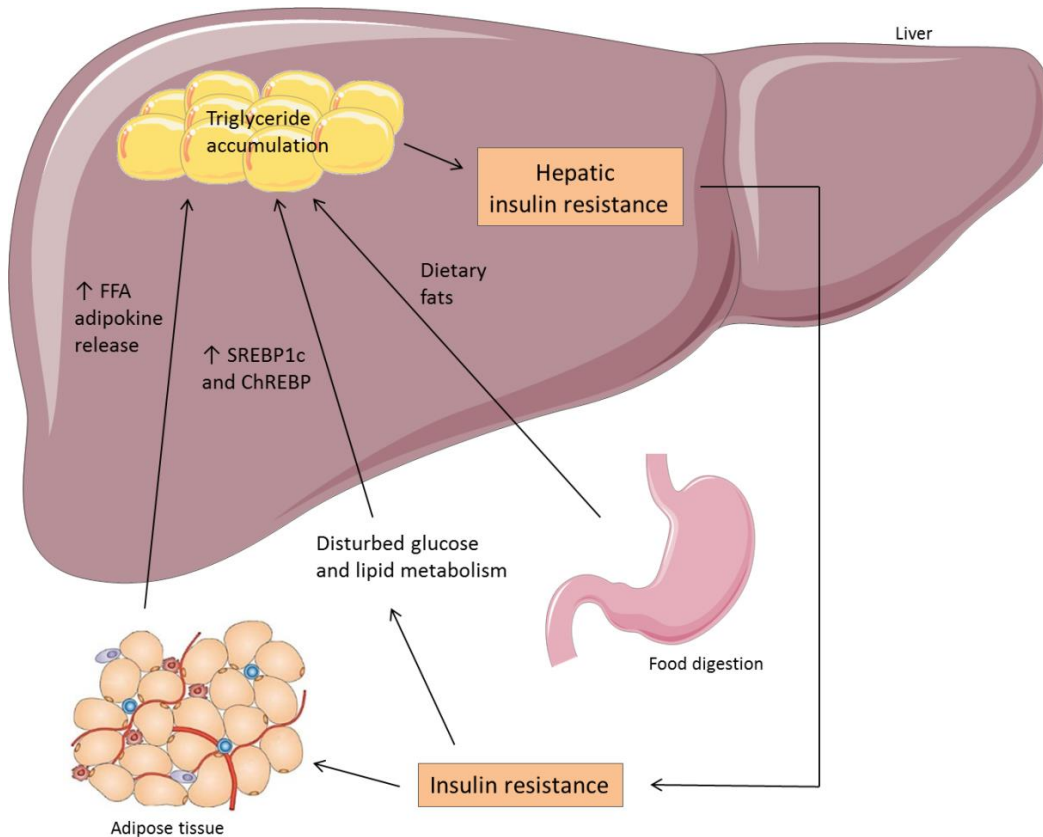


Figure 4 Insulin resistance and hepatic steatosis, a vicious circle. Insulin resistance (IR) results in an elevated release of adipose tissue-derived free fatty acids (FFA) and steatogenic adipokines. Furthermore, the disturbed glucose and lipid metabolism as a result of IR stimulates the pancreas to produce excess insulin (hyperinsulinemia), inducing an elevated hepatic *de novo* lipogenesis by stimulating sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP). Together with an increased dietary fat intake, this excess triglyceride accumulation leads to hepatic steatosis. This hepatic lipid accumulation may in turn result in hepatic IR, exacerbating the overall IR state and leading to a vicious cycle.

Besides from increased *de novo* lipogenesis, hepatic fat accumulation also results from a higher FA influx that originates both from an elevated dietary intake as well as enhanced adipose tissue FA release in case of IR. Furthermore, VAT-derived FA can directly enter the liver via the portal vein [172]. It is thus generally accepted that IR contributes to hepatic steatosis through multiple mechanisms. This hepatic TG accumulation (steatosis) further deteriorates the metabolic balance in the liver, resulting in hepatic IR and activating its resident macrophages, termed Kupffer cells, which results in hepatic inflammation. This suggests that besides the evidence of IR-related development of hepatic steatosis, hepatic lipid accumulation in turn aggravates glucose and lipid metabolism. This contributes to IR leading to a vicious circle that deteriorates metabolic state (Figure 4) [173]. The relation between steatosis and IR has been

emphasized in several mice models with liver-specific defects in genes involved in hepatic lipid metabolism [174, 175].

3.2. Local inflammatory and fibrogenic mechanisms

Similar to adipose tissue, excessive fat accumulation in the liver can lead to hepatic inflammation. Fat accumulation in the liver can generate inflammatory signals by activating JNK and NFκB pathways and can lead to increased ROS production and ER-stress by inadequate lipid oxidation [53]. This inflammatory response amplifies liver injury and may stimulate fibrosis. Activated Kupffer cells are key players in the pathophysiology of NAFLD and NASH, as mice models in which Kupffer cells were depleted showed resistance to steatosis, inflammation, hepatic injury and fibrosis [176, 177]. In case of NAFLD, Kupffer cells are known to shift towards a more inflammatory phenotype in response to several stimuli such as fat accumulation (both by FA and their toxic metabolites), cytokines and IR. These activated Kupffer cells in turn may enhance hepatic steatosis by inhibiting FA oxidation and enhance inflammation by recruiting monocytes via chemokine secretion. In addition, Kupffer cells and the recruited monocytes may further contribute to hepatic fibrosis, both by perpetuating inflammatory cell recruitment and triggering hepatic stellate cells (HSC) activation. HSC can generate pro-fibrotic signals such as elevating the expression of type I collagen and connective tissue growth factor (CTGF) [178]. These HSC are also known to express receptors for insulin, insulin-like growth factor-1 and glycolysed end-products, suggesting that IR and the associated hyperglycemia may also promote fibrosis [179]. A schematic view of the suggested pathways on how lipid accumulation in the liver results in inflammation and fibrosis (steatohepatitis) is presented in Figure 5.

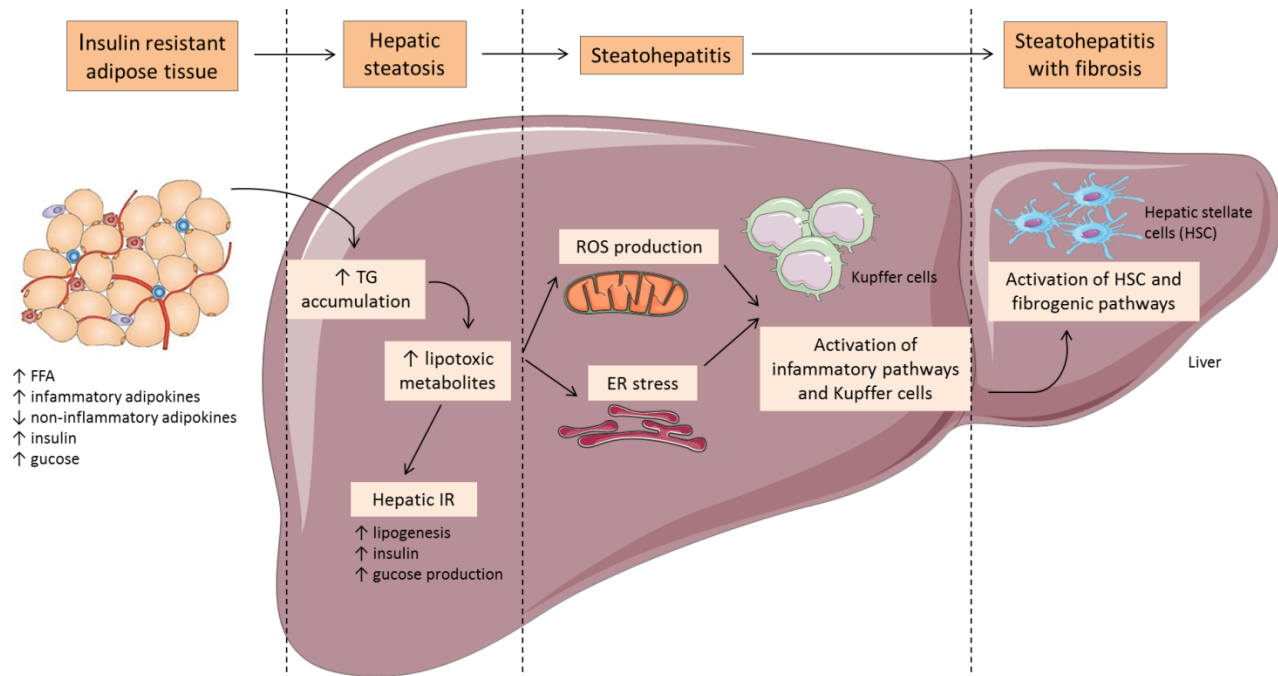


Figure 5 Schematic presentation of the suggested pathways in the development of steatohepatitis with or without fibrosis. Insulin resistance causes the adipose tissue to release more fatty acids (FA) and inflammatory adipokines and less non-inflammatory adipokines (such as adiponectin), resulting in an elevated hepatic accumulation of triglycerides (TG) together with the high circulating levels of glucose and insulin. Consequently, inadequate oxidation of FA leads to the accumulation of lipotoxic metabolites, reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress. All these factors can activate inflammatory pathways as well as the resident hepatic macrophages named Kupffer cells. This escalated inflammatory response in turn may activate hepatic stellate cells (HSC), which promote development of fibrosis. Adapted from [180].

3.3. A pivotal role for adipokines?

Emerging data indicate that an impaired release of adipokines from dysfunctional adipose tissue may underlie NAFLD development and NASH progression. Insulin-sensitizing adipokines such as adiponectin are found to be reduced in NAFLD patients, whereas some inflammatory adipokines including TNF- α and IL-6 are elevated. Inflammatory adipokines are found to be involved in the recruitment and activation of Kupffer cells and are responsible for the transformation of hepatic stellate cells (HSC), contributing to hepatic injury and potentially modulating steatosis, inflammation and fibrosis [181]. Leptin for instance, has been found to promote hepatic inflammation and fibrogenesis, whereas its anti-steatotic effects may be lost in NAFLD patients due to leptin resistance [182]. Reduced adiponectin levels have been frequently reported in NAFLD patients, of which disease severity was inversely associated with adiponectin [183]. Adiponectin infusion in an obese mouse model resulted in reduced steatosis by

activating AMP-activated protein kinase (AMPK; a kinase that elevates lipid oxidation and glucose uptake and inhibits lipogenesis) and attenuated inflammation by suppressing TNF- α expression [184]. Currently, many clinical trials and experimental research are focused on more recently described adipokines, exploring their potential role in NAFLD. Some examples of these adipokines are resistin, vaspin, visfatin, omentin, and chemerin, of which a link with obesity has been reported (section 2.3.1.3.) [181]. Recently, a rather new class of adipokines has been described, which may also be involved in NAFLD development and/or progression, i.e. modulators of the Wnt signaling pathway named secreted frizzled-related proteins (SFRP). The Wnt family of signaling molecules are extracellular ligands that bind to frizzled (Fz) receptors at the cell membrane and induce a canonical or noncanonical signaling cascade. It has been reported that several Wnt pathway components are associated with lipid and glucose metabolism and with inflammation, suggesting a potential involvement in the development of diabetes and NAFLD. SFRP are proteins that can bind Wnt ligands directly and prevent their interaction with Fz receptors [185]. Ehlund *et al.* recently reported that SFRP1, SFRP2 and SFRP4 were expressed and secreted from both SAT and VAT from lean and obese individuals, and that an elevated expression of SFRP4 correlated with IR in patients with obesity [186]. Mice models provided evidence that a downregulation of the noncanonical Wnt pathway may result in a higher risk of NAFLD [187, 188]. This makes SFRP interesting candidate adipokines to investigate their potential relation with NAFLD.

3.4. Current challenges in the diagnosis and treatment of NAFLD

As NAFLD is mainly asymptomatic and its pathophysiology is not completely understood, both the diagnosis and treatment of NAFLD remain true challenges in clinical practice. In sections 3.4.1. and 3.4.2. we describe the methods that are currently used for diagnosing and treating NAFLD patients, including potential pitfalls.

3.4.1. Diagnosis of NAFLD

The diagnosis of NAFLD requires evidence of hepatic steatosis, preferentially on liver biopsy, and the exclusion of other causes of hepatic steatosis such as excessive alcohol consumption or viral infections. Because hepatic steatosis has no specific symptoms, it is often detected incidentally via abnormal liver function tests in serum or by visualization of steatosis on imaging [189]. Abnormal liver function tests in NAFLD are described as mildly raised aminotransferase (with alanine aminotransferase (ALT) > aspartate aminotransferase (AST)) and/or γ -glutamyltransferase (GGT) levels. Although these abnormal liver

function tests have already been associated with presence of NAFLD, they do not specifically distinguish NAFLD from other liver diseases and studies have demonstrated that levels within the normal range do not exclude presence of NAFLD [190, 191]. Detection of hepatic steatosis by imaging is widely used in clinical practice. Especially ultrasound is a low-cost and low-risk widely available diagnostic tool for the assessment of steatosis. However, ultrasound can only detect steatosis when > 33% of hepatocytes are steatotic so that normal liver on ultrasound does not rule out mild steatosis [192]. Other imaging techniques to diagnose hepatic steatosis are CT or MRI. Although they are considered as the most accurate non-invasive tool for the quantification of steatosis, they are not commonly available, expensive and CT imaging leads to radiation exposure [193]. Importantly, none of these imaging techniques were found to be able to distinguish NASH from NAFLD, because they can only detect hepatic steatosis and do not measure inflammation, hepatocyte ballooning or fibrosis. Furthermore, the practical use of those measurement devices is limited in case of morbid obese patients (WHO class II or III obesity) [192]. Transient elastography, also known as FibroScan®, is another non-invasive method to assess liver fibrosis and cirrhosis by specifically measuring liver stiffness using pulsed-echo ultrasound. This method is not able to detect steatosis, inflammation or hepatocyte ballooning and again is less reliable to use in morbidly obese patients [194]. Overall, histological analysis of liver biopsy specimens continues to be the gold standard to diagnose NAFLD and NASH with or without fibrosis, as it provides a definitive assessment of hepatic steatosis, hepatocellular injury, inflammation and fibrosis. Patients with NAFLD are often classified into SS, borderline NASH or NASH based on NAFLD activity score (NAS), a scoring system of Kleiner *et al.* [195]. NAS is the unweighted sum of scores for the intensity of steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2), ranging from 0 to 8. However, performing a biopsy in each patient with suspected NAFLD would not be reasonable and is suggested to be limited to patients with high susceptibility or indeterminate noninvasive measurement [196]. Furthermore, some limitations of liver biopsy are procedure-related complications, sampling error, high costs as well as inter- and intra-observer variability [197]. Since the prevalence of NAFLD continues to rise worldwide it is of great importance to identify high-risk patients with low risk at reasonable costs. Therefore, several non-invasive and routinely measurable parameters such as serum adipokine levels are of interest to develop a potential diagnostic tool.

3.4.2. Promising therapeutic targets

Current treatment of NASH mostly relies on the reduction of body weight, at least by consuming a more healthy diet and increasing physical activity, because there is currently no specific pharmacologic

treatment approved yet. Weight loss of 7-10 % through lifestyle intervention in obese patients with NASH, resulted in improvement of steatosis, lobular inflammation, ballooning injury and overall NAS. The magnitude of weight loss correlated strongly with improvements in histological disease markers of NASH, suggesting an intensive lifestyle intervention with a minimum weight loss goal of 7 % represents an attractive therapy for this disease [198, 199]. Experimental data suggests that the addition of omega-3 fatty acids to the diet may improve hepatic steatosis, IR and inflammation in NAFLD patients. However, evidence in human trials is insufficient due to small sample sizes and limitations in methodology [200]. Some recent studies investigated the effect of exercise without dietary modification on hepatic steatosis using MR spectroscopy. Exercise programs up to 12 weeks resulted in diminished liver fat content without a significant change in body weight, whereas its ability to improve other aspects of liver histology remains unknown [201, 202]. In addition, physical activity with vigorous intensity in combination with dietary restrictions improved grade of inflammation and oxidative stress in obese middle-aged patients with NAFLD, independent of weight reduction [203].

Because of the strong association with IR, insulin-sensitizers are interesting candidate therapies in case of NAFLD and NASH. Meta-analysis and systematic reviews have reported conflicted findings with no significant effects of metformin on liver histology. Thiazolidinedione (TZD) treatment however, showed an elevated adiponectin release and a reduction in transaminase levels, hepatic steatosis and inflammation, whereas effects on fibrosis were inconsistent [204, 205]. More specifically, a RCT with pioglitazone reported improvement of hepatic steatosis, lobular inflammation and hepatocyte ballooning in patients with NASH [206, 207]. Furthermore, GLP-1 receptor agonists may also reverse the progression of NAFLD not only by an indirect incretin effect such as improving insulin sensitivity and weight loss, but also by directly affecting hepatic lipid metabolism, inflammation and inducing adiponectin secretion [208]. Finally, the administration of anti-oxidant supplements is currently of interest to treat patients with NAFLD. However, a systematic review reported that evidence-based data was too limited to draw any definite conclusions on the effectiveness of these agents [209]. Only vitamin E has been found to ameliorate NAFLD histology, with a similar or even better improvement of NAS compared to pioglitazone treatment as shown in a RCT. However, long-term treatment with vitamin E is hampered by adverse effects on insulin sensitivity and TG levels [210, 211]. Altogether, these inconsistent findings suggest that treating solely IR by therapeutic agents may not be sufficient to reverse the complex pathophysiology of NAFLD.

A meta-analysis evaluated the effect of bariatric surgery on liver histology in NAFLD patients in studies using paired liver biopsies before and after surgery. They reported an improvement of steatosis, inflammation and even fibrosis as well as complete resolution of NASH in 69.5 % of cases [212]. However, although beneficial effects of bariatric surgery on NASH have been described, surgery remains an invasive procedure and even progression of steatohepatitis has been reported as a potential complication due to the rapid major weight loss [213].

Because of adiponectin its insulin-sensitizing effects together with the known downregulation in NAFLD patients, this adipokine represents an attractive target for the treatment of NAFLD and/or NASH. Indeed, several drugs including TZD exert at least part of their actions by modulating adiponectin or its intracellular targets (such as AMPK). Once more research proves the role of certain other adipokines in NAFLD, their receptors may be targeted by the use of agonists or antagonists in order to treat the pathology of NAFLD. However, the relevance of findings first needs to be validated in larger prospective trials because often inconsistent results are found throughout literature [178].

4. Obesity and sex steroids: how do they interact?

The widely spread presence of sex steroid receptors throughout many different tissues, indicates the importance of sex steroid exposure on bone, fat and muscle mass in both men and women [214]. It is generally known that features of obesity are associated with disturbed sex steroid levels, both in females and males. While obese women (pre- and postmenopausal) are characterized with higher androgen levels compared to lean controls (hyperandrogenism) [215-217], obese men develop hypogonadism with low levels of androgens [218, 219]. This part of the thesis will only focus on men, because of the complex gender-related differences in sex steroid metabolism and its effects on body composition. In the following section, we will describe the regulation and synthesis of sex steroids in men and focus on their contribution to body composition, fat tissue in particular, as well as on sex steroids in male obesity.

4.1. Sex steroid metabolism in men

Androgens are male sex hormones (sex steroids) that are mainly responsible for the development of primary and secondary sex characteristics. Testosterone (T) is the major biologically active androgen, almost exclusively produced by testes (95%) and to a lesser extent by adrenal cortex (5%). From puberty onwards, a rise in gonadotropins triggers androgen production. Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and stimulates secretion of gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. In turn, LH stimulates Leydig cells to produce T and FSH is mainly involved in spermatogenesis and production of inhibin B by Sertoli cells. This hypothalamic-pituitary-gonadal (HPG) axis is regulated by a negative feedback loop. Circulating T and E₂ inhibit GnRH release on hypothalamic level and LH secretion by the pituitary, whereas inhibin B inhibits FSH secretion of pituitary [220] (Figure 6). In serum, normal total T reference levels in men range from 300 to 1000 ng/dl (10.4 to 34.7 nmol/L) and free T (FT) ranges from 30 to 200 pg/ml, although these values may slightly vary depending on the measurement tool [221].

Synthesis of T involves sequential cytochrome P450-dependent (CYP) and hydroxysteroid dehydrogenase-dependent (HSD) enzymatic reactions, with the first rate-limiting step being the conversion of cholesterol to pregnenolone. Once produced, T can be further metabolized into dihydrotestosterone (DHT) and E₂ [222] (Figure 7). DHT is a biologically potent androgen, of which the conversion from T occurs both in testes (20%) as well as in peripheral tissues (80%) by the enzyme 5 α -

reductase. In some tissues with high 5 α -reductase activity such as the prostate, it has been suggested that DHT amplifies the effects of T on androgen receptor (AR). However, tissues such as skeletal muscle have low expression of 5 α -reductase, suggesting T effects are more important compared to DHT [223].

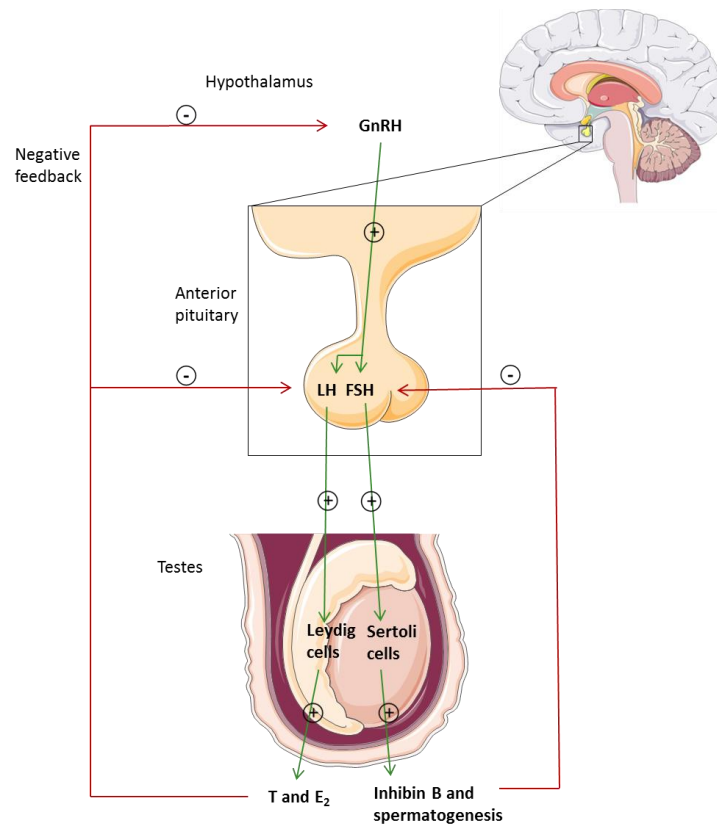


Figure 6 Hypothalamic-pituitary-gonadal axis in men. GnRH: Gonadotropin releasing hormone; LH: Luteinizing hormone; FSH: Follicle stimulating hormone; T: Testosterone; E₂: Estradiol.

In men, E₂ is synthesized in testes as well as in a number of extragonadal sites such as adipose tissue, muscle, brain, and bone, by aromatase conversion of T and acts locally as a paracrine or intracrine factor. The aromatase enzyme, encoded by CYP19 gene, is differentially expressed in many different tissues due to its tissue-specific promoters [224, 225]. Approximately 1% of the daily produced amount of T in men is converted into E₂, accounting for 80% of circulating E₂. The remainder of E₂ is secreted directly by Leydig cells or is converted from estrone, which in turn results from the aromatization of adrenal derived androstenedione [226] (Figure 7). Due to this local aromatization, serum E₂

concentrations do not always reflect tissue exposure to E_2 . In conjunction with T, E_2 is also an important regulator of the negative feedback loop of HPG axis [227].

Sex steroids act on tissue level via binding to their respective nuclear receptor, which is AR for T and DHT and the estrogen receptors α ($ER\alpha$) and β ($ER\beta$) for E_2 . Steroid hormone action thus requires the steroid to enter the cell and bind with high affinity to its specific intracellular receptor, resulting in nuclear transcription of steroid-dependent genes [228]. However, 50-60% of T is strongly bound to sex-hormone-binding globulin (SHBG), a carrier protein secreted by the liver, and is believed not to be readily available for biological action. The remaining fraction of T in circulation is weakly bound to albumin (40-50%) and only 1-2% is circulating freely (FT). Both albumin-bound T and FT represent bio-available T, which can enter target cells rapidly to exert biological effects. Although binding affinity of SHBG for estrogens is lower, circulating E_2 levels have a similar distribution [229, 230]. SHBG is a major determinant of total T and E_2 levels and regulates their bio-availability at tissue levels. Circulating SHBG levels are altered in several conditions such as obesity and polycystic ovarian syndrome (PCOS) and factors that influence hepatic SHBG production may similarly have an impact on total androgen and estrogen levels [231]. Altogether, local steroid action depends on the resultant of freely available and bound circulating levels, expression of nuclear receptors and local steroid metabolism.

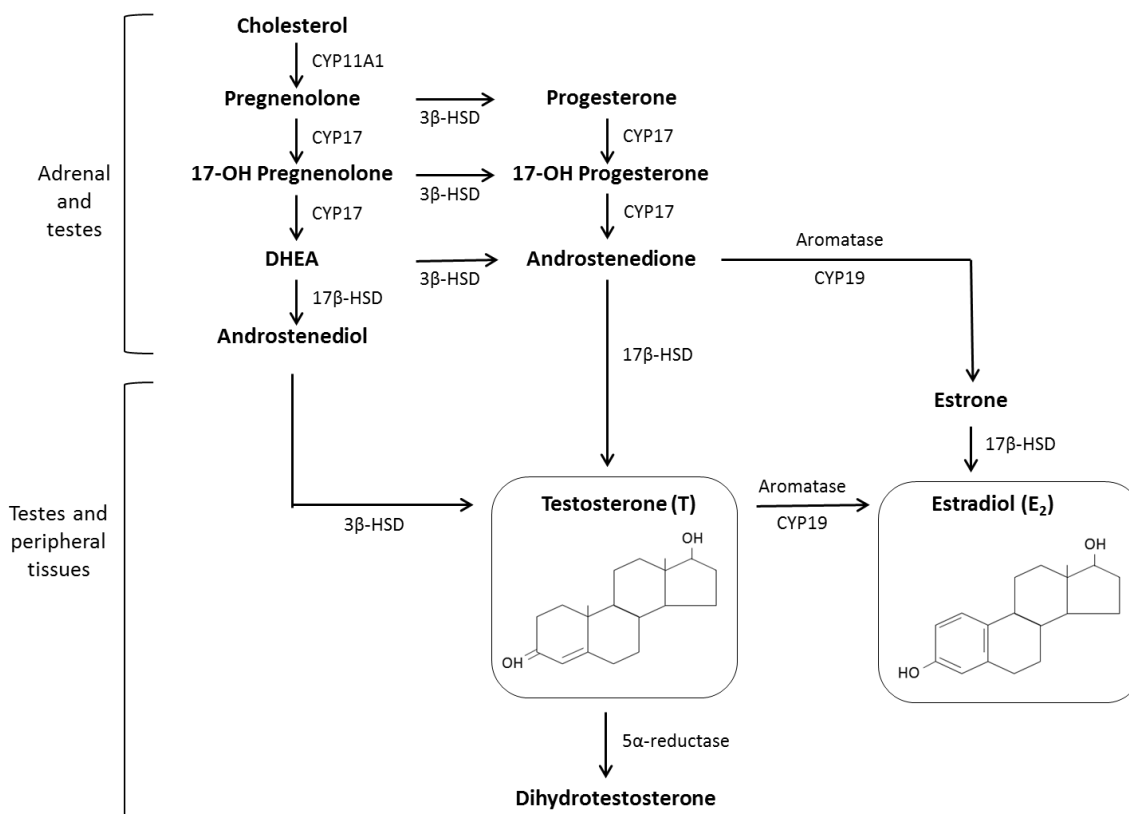


Figure 7 Major pathways in male sex steroid biosynthesis. CYP11A1: Cholesterol side chain cleavage enzyme; CYP17: 17 α -hydroxylase; 3 β -HSD: 3 β -hydroxysteroid dehydrogenase; DHEA: Dehydroepiandrosterone; 17 β -HSD: 17 β -hydroxysteroid dehydrogenase; CYP19: Aromatase enzyme. Data based on Federman *et al.* [222].

4.2. Sex steroids and body composition

Body composition consists of lean mass (skeletal muscle and organs), fat mass and bone mass. Profound differences in body composition exist between men and women, suggesting a contribution of sex steroids to this sexual dimorphism. In adulthood, men are generally characterized with more muscle mass and less fat mass at the limbs, although having similar amounts of absolute abdominal fat mass compared to women. With aging, the amount of fat mass in both sexes increases in a sex-specific manner. Men store fat preferentially in the abdominal region, with a substantial amount of both visceral and subcutaneous fat mass. They are often referred to as having a so-called apple or android body shape, whereas (premenopausal) women are more characterized by a pear or gynecoid body shape with subcutaneous fat stored mainly at the thighs [4]. Men are thus characterized with greater amounts of visceral and abdominal subcutaneous fat, which may at least in part explain the more than twofold

increased risk of developing cardiovascular disease in comparison with women [5]. Moreover, T levels in aging men are known to decrease progressively and are associated with a redistributed body composition. Low T exposure in aging men result from a combined occurrence of testicular failure, disturbed HPG axis, increased SHBG production, lifestyle and disease factors [6, 232]. Overall, we can say that a well-known association between sex steroids and body composition exists, with increasing interest in their potential role in a detrimental body composition as seen within the context of obesity [233]. Furthermore, models of androgen and estrogen insufficiency have revealed new and unexpected roles for sex steroids, of which main effects on fat tissue of men will be described in following sections.

4.2.1. Testosterone and adipose tissue

Adiposity and more specifically the location of adipose tissue appears to be related to total T and also FT levels in men. Indeed, in abdominal obese men with reduced lean mass, a strong inverse association exists between body fat and both total T and FT levels [6, 234]. Using CT and MRI scanning, some study groups found an inverse association between total T levels and VAT accumulation, whereas other fat depots were not or less associated [234-237]. Low total T levels independently predicted an increase in VAT accumulation after 7.5-year follow-up in a community-based population, simultaneously increasing metabolic risk [235]. In contrast, Abate *et al.* reported inverse associations between FT levels and SAT, rather than visceral fat accumulation, in eugonadal diabetic and nondiabetic men [238]. However, these men had FT levels within the normal range, suggesting the impact of T on the accumulation of VAT may only apply to men that have both low total T and low FT levels. AR sensitivity can additionally affect fat accumulation, as a polymorphic CAG repeat sequence within the AR gene has been linked with obesity and type 2 diabetes. It has been reported that an increased CAG repeat length and thus less sensitive AR in men with type 2 diabetes, was associated with a higher WC, BMI and serum leptin levels [239]. These results were confirmed in a mice model with AR deficiency - AR knockout (ARKO) mice, which developed late onset obesity with increased fat mass in subcutaneous and gonadal pads and became insulin resistant [240, 241].

Although the precise role of androgens in regulating body fat and metabolism has not been completely clarified, some experimental and clinical studies have revealed potential mechanisms of T action in adipose tissue. For instance, abdominal adipose tissue LPL activity was inversely associated with circulating bio-available T levels in sedentary obese men [242]. Moreover, androgen replacement therapy in hypogonadal men resulted in a decreased LPL activity and TG uptake, which was more apparent in VAT compared to SAT. This suggests that TG storage in VAT is enhanced due to low T levels

in men [243]. Additionally, T was found to possess both stimulatory effects on lipolysis and inhibitory effects on lipogenesis in mice and rats, also promoting increased fat mass in case of low T levels [244, 245]. Adipogenesis was also influenced by T, normally inhibiting the differentiation and formation of new adipocytes via reduced expression of peroxisome proliferator-activated receptor gamma (PPAR γ) [246]. In models of obesity and MetS, T replacement was able to reverse the negative impact of a high-fat diet on VAT expansion and adipocyte size by increasing the expression of several lipogenic, lipolytic and adipogenic genes to restore visceral adipocyte maturation and correct cell turnover [247]. An overview of the effects of T on targets in adipose tissue is displayed in Table 2. Finally, T levels seem to influence adipokine secretion patterns of adipose tissue, as inverse associations were reported between T and pro-inflammatory adipokines/cytokines (TNF- α , IL-6 and IL1 β) and androgen replacement therapy reduced these cytokines [248, 249]. Although these results indicate a definite contribution of T to metabolic homeostasis, its relationship with fat mass and the effects of T deficiency are complicated and require further investigation.

Table 2 Effects of testosterone on adipose tissue

Target	Abbreviation	Target function	Testosterone action	References
Acyl-CoA synthetase	ACS	<i>de novo</i> lipogenesis	↓	[250]
Acetyl-CoA carboxylase	ACC	fatty acid synthesis	↓	[251]
Adipose triglyceride lipase	ATGL	lipolysis	↓	[252]
Diglyceride acyltransferase 2	DGAT2	triglyceride synthesis	↑	[247]
Fatty acid synthase	FAS	fatty acid synthesis	↓	[251, 252]
Hormone-sensitive lipase	HSL	triglyceride breakdown; lipolysis	↓	[252, 253]
Insulin receptor substrate 1	IRS1	insulin signaling	↑	[252]
Lipoprotein lipase	LPL	triglyceride uptake	↓	[242, 243, 252, 254]
			↑	[247]
Peroxisome proliferator-activated receptor gamma	PPAR γ	adipogenesis	↓	[246]
			↑	[247]

Adapted from [255]

4.2.2. Estradiol and adipose tissue

The major driver of E₂ action in men is the aromatization of androgens to estrogens, of which the responsible aromatase enzyme is highly expressed in adipose tissue. While for decades researchers have focused mainly on the link between androgens and body composition in men, experimental models of E₂ deficiency have revealed effects of E₂ on body composition. For example, a mouse deficient in aromatase - aromatase knockout (ArKO) mouse - and thus also deficient in E₂ developed obesity independent of hyperphagia or reduced energy expenditure. Male ArKO mice developed truncal obesity with an increased adiposity in gonadal and visceral fat pads, IR and even hepatic steatosis, whereas E₂ administration to these mice reduced VAT and hepatic steatosis [256, 257]. Although not obese, an adult man described with a natural mutation in aromatase had an identical phenotype of MetS that recovered after E₂ but not T therapy [258]. Similarly, ER α deficiency in mice - ER α knockout (ER α KO) mice - caused increased body weight and fat mass with an almost doubled VAT depot, though these

mice displayed reduced energy expenditure and a slightly increased food intake [259, 260]. This suggests that E_2 may influence food intake and energy expenditure and indeed, $ER\alpha$ has been localized on several brain areas that control these actions [261]. Based on these results, estrogen is indicated as another hormone that is synthesized by adipose tissue and acts to regulate energy homeostasis [262]. Importantly, both models of E_2 deficiency – ArKO and $ER\alpha$ KO – also exhibited a marked increase in serum T levels. Since $ER\beta$ KO mice were not obese, E_2 induced $ER\alpha$ activation is pointed out as a more important regulator of adipose tissue distribution compared to $ER\beta$ activation [263]. Concerning human studies, Finkelstein *et al.* highlighted the role of estrogens in fat mass distribution/accumulation in a group of healthy men with suppressed endogenous sex steroids receiving a variety of T doses with and without concomitant aromatase inhibition. They reported that changes in lean mass were mainly attributable to variations in T levels, whereas changes in fat mass measures were primarily related to variations in E_2 levels [264]. Furthermore, administration of E_2 to individuals (and rodents) with IR has resulted in increased insulin sensitivity, though physiological and genetic evidence argue that E_2 and ER favor insulin sensitivity only when E_2 concentrations stay within a tight physiological range [265, 266]. Experimental studies have evidenced the direct effects of E_2 on cultured adipocytes. E_2 is found to inhibit lipogenesis and adipogenesis, by suppressing LPL, fatty acid synthase (FAS), ACC and PPAR γ [267].

Overall, although not completely clarified we can say that both androgens and estrogens exhibit regulatory effects on adipose tissue deposition and distribution. Potentially, both the presence of androgens and estrogens is needed to obtain a normal body fat distribution. Clearly, additional studies - both experimental as well as studies in rodents and humans - are necessary not only to better understand pure androgen and estrogen effects on adipose tissue and metabolic risk, but also to unravel the interplay of sex steroids between themselves and their actions.

4.3. Low testosterone levels in male obesity

It is generally known that features of obesity are negatively associated with total T levels, FT and bio-available T fractions in men [218, 219, 268-270], which is maintained throughout all age groups and is independent of the presence of MetS [271, 272]. T deficiency or hypogonadism, characterized by low levels of total T and FT measured before 10:00 am, is considered a common comorbidity in male obesity and is associated with BMI, WC, IR and type 2 diabetes [236, 270, 273-275]. A bidirectional relationship

between T and obesity underpins these associations, as indicated by the “hypogonadal-obesity cycle” [255]. Low T levels are suggested to be a consequence of obesity, as evidenced by weight loss interventions such as diet, exercise or bariatric surgery that resulted in significantly increased T levels in men [276]. This increase in T levels was proportional to the amount of weight lost, as reviewed recently and presented in Figure 8 [255, 276, 277]. However, minor weight loss is only associated with increased SHBG and total T levels, whereas increased FT levels, suggesting a recovered HPG axis, were only reported after more pronounced weight loss [278].

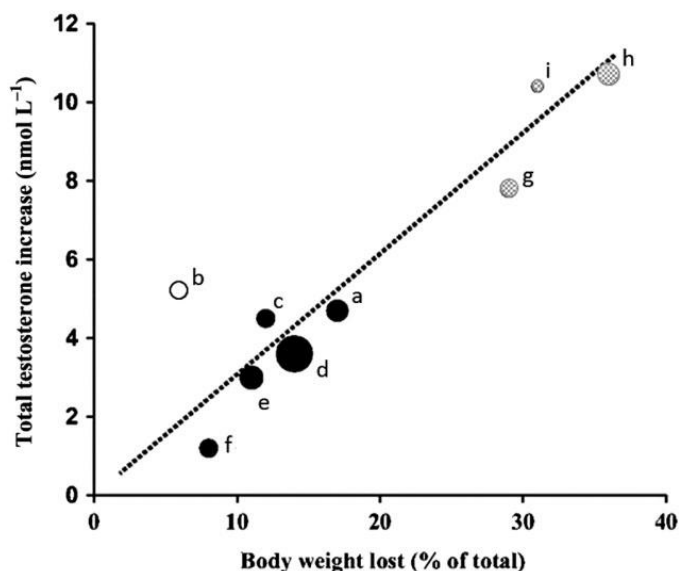


Figure 8 Effect of weight loss on serum total testosterone levels. Each data point represents an individual study, of which the size of the point is proportional to the size of the study that ranges from 10 (i) to 58 (d) men. Black points indicate studies reporting diet-induced weight loss, white points indicate exercise-induced weight loss and gray points indicate bariatric surgery-induced weight loss. a, Stanik *et al.* [279]; b, Pritchard *et al.* [280]; c, Kaukua *et al.* [281]; d, Niskanen *et al.* [282]; e and f, Khoo *et al.* [283] (Khoo *et al.* included 2 distinct obese male populations, with (f) and without (e) diabetes); g, Globerman *et al.* [284]; h, Hammoud *et al.* [285]; i, Omana *et al.* [286]. Adapted from [255, 277].

Several mechanisms contribute to low T levels in male obesity. Firstly, obesity-related hepatic IR is associated with reduced hepatic expression and production of SHBG, which in turn is a major determinant of circulating total T levels. This explains why men with mild obesity are reported to have decreased total T and SHBG levels. Only with increasing degrees of obesity, additional reduction in FT levels occurs by a downregulated HPG axis [287, 288]. One hypothesis explaining this downregulation involves the high expression of aromatase in adipocytes. Studies have suggested that the increased

amount of body fat in obese subjects results in elevated aromatase activity, leading to a higher T to E₂ conversion rate. Elevated E₂ levels in turn act upon the HPG axis as a negative feedback regulator to suppress GnRH and subsequent LH release, reducing gonadal T production [227, 289, 290]. However, data on E₂ levels in obese men are inconsistent, as reports on increased E₂ levels have not been confirmed by more recent studies reporting normal or even lower E₂ levels [238, 291-294]. Next, pro-inflammatory adipokines such as TNF- α , IL-1 β and IL-6, of which the expression is elevated in adipose tissue of obese subjects, have been found to inhibit T secretion by suppressive effects both on HPG axis as well as directly on testicular level [295-297]. Indeed, decreased T production in men has been described in acute and chronic inflammatory states [249]. Other adipose tissue-derived adipokines that have been reported to influence T secretion are leptin, adiponectin and chemerin. Especially leptin has been indicated as an important regulator of reduced androgens in male obesity, as influences of elevated leptin on the HPG axis and its direct testicular effects were reported by *in vitro* and *in vivo* studies [298-300]. Adiponectin normally stimulates Leydig cell steroidogenesis and T production, which is reduced as a result of obesity-related lowering of adiponectin levels [301]. A recent study also reported the suppression of T secretion in primary Leydig cells after the addition of chemerin, a novel adipokine of which elevated levels have been associated with both obesity and diabetes in humans [302].

Extending the “hypogonadal-obesity cycle” theory, sex steroids themselves are known to influence body composition as described above, suggesting their potential role in detrimental body compositions like obesity or sarcopenia. Men with prostate cancer undergoing androgen deprivation therapy (ADT) constitute a well-described cohort that allows investigating the direct effects of lowering T levels. In these patients, ADT leads to a decreased lean mass together with an increased total fat mass and is associated with a lowered insulin sensitivity that may evolve to the development of MetS after long-term treatment [303-305]. Recently, ADT was reported to result in increased subcutaneous abdominal as well as visceral fat mass after 12 months of therapy, and total T levels were inversely associated with visceral fat independent of E₂ [306].

Similar effects were reported in male-to-female trans persons who receive anti-androgen therapy in combination with estrogens to induce feminization. In general, at least one year of treatment resulted in an increased body weight or BMI, with a higher total and regional fat mass and a decreased lean body mass compared to age- and height matched control men [307-309]. Elbers *et al.* additionally reported higher SAT and VAT levels after one year of treatment in these male-to-female trans persons [307].

Furthermore, trials with androgen replacement therapy in (obese) hypogonadal men with MetS reported that the correction of T levels resulted in a lowered body weight, BMI, WC, VAT and total fat mass [254, 310-313].

In summary, different mechanisms seem to contribute to the obesity-related disturbed sex steroid profile in men including changes in local sex steroid metabolism, IR, impaired adipokine secretion patterns and inflammation. Furthermore, lowering T levels in men causes loss of lean mass and gain of fat mass, together with an increased incidence of IR and MetS. This increase in (especially visceral) fat mass followed by increased metabolic risk itself again contributes to a disturbed sex steroid profile, terminating the “hypogonadal-obesity cycle” (Figure 9). Up till now, it is unclear to what extent each of these mechanisms individually contributes and whether the effects on body composition are pure androgen or estrogen effects or a combination of both.

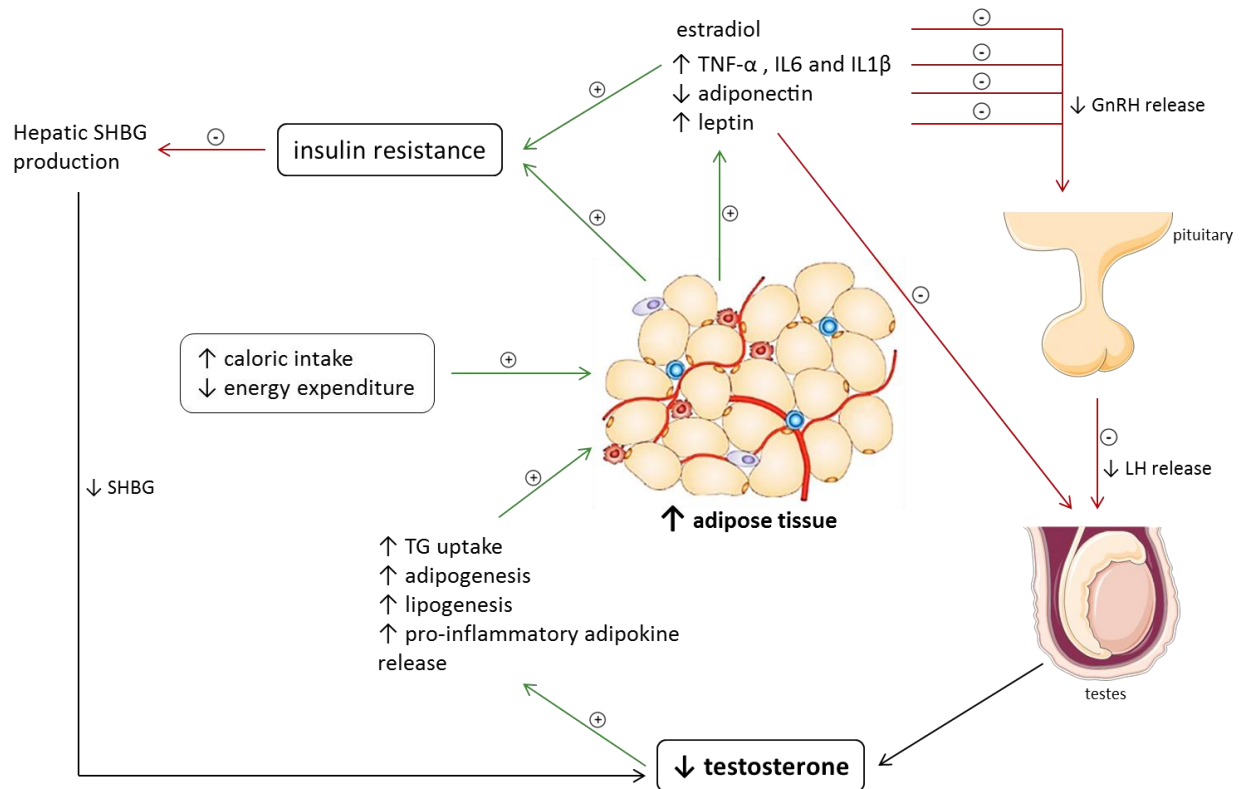


Figure 9 The hypogonadal-obesity cycle. Obesity develops when caloric intake increases and energy expenditure decreases, resulting in an expanded adipose tissue. This higher amount of adipose tissue may lead to a higher aromatase activity, impaired adipokine secretion pattern and insulin resistance, which in turn results in the lowering of testosterone (T) levels. Low T levels in obese men have been associated with an elevated triglyceride (TG) uptake, adipogenesis, lipogenesis and the secretion of pro-inflammatory adipokines, leading to a higher fat mass and thus terminating the hypogonadal-obesity cycle. +: positive effect; -: negative effect. SHBG: sex-hormone-binding globulin; GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; IL: interleukine; TNF- α : tumor necrosis factor- α . Adapted from [314].

5. Aims

5.1. General aims

Overall it can be stated that the maintenance of metabolic homeostasis is critical for health. However, from this introduction it became clear that major inconsistency still exists regarding the pathophysiology of metabolic comorbidities resulting from obesity. Although adipose tissue represents a central culprit within this topic, it comprises an embroiled complex of several pathological mechanisms such as decreased insulin sensitivity of fat cells, peripheral lipotoxicity, inflammation and changes in adipokine secretion. Elucidating the pathways on how each of these dysregulations contributes to the overall disturbed metabolic profile is of major importance given the obesity epidemic. The combination of human data together with experimental data investigating the molecular mechanisms involved in the pathophysiology, can lead to the development of effective therapeutic or even preventive strategies.

We have conducted human studies in order to gain more insight into some specific aspects of the broad scope of obesity-related consequences. Our intention was to examine adipose tissue characteristics specifically in adipose tissue biopsies of obese subjects and to investigate its possible links with biopsy-proven NAFLD and disturbed sex steroid levels. In particular, we aimed to investigate the potential role of adipokines in NAFLD and to gain a better view on the complex relationship between body composition, sex steroid levels and metabolic parameters in obese men.

5.2. Specific research aims

Both VAT and SAT biopsies were collected in a cohort of morbidly obese (WHO class II and III obesity) and control men to investigate adipose tissue characteristics of both fat depots. More specifically, we wanted to explore how these characteristics and the degree of NAFLD and IR are linked. Especially the adipokine secretion of VAT, considering direct portal delivery of these secretory factors to the liver, was investigated in an obese cohort with biopsy-proven NAFLD. This topic is explored in **chapter III**. While classic adipokines such as adiponectin, leptin and the cytokines TNF- α and IL-6, have been extensively investigated within the context of NAFLD, knowledge on other recently described adipokines remains scarce. Therefore, literature was first reviewed to summarize current knowledge on the potential link between these recently described adipokines and NAFLD. More specifically, all human studies that investigated non-classical adipokines and their association with histopathological severity in biopsy-proven NAFLD patients were collected and reviewed. Results were combined into a systematic review,

as discussed in **chapter 3.1**. Secondly, a selection of more novel adipokines was made, of which we suspected a contribution to NAFLD, to test their association with hepatic histological parameters in a cross-sectional cohort of obese middle-aged NAFLD patients. We aimed to investigate whether the adipokines were independently associated with steatosis grade, inflammation, hepatocyte ballooning and/or fibrosis grade of liver biopsies, in order to clarify their potential contribution to NAFLD development and/or progression. These findings are reported in **chapter 3.2**.

Finally, we aimed to examine the impact of (dysfunctional) adipose tissue on sex steroid levels in a cohort of morbidly obese men. In order to investigate this and other potential determinants of low T levels in obese men, focus was made on several events that have been associated with sex steroid levels such as adipose tissue characteristics and aromatase expression, parameters of the HPG axis and metabolic alterations. Although the contribution of higher aromatase activity to lowering T levels in obese men has been hypothesized by many researchers since decades, few have truly examined *in situ* aromatase expression in hypertrophied adipocytes of obese men. Adipose tissue and metabolic characteristics as well as sex steroid levels were determined in a cross-sectional study cohort of obese men (BMI > 30 kg/m²) and healthy control men, as discussed in **chapter IV**.

II. STUDY DESIGN AND METHODS

This chapter describes the study populations and methodology used throughout this thesis. For more technical details on the methodology, we refer to chapter III and IV. Depending on the research question and the time point of the analysis, the number of included patients will vary in the following chapters.

1. Study populations

Throughout this thesis three different study populations were analyzed, which included obese patients that were scheduled for bariatric surgery. In two studies, (non-obese) healthy controls scheduled for elective abdominal surgery were included in addition to the obese patients. A general overview of the study populations is given in Figure 10, with a description of the participants as well as the collected samples for analysis. All three study protocols were approved by the institutional ethics committee and were conducted according to the principles of the Declaration of Helsinki. Participants gave their written informed consent before inclusion.

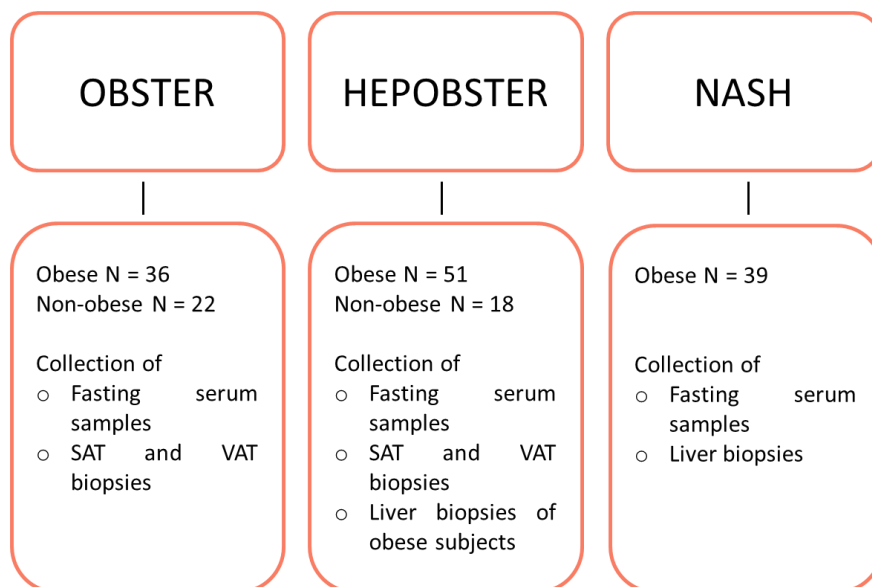


Figure 10 Overview of the study populations analyzed throughout this thesis. OBSTER, OBesity and sex STERoids; HEPOBSTER, HEPatic disease in OBesity and sex STERoids; NASH, Non-Alcoholic SteatoHepatitis; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

Analyses presented in chapter III were performed on the Hepobster and NASH study cohorts, whereas chapter IV describes analyses performed on the Obster study cohort and a subgroup of the Hepobster study cohort (N = 24; the amount of patients recruited in Hepobster study up till then).

1.1. Obster study

The Obster study is a case-control study, established at the Department of Endocrinology of Ghent University Hospital to investigate SAT and VAT depots as well as sex steroid levels in obese middle-aged men. The study cohort consisted of 36 obese men scheduled for gastric bypass surgery and 22 normal-weight control men undergoing surgery for adhaesiolysis, rupture of the stomach, intestinal resection, stomach closing or Nissen fundoplication, all between 22 and 70 years old. Obese (BMI > 30 kg/m²) participants were recruited at the entry of a multidisciplinary care plan for morbidly obese patients, if they were found suited to undergo bariatric surgery at the Department of Gastrointestinal Surgery of Ghent University Hospital. Participants with primary hypogonadism, abnormal thyroid function, hepatitis or malignancies, serum total cholesterol > 300 mg/dl and/or serum TG > 450 mg/dl were excluded. None of the subjects used steroids or insulin, and oral glucose lowering-medication were discontinued prior to surgery.

1.2. Hepobster study

The Hepobster study sequels the Obster study. Otherwise having an overall identical study protocol, an additional liver biopsy is performed during surgery of obese participants in order to obtain histological NAFLD status. Between 2011 and 2015, we recruited 51 obese men undergoing gastric bypass surgery and 18 control men undergoing Nissen fundoplication surgery between 20 and 66 years of age. In- and exclusion criteria were similar to the Obster study but additionally, patients with other causes of liver disease, e.g. hepatitis B or C, viral or autoimmune hepatitis, Wilson disease or any drug-induced liver disease, or evidence of excessive alcohol consumption (≥ 20 g per day) were specifically excluded for this study. None of the subjects used steroids, insulin or thiazolidinediones and other oral glucose lowering-medication were discontinued prior to surgery.

1.3. NASH study

The Department of Gastroenterology and Hepatology of Ghent University Hospital recruited 39 obese patients (6 males and 33 females) between 32 and 68 years old, that were scheduled for gastric banding or gastric bypass surgery for inclusion in the NASH study. They all met the criteria for bariatric surgery of the International Federation for the Surgery of Obesity: BMI ≥ 40 kg/m² or BMI ≥ 35 kg/m² in conjunction with obesity-induced co-morbid conditions. Again, patients with other causes of liver disease, e.g. hepatitis B or C, viral or autoimmune hepatitis, Wilson disease or any drug-induced liver disease, evidence of excessive alcohol consumption (≥ 20 g per day), abnormal thyroid function, malignancies or cancer, serum total cholesterol > 300 mg/dl and/or serum TG > 450 mg/dl were excluded for this study.

2. Methods

2.1. Anthropometry and biochemical analysis

Anthropometrics were measured in the three study populations during a pre-operative examination. Body weight (kg) was measured to an accuracy of 0.1 kg in light indoor clothing without shoes. Standing height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. BMI was calculated as body weight divided by squared height (kg/m²). Blood samples were collected after overnight fasting, prior to surgery, and were centrifuged, fractionated and stored at -80°C until batch analysis. Measurement of TG, glucose, insulin, AST, ALT, GGT and CRP was routinely performed using a commercially available immunoassay or using a conventional automated analyzer. Homeostasis model of the assessment for insulin resistance (HOMA-IR) was calculated with the following formula: (fasting glucose [mmol/L] x fasting insulin [μ U/mL])/22.5 [315].

2.1.1. Measurement of adipokines

Serum levels of adipokines including chemerin, omentin, MCP-1, adiponectin and SFRP4 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions. Measurements were performed at the Institute for Clinical Biochemistry and Pathobiochemistry in the German Diabetes Center at Duesseldorf (Deutsches Diabetes Zentrum, DDZ) supervised by Prof. Dr. Ouwens.

2.1.2. Measurement of sex steroids

Total T and E₂ were measured in serum using liquid chromatography tandem mass spectrometry (LC-MS/MS). LC-MS/MS is a technique that combines the physical separation of components in a sample according to their polarity, electrical charge or molecular size (liquid chromatography (LC)), with measurement of the mass-to-charge ratio of charged particles after ionization (mass spectroscopy (MS)). The advantages of LC-MS/MS are the high sensitivity and specificity and limit of quantification (LOQ) is very low. Unlike immunoassays, LC-MS/MS allows for measuring whole steroid profiles in one single run for one sample and requires only small sample volumes (50 to 200 µl) [316].

Free T levels were measured using a validated equilibrium dialysis method, as described previously by Vermeulen *et al.* [317]. Free E₂ levels were calculated from total E₂, SHBG, and albumin concentrations as described elsewhere [318]. Commercial immunoassays were used to determine SHBG, LH, and FSH.

2.2. Adipose tissue processing

At the end of the surgical intervention, abdominal SAT and VAT biopsies were obtained from the male subjects of the Obster and Hepobster study. Biopsies were immediately stored at -80 °C until further gene expression analysis or fixated in formol (buffered 4 % paraformaldehyde solution; Klinipath, Belgium) at room temperature for microscopic analysis.

2.2.1. Subcutaneous adipocyte cell size assessment

Adipocyte cell size was measured in SAT samples that were obtained in the Obster study. After paraffin impregnation, the SAT samples were stained with Hematoxylin-eosin and were completed into 3 µm slides. Digital photographs of the paraffin slides were taken with an Axioskop 20 light microscope (Zeiss, Jena, Germany), on which the mean surface area of adipocytes was measured by indicating the margins of the cell membrane of all complete adipocytes. As adipocytes were assumed to be spheres, as many as possible complete imaged adipocytes were measured in order to calculate the median surface area as expressed in µm² per study patient, followed by calculation of the median SAT cell size per study group. Cell size assessment was blinded to grouping and was determined in 50 subjects of the Obster study.

2.2.2. Gene expression analysis of aromatase and adipokines

Aromatase expression was determined in the frozen SAT samples of 36 subjects (a subgroup of subjects from the Obster and Hepobster study cohort). Expression of the adipokines chemerin, omentin, MCP-1

and SFRP4 was determined in the frozen VAT samples of all 69 subjects of the Hepobster study cohort. Gene expression analysis was measured using real-time polymerase chain reaction (real-time PCR). After RNA isolation from 100 mg of the frozen fat biopsies, contaminating genomic DNA was removed and cDNA was synthesized. Next, aromatase and adipokine expression was quantified by real-time PCR using specific primer assays and PCR Master Mix on a StepOne Plus system (Applied Biosystems). Obtained threshold cycle (Ct) values were normalized for the expression of the stable reference genes.

2.3. Liver tissue processing and histopathological analysis

In the Hepobster and NASH study, liver biopsies were obtained during surgery and immediately processed. After formalin fixation, biopsies were routinely processed and stained with hematoxylin-eosin and Masson trichrome. An experienced pathologist from the Department of Pathology at Ghent University Hospital established the histological diagnosis of NAFLD according to the scoring system of Kleiner *et al.* [195], blinded to characteristics of participants. At least 6 complete portal tracts in liver specimen are required for adequate histological evaluation. Steatosis was assessed as the percentage of hepatocytes containing fat droplets, with a minimum of 5 %. NAS has been introduced as the unweighted sum of scores for the intensity of steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2), ranging from 0 to 8. NAS of 0 – 2 was considered as SS and scores of 5 or greater were considered as definite NASH. Intermediate scores of 3 or 4 were considered as borderline NASH. Additionally, stage of fibrosis was also scored using a 4-point scale.

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III. POTENTIAL ROLE OF ADIPOKINES IN NAFLD

3.1. Association of recently described adipokines with liver histology in biopsy-proven non-alcoholic fatty liver disease: a systematic review

3.2. Reduced expression of chemerin in visceral adipose tissue associates with hepatic steatosis in patients with obesity

3.1. Association of recently described adipokines with liver histology in biopsy-proven nonalcoholic fatty liver disease: a systematic review

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Abstract

The prevalence of non-alcoholic fatty liver disease (NAFLD) is rising, as is the prevalence of obesity and type 2 diabetes. It is increasingly recognized that an impaired pattern in adipokine secretion could play a pivotal role in the development of NAFLD. We performed a systematic review to evaluate the potential link between newly described adipokines and liver histology in biopsy-proven NAFLD patients. A computerized literature search was performed in PubMed, EMBASE and Web of Science electronic databases. Thirty-one cross-sectional studies were included, resulting in a total of seven different investigated adipokines. Studies included in this review mainly had a good methodological quality. Most adipokines were suggested to be involved in the inflammatory response that develops within the context of NAFLD, either at hepatic or systemic level, and/or hepatic insulin resistance. Based on literature, clinical studies suggest that chemerin, resistin and AFABP potentially are involved in NAFLD pathogenesis and/or progression. However, major inconsistency still exists and there is a high need for larger studies, together with the need of standardized assays to determine adipokine levels.

Introduction

The growing epidemics of obesity and type 2 diabetes are associated with an increased incidence of non-alcoholic fatty liver disease (NAFLD) in the Western world. NAFLD is becoming one of the leading causes of chronic liver disease and can present as a broad spectrum of liver diseases, ranging from a simple fatty liver (steatosis) with a benign non-progressive clinical course to non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis and ultimately end-stage liver disease or development of hepatocellular carcinoma (HCC). It is estimated that the prevalence of NAFLD has reached up to 75% in a high-risk Western population (1-3). The widespread increasing rates of obesity-related NAFLD urges for a better understanding of its pathophysiology. NAFLD is associated with visceral obesity and is considered to be the hepatic component of metabolic syndrome, with insulin resistance (IR) playing a major role (1, 4). However, the pathogenesis remains incompletely understood.

Adipose tissue has been found to secrete multiple regulating proteins, so called adipokines, as an endocrine gland, which exert local, peripheral and central effects. It is increasingly recognized that an impaired pattern in adipokine secretion could play a pivotal role in development of metabolic syndrome, including NAFLD and the progression to NASH. Visceral adipose tissue (VAT) has been proposed as a major contributor to NAFLD (5, 6). A study in Japan suggested that hepatic steatosis may be influenced by visceral fat accumulation regardless of body mass index (BMI), as the severity of hepatic steatosis by ultrasound was positively associated with visceral fat accumulation and IR in both obese and non-obese subjects (7). Furthermore, adipokines derived from visceral adipose tissue are considered to be delivered directly to the liver via its portal vein. Many investigators have attempted to demonstrate their role in the pathogenesis and progression of NAFLD and/or NASH. As an example, hypoadiponectinemia has been suggested to play a role in the progression from NAFLD to NASH, because of adiponectin its anti-inflammatory, antifibrogenic and insulin-sensitizing effects in the liver as well as its potential role in hepatic fatty acid metabolism (8-12). Also, several cross-sectional studies have indicated associations between NAFLD and serum levels of inflammatory cytokines including tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) (13, 14). The role of these more classic adipokines has been reviewed frequently (12, 15-19). Relevance on the contribution of other known adipokines however, continues to be unclear. In view of this, the present review summarizes current knowledge on recently described adipokine levels in patients with biopsy-proven NAFLD and NASH, of which reports in the clinical setting of NAFLD is scarce. Whether or not these adipokines associate with liver histology and more specifically with the degree of steatosis, ballooning, fibrosis and/or

inflammation, could provide useful information and form the basis for experimental trials, leading to investigating new treatment approaches.

Methods

Search strategy and study selection

Search objectives included human studies with biopsy-verified NAFLD classification, the determination of adipokines and association analysis between adipokine levels and liver histopathologic severity. A computerized literature search was performed in PubMed, EMBASE and Web of Science electronic databases. Medical Subject Headings were used to form the combination of terminological terms, in order to retrieve relevant journal articles. After a preliminary search, a more specific query was used including ‘(((liver) AND histology) AND (adipokines OR adipocytokines)) AND (NAFLD OR NASH OR non-alcoholic liver disease)’. Search was limited to human studies using the ‘Humans’ search filter but was not limited by publication time. After removing duplicates, the literature search was extended by checking references and citations of the retrieved articles.

Articles were excluded from the systematic review if (i) article types were reviews, conference abstracts, case reports, editorials or letters; (ii) adipokine information was lacking; (iii) studies investigated gene polymorphisms; (iv) studies were interventional; (v) studies investigated other hepatic diseases (alcoholic fatty liver disease, unspecified cirrhosis, viral or autoimmune hepatitis), and adipokine data was missing for NAFLD subjects; (vi) patients were not subjected to liver biopsy; (vii) data on relations between adipokine levels and liver histology were absent; (viii) studies contained only paediatric data or (ix) there was a patient overlap. (x) Because this review was composed with the objective to investigate only recently described adipokines, articles were excluded when focusing on adiponectin, leptin or cytokines such as TNF- α and interleukines (IL-6), which have been extensively studied and/or reviewed over the last years (12, 13, 18-20). (xi) Finally, three out of ten found adipokines were additionally excluded because they were reported by only one respective study, leading to incomparable results. An overview of the search strategy is depicted in Fig. 1.

Data on selected studies

This review included studies reporting serum or expression levels of recently described adipokines in biopsy-proven NAFLD subjects. In most studies, histological classification was based on Brunt *et al.* (21) or Kleiner *et al.* (22) and controls were included when having a normal liver ultrasound and normal serum aminotransferases levels. The scoring system of Kleiner is known to be the successor of Brunt, with a more extensive range of histological parameters studied. According to the scoring system of Kleiner *et al.*, a NAFLD activity score (NAS) has been introduced as the unweighted sum of scores for the intensity of steatosis, lobular inflammation, and hepatocellular ballooning, ranging from 0 to 8. Scores of

0 – 2 were considered as simple steatosis, and scores of 5 or greater were diagnosed with definite NASH. Intermediate scores of 3 or 4 were considered as borderline NASH (22). Three studies did not mention the scoring system used to define NAFLD and/or NASH, whereas one study referred to an alternative scoring system. The latter study performed histological classification according to Dixon *et al.* (23), of which criteria were steatosis (0-4), inflammation (0-4) and fibrosis (0-4) as a modification of the scoring system published by Brunt *et al.* (21) and Lee *et al.* (24).

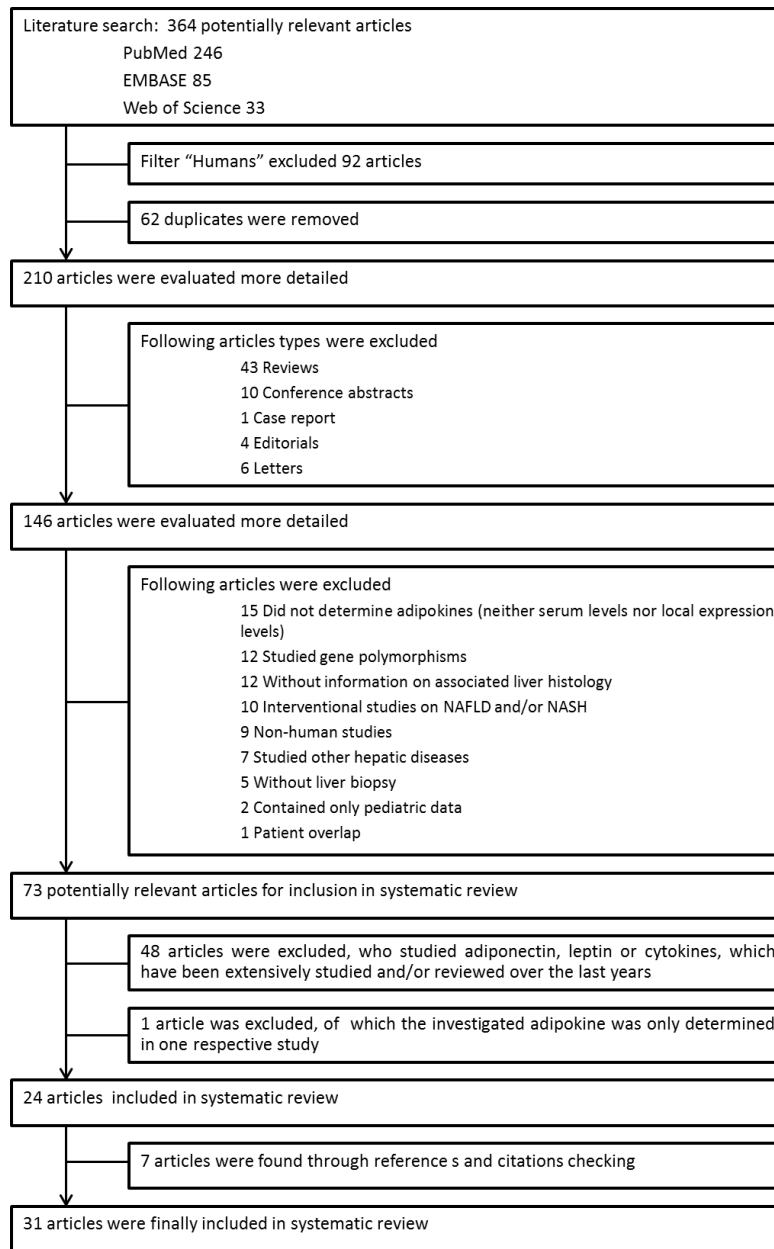


Figure 1 Flowchart of the search strategy

Serum adipokine levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits in 26 studies, quantikine immunoassay in one study and nephelometry in one study. Furthermore, liver adipokine expression was determined in five studies, subcutaneous adipose tissue (SAT) adipokine expression was determined in two studies and VAT adipokine expression was determined in three studies. Comparison groups were mostly (i) control versus NAFLD subjects; (ii) control versus NASH subjects and/or (iii) NAFLD versus NASH subjects.

Risk of bias

Methodological quality of individual studies was evaluated by two independent researchers (M.B. and P.C.). Results of both researchers were compared, and differences were discussed and analyzed in a consensus meeting. Methodological quality of the studies was assessed using a standardized checklist for cohort studies of the Dutch Institute for Healthcare Improvement CBO (www.cbo.nl/en/). The checklist is presented in Table 1 and consists of seven criteria evaluating risk of bias. The checklist included questions concerning the description of study groups, risk of selection bias, clearly defined methods and risk of confounding factors. Every positive question was awarded by one point, leading to a maximum score of seven if all criteria were applicable. Finally, scores were converted into percentages to allow comparison of studies.

Table 1 Methodological quality assessment of individual studies

Studies	1	2	3	4	5	6	7	Score /7	Score %
Sell H <i>et al.</i> , 2010, Germany (25)	+	+	+	+	-	+	+	6	86
Kukla M <i>et al.</i> , 2010, Poland (26)	+	+	+	+	-	-	+	5	71
Yilmaz Y <i>et al.</i> , 2011, Turkey (27)	+	+	+	+	+	+	+	7	100
Döcke S <i>et al.</i> , 2013, Germany (28)	+	-	+	+	+	+	+	6	86
Musso G <i>et al.</i> , 2005, Italy (29)	+	+	+	-	-	+	+	5	71
Pagano C <i>et al.</i> , 2006, Italy (30)	+	+	+	+	-	-	+	5	71
Baranova A <i>et al.</i> , 2006, USA (31)	+	+	+	+	-	-	+	5	71
Wong VW <i>et al.</i> , 2006, China (32)	+	+	+	+	+	+	+	7	100
Younossi ZM <i>et al.</i> , 2008, USA (33)	+	+	+	+	-	+	+	6	86
Jarrar MH <i>et al.</i> , 2008, USA (34)	-	-	+	+	-	+	+	4	57
Aller R <i>et al.</i> , 2008, Spain (35)	+	+	+	+	-	+	+	6	86
Krawczyk K <i>et al.</i> , 2009, Poland (36)	+	-	+	-	-	-	-	2	29
Argentou M <i>et al.</i> , 2009, Greece (37)	+	+	+	+	-	-	+	5	71
Cengiz C <i>et al.</i> , 2010, Turkey (38)	+	-	+	+	+	+	+	6	86
Senates E <i>et al.</i> , 2012, Turkey (39)	+	+	+	+	+	-	+	6	86
Koehler E <i>et al.</i> , 2012, USA (40)	+	+	+	+	+	+	+	7	100
Petta S <i>et al.</i> , 2008, Italy (41)	+	+	+	+	+	+	+	7	100
Alkhouri N <i>et al.</i> , 2009, USA (42)	+	-	+	+	-	+	+	5	71
Kashyap SR <i>et al.</i> , 2009, USA (43)	+	+	+	+	-	+	+	6	86
Schina M <i>et al.</i> , 2009, Greece (44)	+	+	+	+	-	-	+	5	71
Milner KL <i>et al.</i> , 2009, Australia (45)	+	+	+	+	-	+	+	6	86
Terra X <i>et al.</i> , 2013, Spain (46)	-	-	+	-	-	-	+	2	29
Aller R <i>et al.</i> , 2009, Spain (47)	+	+	+	+	-	+	+	6	86
Gaddipati R <i>et al.</i> , 2010, India (48)	+	-	+	+	+	-	-	4	57
Kukla M <i>et al.</i> , 2010, Poland (49)	-	+	+	+	-	-	+	4	57
Dahl TB <i>et al.</i> , 2010, Norway (50)	+	+	+	+	+	+	+	7	100
Genc H <i>et al.</i> , 2013, Turkey (51)	+	+	+	+	+	+	+	7	100
Shen J <i>et al.</i> , 2012, China (52)	+	-	+	+	+	+	+	6	86
Genc H <i>et al.</i> , 2011, Turkey (53)	+	+	+	+	+	+	+	7	100
Aktas B <i>et al.</i> , 2011, Turkey (54)	+	+	+	+	+	+	+	7	100
Ercin CN <i>et al.</i> , 2010, Turkey (55)	+	+	+	+	+	+	+	7	100

1, study groups are clearly defined; 2, selection bias can be sufficiently excluded; 3, exposure (adipokine levels) and method of assessment are clearly described; 4, output (liver histology) and method of assessment are clearly described; 5, output is blindly assessed; 6, potential confounders are identified and corrected for analysis; 7, results are valid and applicable.

Results

Search results

A flowchart summarizing search results is depicted in Fig. 1. The 31 included studies were published between 2005 and 2013 and had a cross-sectional study design (25-55). Table 2 shows the main clinical and biochemical data of all 31 included studies, grouped per adipokine. Apart from adipokine levels, method of histological classification, group classification, male-to-female ratio, number of patients with type 2 diabetes, adjusted confounding factors and main conclusions were summarized. Following adipokines were described: chemerin in four studies, resistin in 12 studies, retinol-binding protein 4 (RBP4) in seven studies, visfatin in seven studies, adipocyte-fatty-acid-binding protein (AFABP) in two studies, vaspin in three studies and apelin in two studies.

Risk of bias

The risk of bias of the individual studies was evaluated using a seven-item checklist, of which results are presented in Table 1. The final score of each study was calculated and converted into a percentage. Methodological quality varied between 29% and 100%, with only two studies scoring beneath 50%. In general, 26 out of 31 included studies had good quality with a score above 70%. Most studies lost points on blinding of output assessment and on correcting for potential confounders.

Chemerin

Chemerin was suggested to be related with the progression of NAFLD in four studies reporting associations with histological parameters that may indicate disease progression. Presence of portal inflammation and the combined severity of fibrosis and inflammation were positively associated with serum chemerin levels, a correlation that persisted after BMI adjustments, in a study cohort of morbidly obese women by Sell *et al.* (25). A positive association between chemerin levels and both NAS and hepatocyte ballooning was shown in obese NAFLD and NASH patients by the study of Kukla *et al.* (26). Although Yilmaz *et al.* (27) suggested a positive association with liver fibrosis in non-obese NAFLD patients, significance did not persist after allowance for potential confounders. In a cohort of obese undefined and defined NASH patients, Döcke *et al.* (28) reported a positive association of hepatic chemerin expression with overall histological NAS, as well as an independent association with liver fibrosis, steatosis, inflammation and hepatocyte ballooning.

Table 2 Main adipokine-related data and conclusions of all studies included in the systematic review, grouped per adipokine

Adipokine	Reference	Histology	Group	M/F	Adipokine determination	T2D patients	Adjusted confounding factors	Main conclusions
Chemerin	Sell <i>et al.</i> , 2010, Germany (25)	Kleiner and fibroinflammation score	Healthy control	0/13	191 ± 14 ng/ml (vs total obese group 354 ± 18 ng/ml*)	21	BMI	Chemerin serum concentrations were highly associated with the presence of portal inflammation and the combined severity of fibrosis and inflammation, a correlation that persisted after BMI adjustments. Authors suggest that chemerin could be a link between adipose tissue inflammation and liver pathology in obesity.
			Obese non-NAFLD	0/13	NA			
			Obese NAFLD	0/44	NA			
			Obese NASH	0/3	487 ± 18 ng/ml*			
Kukla <i>et al.</i> , 2010, Poland (26)	Kleiner	Healthy control	10/10	6.1 ± 2.5 ng/ml (vs total NAFLD group 24.7 ± 17.1 ng/ml**)	10	/	Chemerin was positively associated with NAS and hepatocyte ballooning. No association between the severity of hepatic fibrosis and chemerin levels was found. Authors suggest that chemerin and IR play a role in NAFLD progression.	
		Obese NAFLD	NA (n=21)	16.3 ± 10.9 ng/ml**				
		Obese NASH	NA (n=20)	33.0 ± 18.5 ng/ml ^{###}				
Yilmaz <i>et al.</i> , 2011, Turkey (27)	Kleiner	Healthy control NAFLD	37/38 50/49	159 ± 43 ng/ml 219 ± 83 ng/ml ***	29	Age, sex, BMI, HOMA-IR, T2D, MetS, systolic and diastolic BP, AST, ALT, TG, total, HDL and LDL Chol, hs-CRP	Chemerin serum levels were associated with the degree of liver fibrosis. After allowance for potential confounders, however, this result did not persist. Chemerin may play a role in the pathophysiology of NAFLD but does not seem to be a key biomarker of liver fibrosis in this entity.	
Döcke <i>et al.</i> , 2013, Germany (28)	Kleiner and Ishak fibrosis scoring system	No NASH Obese undefined NASH Obese definite NASH	14/20 4/6 1/2	Liver mRNA (qPCR): ^A 1.06 AU 1.3 AU 2.76 AU** ^{###}	9	Age, sex, BMI, alcohol consumption, oral anti-diabetics	Although circulating chemerin levels were not correlated with hepatic histology and mRNA levels, its mRNA expression was correlated with NAS. Linear regression analysis confirmed an independent association of liver fibrosis, steatosis, inflammation, and hepatocyte ballooning with hepatic chemerin mRNA expression.	
Resistin	Musso <i>et al.</i> , 2005, Italy (29)	Brunt	Healthy controls NASH	23/2 23/2	4.28 ± 0.21 ng/ml 4.12 ± 0.37 ng/ml	Excluded	Age, BMI, WC, WHR, insulin, ISI, TNF-α, adiponectin, leptin	No statistical correlations were found between resistin and histological findings of NASH patients.

Pagano <i>et al.</i> , 2006, Italy (30)	Brunt	Healthy control Obese non-NAFLD NAFLD	ELISA: 30/3 26/4 26/2 qPCR: NA (n=9) NA (n=9) NA (n=10)	ELISA: 4.30 ± 0.20 ng/ml 4.37 ± 0.27 ng/ml 5.87 ± 0.49 ng/ml** ^{§§} SAT mRNA (qPCR): ^A 7 AU 4 AU 20 AU* [§]	Excluded	/	In patients with NAFLD, a positive correlation was found between resistin and histological NASH score and not with steatosis score, suggesting resistin is related to histological severity of liver disease but does not support a link with IR or BMI in these patients
Baranova <i>et al.</i> , 2006, USA (31)	NA	Obese IR (4 NASH) Obese non-IR (0 NASH)	2/9 1/9	ELISA: 6.91 ng/ml 3.9 ng/ml** VAT mRNA (qPCR): 9.04 AU 9.23 AU	NA	NA	Resistin did not correlate with histological parameters but serum resistin associated with markers of IR.
Wong <i>et al.</i> , 2006, China (32)	Brunt	Healthy controls SS NASH	17/24 19/9 33/19	Quantikine immunoassay: 15 ± 8 ng/ml 18 ± 14 ng/ml 14 ± 13 ng/ml	47	Age, sex, BMI, T2D, WHR, TNF- α , adiponectin, leptin	Resistin levels did not correlate with histological parameters.
Younossi <i>et al.</i> , 2008, USA (33)	NA	Obese controls Obese SS Obese definite NASH	3/29 1/14 9/13	7.9 ± 4.1 ng/ml 7.9 ± 3.4 ng/ml 5.9 ± 3.0 ng/ml	NA	M30, M65-M30, adiponectin	Multivariate analysis revealed that histological NASH could be predicted by a combination of Cleaved CK-18 (marker of apoptosis), a product of the subtraction of Cleaved CK-18 level from Intact CK-18 level (marker of necrosis), serum adiponectin and serum resistin.
Jarrar <i>et al.</i> , 2008, USA (34)	Kleiner	Healthy controls Obese non-NAFLD Obese SS Obese NASH	6/6 5/33 2/17 11/15	NA 7.6 ± 3.8 ng/ml 7.8 ± 4.3 ng/ml 6.0 ± 2.9 ng/ml	NA	Age, sex, BMI, ethnicity, WHR, AST, ALT, TG, Chol, glucose, insulin, TNF- α , adiponectin, IL-6, IL-8, visfatin	No statistically significant findings related to serum resistin levels were found in NASH, SS and obese controls. This study confirmed the absence of an association between NAFLD subtypes and resistin and showed the complexity of the interactions between various adipokines, IR and the pathogenesis of NAFLD.
Aller <i>et al.</i> , 2008, Spain (35)	Brunt	Low grade steatosis High grade steatosis	10/2 10/2	2.36 ± 0.6 ng/ml 2.8 ± 0.6 ng/ml*	Excluded	Age, sex, BMI, HOMA-IR, dietary intake	High levels of resistin could be found as a surrogate marker of IR, as including HOMA-IR to multivariate logistic analysis resulted in a lack of association between resistin and steatosis.
Krawczyk <i>et al.</i> , 2009, Poland (36)	Dixon	Healthy controls NASH	NA (n=16) 16/2	25.78 ± 26.02 ng/ml 22.94 ± 6.3 ng/ml	3	/	Resistin did not correlate with histological findings of NASH patients. However, a correlation between resistin and TNF- α suggests a role for resistin in the inflammatory process.

	Argentou <i>et al.</i> , 2009, Greece (37)	Kleiner	Obese controls Obese SS Obese NASH	2/7 9/22 6/4	0.94 ± 0.31 ng/ml 0.91 ± 0.33 ng/ml 0.98 ± 0.55 ng/ml	18	/	In the NAFLD group, resistin levels were negatively associated with the grade of steatosis and percentage of liver parenchyma involved by steatosis. Resistin levels are related to liver histology in bariatric patients and may be indicative of the extent of hepatic steatosis.
	Cengiz <i>et al.</i> , 2010, Turkey (38)	Kleiner and Brunt	Healthy controls Ultrasound steatosis Biopsy-proven NAFLD	NA (n=24) NA (n=76) NA (n=34)	1.3 ± 0.9 ng/ml 2.2 ± 0.9 ng/ml*** NA	Excluded	Age, BMI, HOMA-IR, AST, ALT, GGT, TG, Chol, adiponectin, leptin, RBP4	Although resistin was not related to HOMA-IR, on multivariate logistic regression analysis resistin remained a risk factor for the development of steatosis. Furthermore, resistin was found as a predictive factor independently associated with the degree of necroinflammation.
	Senates <i>et al.</i> , 2012, Turkey (39)	Kleiner	Healthy controls SS Borderline NASH Definite NASH	33/33 NA (n=12) NA (n=34) NA (n=51)	26.57 ± 13.60 ng/ml (vs NAFLD 32.10 ± 10.0ng/ml**) 20 ± 10 ng/ml 26 ± 15 ng/ml 29 ± 13 ng/ml [#]	24	/	Resistin levels were associated with histological steatosis, portal inflammation and NASH scores, regardless of potential confounders. Although subject to future confirmation, this preliminary data suggests resistin could discriminate SS from definite NASH and may be useful as a non-invasive marker for more advanced forms of NAFLD.
	Koehler <i>et al.</i> , 2012, USA (40)	Brunt	Obese no NAFLD Obese SS Obese NASH with fibrosis stage 0-1 Obese NASH with fibrosis stage ≥ 2	4/12 8/64 8/52 4/8	15.1 (13.7-23.4) ng/ml 15.3 (12.7-19.0) ng/ml 14.9 (12.6-17.8) ng/ml 17.5 (14.2-19.7) ng/ml	59	QUICKI, GH, CK-18, adiponectin, IL-6	Resistin levels were not correlated with histological findings of NASH patients. However, authors did not look for predictors or associations with necroinflammation grade.
RBP4	Petta <i>et al.</i> , 2008, Italy (41)	Kleiner and Brunt	Healthy controls Hepatitis C NAFLD	19/11 76/67 29/8	28.9 ± 12.1 µg/L 36.8 ± 17.5 µg/L* 35.2 ± 9.3 µg/L*	Excluded	Age, sex, HOMA-IR, WC, HC, ALT, GGT, total and HDL Chol, TG, glucose, insulin, ferritin, platelet count	In the NAFLD group, low degree of fibrosis and necroinflammation were associated with high RBP4 levels, but only low necroinflammatory activity was maintained in multiple linear regression analysis. Authors suggest that in NAFLD the elevated RBP4 levels are more closely associated with IR and obesity, in keeping with a general metabolic disorder.
	Alkhoury <i>et al.</i> , 2009, USA (42)	Kleiner	Obese SS Obese NASH	9/7 15/18	26.9 ± 13.4 mg/L 21.4 ± 10.3 mg/L	15	Age, BMI, HOMA-IR, AST, ALT, glucose, insulin	There was an inverse association between stage of fibrosis and RBP4 levels, with the lowest levels in patients with advanced fibrosis or cirrhosis. These results identify RBP4 as a potential novel marker to assess fibrosis progression in patients with NAFLD.

Kashyap <i>et al.</i> , 2009, USA (43)	Kleiner	Obese no NAFLD Obese SS Obese borderline NASH Obese NASH	6/37 10/23 9/31 5/21	41.61 ± 21.18 mg/L 41.63 ± 13.61 mg/L 39.24 ± 10.57 mg/L 44.80 ± 15.83 mg/L	55	Age, BMI, systolic BP, AST, ALT, TG, HDL Chol, TNF- α , glucose, leptin, adiponectin	RBP4 could be linked to BMI and TG levels but not to liver histology, suggesting that these levels relate to IR indexes but not to severity of liver injury.
Schina <i>et al.</i> , 2009, Greece (44)	Kleiner and Brunt	Healthy controls SS NASH	ELISA: 13/17 5/8 13/4 IHC: 3/2 5/8 13/4	ELISA: 34.66 (27.04-43.6) μ g/ml 22.9 (19.5-33) μ g/ml*** 25.2 (22.9-26.9) μ g/ml*** IHC: 1.7 ± 0.67 4.69 ± 0.95*** 6.35 ± 0.99***###	Excluded	/	No correlation was found with respect to serum RBP4 and disease activity or metabolic parameters. However, liver expression of RBP4 was found to correlate with the extent of steatosis, grade of NASH activity and fibrosis stage. Serum RBP4 is not a clinically valid marker of IR in patients with NAFLD, but the hepatic expression of RBP4 probably is.
Milner <i>et al.</i> , 2009, Australia (45)	Kleiner and Brunt	Healthy controls SS NASH	71/58 22/9 38/31	16.9 ng/ml (vs total NAFLD group 16.3 ng/ml) 16.9 ± 3.6 ng/ml 16.0 ± 4.6 ng/ml	28	BMI, HOMA-IR, WC, TG, HDL, Chol	Serum RBP4 levels did not correlate with the degree of liver steatosis, inflammation or fibrosis in patients with NAFLD.
Cengiz <i>et al.</i> , 2010, Turkey (38)	Kleiner and Brunt	Healthy controls Ultrasound steatosis Biopsy-proven NAFLD	NA (n=24) NA (n=76) NA (n=34)	18.0 ± 0.6 μ g/ml 18.1 ± 0.7 μ g/ml NA	Excluded	Age, BMI, Chol, HOMA-IR, AST, ALT, GGT, TG, adiponectin, leptin, resistin	This study suggests that there is no significant relationship between NAFLD, as determined by both ultrasound and liver histology, and serum RBP4 levels, although it may be correlated with IR.
Terra <i>et al.</i> , 2013, Spain (46)	Kleiner and Brunt	Healthy controls Obese non-NAFLD Obese SS Obese NASH	0/4 0/11 0/13 0/21	<u>Serum nephelometry:</u> 2.5 ± 0.2 mg/dl 2.7 ± 0.2 mg/dl 3.8 ± 0.4 mg/dl* [§] 3.3 ± 0.2 mg/dl* [§] <u>SAT mRNA (qPCR):</u> 2.22 ± 0.24 AU ^{&} 4.17 ± 1.24 AU 4.09 ± 1.12 AU 2.77 ± 0.47 AU <u>VAT mRNA (qPCR):</u> 3.73 ± 0.36 AU ^{&} 1.72 ± 0.27 AU 2.38 ± 0.34 AU 1.88 ± 0.22 AU <u>Liver mRNA (qPCR)[§]:</u> 8.85 ± 0.56 AU 10.51 ± 2.28 AU 7.34 ± 0.83 AU 6.57 ± 0.72 AU	10	NA	Authors only found an association between systemic levels of RBP4 and TG levels in MO women but not with histological findings. This study also indicated that mRNA RBP4 expression in SAT and VAT appeared to be unrelated to IR or NAFLD histology and liver had the highest expression of RBP4 in all physiological states studied, but there was a lack of correlation between hepatic expression and systemic levels.

Visfatin	Younossi <i>et al.</i> , 2008, USA (33)	NA	Obese controls Obese SS Obese definite NASH	3/29 1/14 9/13	25.8 ± 18.0 pg/ml 52.5 ± 67.0 pg/ml 16.7 ± 6.3 pg/ml ^{###}	NA	M30, M65-M30, adiponectin	Visfatin levels did not correlate with histological findings in patients with NAFLD.
	Jarrar <i>et al.</i> , 2008, USA (34)	Kleiner	Healthy controls Obese non-NAFLD Obese SS Obese NASH	6/6 5/33 2/17 11/15	11.4 ± 5.7 pg/ml (vs NAFLD 28.9 ± 41.6 ^{**}) 26.8 ± 19.0 pg/ml ^{**} (vs NAFLD 28.9 ± 41.6 NS) 45.1 ± 60.9 pg/ml (vs non-obese controls ^{**}) 17.1 ± 6.2 pg/ml [#]	NA	Age, sex, BMI, ethnicity, WHR, AST, ALT, TG, Chol, glucose, insulin, TNF- α , adiponectin, IL-6, IL-8, resistin	Although visfatin was not independently associated with NAFLD, multivariate analysis showed a positive correlation between TNF- α and NAFLD that was interdependent of visfatin. Visfatin may play a protective role in NAFLD and this study showed the complexity of the interactions between various adipokines, IR and the pathogenesis of NAFLD.
	Aller <i>et al.</i> , 2009, Spain (47)	Brunt and Masson trichome stain (fibrosis)	Obese with low grade steatosis Obese with high grade steatosis	21/10 16/8	14.1 ± 6.6 ng/ml 15.7 ± 7.3 ng/ml	Excluded	Age, sex, fat mass, insulin	Serum visfatin levels were not related to steatosis grade or fibrosis in overweight and obese patients with NAFLD. However, visfatin predicted the presence of portal inflammation.
	Gaddipati <i>et al.</i> , 2010, India (48)	Brunt	Non-NAFLD SS Moderate steatosis NASH	NA (n=38) NA (n=35) NA (n=30) NA (n=12)	VAT ELISA: 210.4 ± 93.2 ng/ml 87.3 ± 75.2 ng/ml* 61.4 ± 54.5 ng/ml ^{***} 31.8 ± 29.4 ng/ml ^{***}	Excluded	/	There was an inverse association between the level of visceral visfatin and occurrence of SS in NAFLD. These findings point out the protective role of visfatin in NAFLD.
	Kukla <i>et al.</i> , 2010, Poland (49)	Kleiner and Scheuer (inflammatory)	Obese SS Obese NASH OR Obese non fibrosis Obese fibrosis	NA (n=16) NA (n=24) OR NA (n=7) NA (n=33)	Liver IHC: 0.62 ± 0.19 AU 1.11 ± 0.71 AU OR 0.36 ± 0.03 AU 1.09 ± 0.65 AU*	NA	/	This study showed a positive association between liver visfatin expression and fibrosis stage in NAFLD patients, but not with liver steatosis and inflammation. This observation suggests a potential role of visfatin in the pathogenesis and progression of fibrosis in NAFLD patients.
	Dahl <i>et al.</i> , 2010, Norway (50)	Brunt	Healthy controls Obese SS Obese NASH	14/13 12/14 19/13 qPCR: NA (n=6) NA (n=6) NA (n=7)	ELISA: ELISA: ^A 15 ng/ml 7 ng/ml ^{***} 7.5 g/ml ^{***} Liver mRNA (qPCR): ^A 2.5 AU 1 AU ^{**} 0.9 AU ^{**}	26	Age, sex, BMI, ALT	NAFLD was a significant predictor of serum visfatin levels, independent of all the measured metabolic parameters. This study suggests a role for decreased visfatin levels in hepatocyte apoptosis in NAFLD-related disease

	Genc <i>et al.</i> , 2013, Turkey (51)	Kleiner	Healthy controls SS Borderline NASH NASH	60/0 31/0 35/0 48/0	13.33 ± 2.73 ng/ml 13.2 (10.8-17.0) ng/ml 14.3 (9.2-19.1) ng/ml 14.0 (8.6-21.2) ng/ml	Excluded	BMI	Plasma visfatin levels were not associated with histological findings of NAFLD patients. However, it is inversely associated with TNF-α, suggesting a role for visfatin in protection against liver injury during the inflammatory process.
AFABP	Milner <i>et al.</i> , 2009, Australia (45)	Kleiner and Brunt	Healthy controls SS NASH	71/58 22/9 38/31	23.1 ng/ml (vs total NAFLD group 33.5 ng/ml***) 28.5 ± 11.8 ng/ml 35.8 ± 15.0 ng/ml***#	28	BMI, HOMA-IR, WC, TG, HDL, Chol	Serum AFABP predicted inflammation (lobular inflammation and ballooning) and fibrosis in NAFLD patients, even when measurements of IR, VAT and other adipokines were considered. These results suggest AFABP may have a direct pathogenic link to disease progression.
	Shen <i>et al.</i> , 2012, China (52)	Kleiner	Healthy controls Simple NAFLD NASH	40/34 36/28 45/37	15.4 (12.4-19.1) ng/ml (vs total NAFLD group 19.2 (14.9-25.8) ng/ml***) 18.9 (13.1-25.4) ng/ml 19.4 (16.0-27.4) ng/ml	71	Age, BMI, MetS, TG, Chol, glucose	AFABP positively associated with lobular inflammation, ballooning and NAS, of which ballooning remained independently associated with AFABP on multiple linear regression. The utility of AFABP in diagnosing NAFLD is limited by the relatively low accuracy.
Vaspin	Kukla <i>et al.</i> , 2010, Poland (26)	Kleiner	Healthy control Obese NAFLD Obese NASH	10/10 NA (n=21) NA (n=20)	1.6 ± 0.8 ng/ml (vs total NAFLD group 1.0 ± 0.7 ng/ml) 0.9 ± 0.6 ng/ml 1.2 ± 0.7 ng/ml	10	/	A relationship between serum vaspin and hepatocyte ballooning was found in NAFLD patients, a known hallmark of NASH. Up-regulation of vaspin could be a compensatory mechanism associated with obesity and IR.
	Genc <i>et al.</i> , 2011, Turkey (53)	Kleiner	Healthy control NASH	30/0 50/0	0.44 ± 0.29 ng/ml 0.99 ± 0.84 ng/ml**	Excluded	BMI, HOMA-IR, WC, lipid parameters, glucose	Vaspin levels did not associate with histological findings namely steatosis, ballooning degeneration, lobular inflammation and fibrosis. These results suggest that in the absence of metabolic risk factors, vaspin <i>per se</i> may not be involved in the pathogenesis of NASH.
	Aktas <i>et al.</i> , 2011, Turkey (54)	Kleiner	Healthy control NAFLD	39/42 43/48	0.4 (0.2-0.7) ng/ml 0.6 (0.3-1.1) ng/ml**	22	Age, sex, BMI, HOMA-IR, T2D, MetS, systolic and diastolic BP, AST, ALT, TG, total, HDL and LDL Chol, hs-CRP, albuminuria, smoking	Results of the present study indicate that vaspin is a predictor of liver fibrosis, independent of potential confounders including metabolic parameters.

Apelin	Ercin <i>et al.</i> , 2010, Turkey (55)	Kleiner	Healthy control NAFLD	30/0 50/0	0.21 (0.2-0.4) ng/ml 0.26 (0.09-0.5) ng/ml***	Excluded	Age, BMI, HOMA- IR	Although apelin-12 concentration was associated with the degree of IR and body composition, no relation with liver histology could be found.
	Aktas <i>et al.</i> , 2011, Turkey (54)	Kleiner	Healthy control NAFLD	39/42 43/48	1.1 (0.8-1.5) ng/ml 1.4 (0.9-2.1) ng/ml**	22	Age, sex, BMI, HOMA-IR, T2D, MetS, systolic and diastolic BP, AST, ALT, TG, total, HDL and LDL Chol, hs-CRP, albuminuria, smoking	Apelin-36 could be linked to IR but not with liver histology. However, after allowance for potential confounders this relation did not persist. Findings suggest that apelin does not seem to play a major role in the pathophysiology of NAFLD and does not point towards a major independent association of apelin with metabolic liver disease.

Adipokine serum levels were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits, unless mentioned otherwise.

AFABP, adipocyte fatty acid binding protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AU, arbitrary units; BMI, body mass index; BP, blood pressure; Chol, cholesterol; GGT, gamma-glutamyl transferase; GH, growth hormone; HC, hip circumference; HDL, high-density lipoprotein; HOMA-IR, homeostasis model of assessment for insulin resistance; hs-CRP, high-sensitivity C-reactive protein; IHC, immunohistochemistry; IL, interleukin; IR, insulin resistance; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein-1; MetS, metabolic syndrome; M/F, male-to-female ratio; MO, morbidly obese; NA, not available; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NS, not significant; RBP4, retinol binding protein-4; SAT, subcutaneous adipose tissue; SS, simple steatosis; T2D, type 2 diabetes; TNF- α , tumor necrosis factor- α ; VAT, visceral adipose tissue; WC, waist circumference.

* vs. corresponding (obese) controls; # vs. simple NAFLD; \$ vs. obese non NAFLD, & vs. whole obese group; § vs. SAT and VAT mRNA

*, < 0.05; **, < 0.01; ***, < 0.001

^A estimation based on Figure.

All studies except for Döcke *et al.* reported significantly elevated serum chemerin levels in NAFLD patients compared with healthy or obese controls, whereas Döcke *et al.* found significantly elevated hepatic chemerin expression in patients with definite NASH versus no or undefined NASH. Overall, although inconsistency exists in histological parameters, all studies had a good methodological quality above 70% and suggest that chemerin may be involved in NAFLD progression with regard to liver inflammation and hepatocyte ballooning. Although results were similar, the study of Kukla *et al.* used a different ELISA and did not adjust analysis for potential confounders in comparison with the other studies. Based on these results, contribution of chemerin in the development of NASH is possible and may involve its hepatic expression, as reported by the study group of Döcke.

Resistin

We found 12 studies in literature evaluating the association between resistin and liver histology in patients with NAFLD and/or NASH, of which six showed statistically significant results. Circulating resistin levels were positively associated with histological steatosis, portal inflammation and NAS in patients with NAFLD and NASH, reported by Senates *et al.* (39). On multivariate regression analysis, Cengiz *et al.* (38) also found serum resistin as a positive correlate for the development of steatosis and degree of necroinflammation in non-diabetic NAFLD patients. The association between resistin and the grade of hepatic steatosis was additionally analyzed by two other study groups, Aller *et al.* and Argentou *et al.* Although initially associated, including homeostasis model of assessment for insulin resistance (HOMA-IR) to the multivariate logistic analysis resulted in a loss of the association between resistin and the grade of steatosis in non-diabetic NAFLD patients who participated the study of Aller *et al.* (35). Because they found a positive association between resistin and HOMA-IR, resistin was suggested to be a mere surrogate marker of IR. Besides Senates *et al.* (39), two studies similarly reported a positive association between serum resistin and histological NAS. In the first study, by Pagano *et al.* (30), univariate correlation analysis revealed that serum resistin was associated with the histological NAS in non-diabetic patients with NAFLD, whereas no association was found with steatosis score. Secondly, Younossi *et al.* (33) proposed a diagnostic biomarker panel to predict histological NASH within a cohort of obese NAFLD patients and obese controls, including serum resistin levels next to serum adiponectin, cleaved CK-18 (a marker of apoptosis) and a product of the subtraction of cleaved CK-18 from intact CK-18 (a marker of necrosis). In contrast, while all other studies reported positive associations concerning resistin and liver histology, Argentou *et al.* (37) was the only study group indicating a negative association between resistin serum levels and the grade of steatosis as well as percentage of liver parenchyma involved by

steatosis in obese patients with NAFLD. Despite these inconsistent findings, the differences in associations between resistin and histological parameters could not be reflected to patterns found in study design of the different studies. Among the 12 studies, five different ELISA were used. However, usage of the same assay among independent studies did not result in similar results. Significantly elevated circulating resistin levels in patients with NAFLD were described in four studies, compared with healthy or obese controls. The presence of definite NASH resulted in even higher serum levels compared with patients with simple steatosis in two of these studies. Pagano *et al.* also reported an up-regulated resistin mRNA expression in SAT of patients with NAFLD, although they were not associated with histological parameters. Finally, the remaining six studies that were found to investigate resistin in relation to NAFLD did not show a correlation between this adipokine and liver histological parameters in obese and non-obese patients with NAFLD and/or NASH (29, 31, 32, 34, 36, 40). Baranova *et al.* (31) was the only study group that measured resistin mRNA expression in VAT of obese patients with NAFLD, although expression levels were also not associated with liver histology. Altogether, of the six studies reporting relations between resistin and liver histology in NAFLD patients, grade of steatosis was presented as the strongest associated parameter in most studies followed by portal inflammation and necroinflammation as well as histological NAS. Contribution of adipose tissue (SAT or VAT) to circulating resistin levels and liver histology of NAFLD patients could not be confirmed by the studies described earlier. Four out of 12 studies did not adjust analysis for confounders, and the potential link between IR and resistin was also inconsistent among different studies. All positive studies had a methodological quality above 70%, while only two studies that did not report significant results scored below 70%.

Retinol-binding protein 4

Of all seven studies from our search results, only three effectively reported an association between serum RBP4 levels and liver histology in patients with biopsy-proven NAFLD. Liver RBP4 expression but not serum levels was shown to positively associate with the extent of steatosis, grade of NASH activity and fibrosis stage in non-diabetic non-obese patients with NAFLD, reported by Schina *et al.* (44). Petta *et al.* (41) was the first study group that found a negative association between serum RBP4 and the stage of fibrosis as well as necroinflammation in non-diabetic non-obese NAFLD patients. However, after multivariate regression analysis including BMI, alanine aminotransferase (ALT), γ -glutamyltransferase (GGT) and cholesterol, only necroinflammation remained to be negatively associated with serum RBP4. Subsequently, in obese NAFLD patients participating the study of Alkhoury *et al.* (42), serum RBP4 levels were inversely associated only with the stage of fibrosis. Only two study groups indicated significantly

altered RBP4 levels in NAFLD patients, whereas other studies reported no differences. Petta *et al.* reported higher serum RBP4 levels in NAFLD patients versus healthy controls, whereas Schina *et al.* reported lower serum levels but an up-regulated hepatic RBP4 expression in patients with simple steatosis or NASH versus healthy controls. Four studies did not demonstrate any association between RBP4 and NAFLD-related histological parameters in both obese and non-obese patients with NAFLD and/or NASH (38, 43, 45, 46). Besides serum RBP4 levels, Terra *et al.* (46) measured liver, SAT and VAT RBP4 mRNA expression levels in morbidly obese women with NAFLD but could not relate them to liver histology. They reported significantly higher serum levels in obese patients with NAFLD compared with obese and healthy controls but found no significant results in tissue expression. This last study indicated that liver had the highest expression of RBP4 in all physiological states studied, but there was a lack of correlation between hepatic expression and systemic levels. In summary, considering results from the literature, the contribution of circulating RBP4 to NAFLD progression appears to be unlikely, as two out of seven studies found associations with divergent histological parameters. Furthermore, five different ELISA were used among seven studies, and two studies did not mention potential confounding factors. Presence of this adipokine in the liver, however, could contribute to the extent of steatosis, NAS level and fibrosis stage but needs confirmation in more (prospective) studies. Except for Terra *et al.*, all studies had a good methodological quality score above 70%.

Visfatin

Although four studies out of seven search results reported associations of visfatin with NAFLD histological parameters, results were inconsistent among the different studies. Gaddipati *et al.* (48) examined visfatin levels in VAT biopsies of non-diabetic patients with and without NAFLD and found a significant decline in patients with NAFLD versus controls, which was positively associated with the degree of steatosis. One year before, Aller *et al.* (47) had indicated that serum visfatin levels may predict the presence of portal inflammation in non-diabetic obese patients with NAFLD, while they could not demonstrate relations with steatosis or fibrosis. Kukla *et al.* (49) only measured hepatic visfatin expression in relation to NAFLD and showed a positive association with fibrosis stage in morbidly obese patients with NAFLD, but not with liver steatosis and inflammation. They could not demonstrate significant differences in hepatic visfatin expression between patients with simple steatosis and NASH, although patients with fibrosis had a significantly higher visfatin expression versus no fibrosis. The presence of NAFLD itself has been proposed to be a significant predictor of serum visfatin levels by Dahl *et al.* (50), independent of sex, BMI, age and serum ALT. Within this obese NAFLD patient group, liver

visfatin mRNA expression and its serum levels were markedly decreased compared with healthy controls, with no difference between simple steatosis and NASH. The three remaining studies that investigated visfatin in both obese and non-obese patients with biopsy-proven NAFLD did not find significant associations with liver histology (33, 34, 51). While one study reported no differences in concentration among patient groups, both Jarrar *et al.* (34) and Younossi *et al.* (33) showed significantly lower serum visfatin levels in NASH patients compared with patients with simple steatosis or (obese) controls. Overall, three studies suggested a protective role of visfatin in NAFLD progression, of which one study had a methodological quality score of 100% and others only had 57%. Two other study groups described hepatic visfatin levels as a potential contributor of fibrosis progression or hepatocyte apoptosis, with a quality score of 57% and 100%, respectively, while another study group suggested that serum visfatin may predict portal inflammation, which had a quality score of 86%. Potential contribution of serum, adipose tissue or liver visfatin to NAFLD progression could not be concluded from literature results. Confounding factors such as BMI, age and sex were mostly taken into account, whereas insulin (resistance) was only included in two studies, and two remaining studies indicated no confounding factors. Most studies used the same ELISA.

Adipocyte-fatty-acid-binding protein

Two study groups suggested that AFABP may play a role in NAFLD progression. Milner *et al.* (45) indicated serum AFABP as an independent positive correlate of lobular inflammation, hepatocyte ballooning and fibrosis in non-obese patients with NAFLD, even when measurements of IR, visceral fat and other adipokines were considered. In another study, serum AFABP had a positive association with lobular inflammation, hepatocellular ballooning and NAS, of which hepatocellular ballooning remained independently associated with AFABP on multiple linear regression also correcting for age, BMI, fasting glucose, total cholesterol, triglyceride, MetS, steatosis and fibrosis. These findings were reported by Shen *et al.* in a non-obese cohort of NAFLD patients (52). Both studies showed significantly elevated serum AFABP levels in patients with NAFLD versus healthy controls, although only Milner *et al.* reported additionally elevated levels in patients with NASH compared with simple steatosis. Both studies applied the same ELISA. Overall, according to literature, AFABP was suggested to associate most strongly with hepatocyte ballooning degeneration, and methodological quality of both studies was equally very good, with a score of 86%.

Vaspin

Vaspin was suggested to associate with liver histology in two studies with biopsy-proven NAFLD patients. The first study was performed by Kukla *et al.* (26), who found a positive relationship between serum vaspin levels and hepatocyte ballooning degeneration in an obese NAFLD cohort. Secondly, Aktas *et al.* (54) indicated vaspin as a positive correlate of liver fibrosis, independent of potential confounders such as sex, age, metabolic and histological parameters. While the latter study group described significantly elevated serum vaspin levels in NAFLD patients compared with healthy controls, Kukla *et al.* only found significantly higher levels in NAFLD patients with versus without hepatocyte ballooning. Serum vaspin levels were not found to be associated with histological findings in a last study with non-diabetic non-obese NASH patients by Genc *et al.* (53). Although they also reported significantly higher levels in NASH patients versus healthy controls, significance disappeared after adjustment with metabolic risk factors. Altogether, out of three studies that evaluated associations of vaspin with liver histology, each study was reporting different outcomes. Therefore, the role of vaspin in NAFLD, if any, remains unclear. While the study of Kukla *et al.* had a methodological quality score of 71% and used a different ELISA, the two remaining studies both had a maximum score of 100% and applied similar ELISA.

Apelin

Two studies that investigated apelin in relation to liver histology in NAFLD patients did not show significant associations with histological parameters. Ercin *et al.* (55) have indicated in their preliminary study that serum apelin-12 levels were not altered in male non-diabetic subjects with NAFLD when findings were adjusted according to BMI and HOMA-IR indexes. Also apelin-36, the longest biologically active secreted form of apelin, could not be linked to histological findings of NAFLD patients, reported by Aktas *et al.* (54). In this study, serum apelin-36 levels were significantly higher in patients with NAFLD versus healthy controls. Concluding from these two studies, the contribution of apelin to NAFLD progression is suggested to be negligible. Both studies used the same ELISA and had a maximum score of 100% on methodological quality.

Major findings

Except for two, studies included in this review had a good or average (>50%) methodological quality. Furthermore, they all had category B in level of evidence because of the cross-sectional study design, meaning only suggestive and no conclusive findings can be made. Of all seven adipokines described earlier, findings with chemerin and AFABP were most persisting with at least two study groups reporting similar results. Although only half of the studies reported significant associations with resistin, findings

were also promising with consistent results in different independent studies. Therefore, these three adipokines are suggested to be most probably related to NAFLD progression and/or pathogenesis. Omentin was also pointed out as a potential modulator of NAFLD by Yilmaz *et al.* (27), although they were the only study group investigating this adipokine. These results could thus not be confirmed by other independent studies and were omitted from this review. Visfatin, RBP4 and vaspin were investigated by several studies (visfatin and RBP4, N = 7; vaspin, N = 2), with each study reporting different or no associated histological parameters. These results were too divergent to suggest any potential involvement of the adipokines in NAFLD. Finally, no significant findings were found concerning apelin. Altogether, major inconsistent results were found among different studies, which impede making conclusions or even suggestions from these findings. An overview of the positively and/or negatively associated histological parameters among the different studies is presented in Table 3, grouped per adipokine. Efforts on finding any possible causes of this inconsistency revealed no results, as no patterns among the studies were found in, e.g. inclusion and exclusion criteria, age, number of patients, male-to-female ratio, study groups or methods.

Table 3 Overview of the positively (+) or negatively (-) associated histological parameters, grouped per adipokine

	Steatosis	Portal inflammation	Lobular inflammation	Necroinflammation	Hepatocellular ballooning	Fibrosis	Histological NAS
Chemerin	+	++			++	++	++
Resistin	++ -	+		+			+++
RBP4	+			-		+ -	+
Visfatin	-	+				+	-
AFABP			+		++	+	
Vaspin					+	+	
Apelin							

+, positive association; -, negative association; AFABP, adipocyte fatty acid binding protein; MCP-1, monocyte chemoattractant protein-1; NAS, nonalcoholic fatty liver disease (NAFLD) activity score RBP4, retinol binding protein-4.

Discussion

This systematic review summarizes the growing body of literature suggesting that novel adipokines may modulate the clinical course of NAFLD. Of all seven novel adipokines described in literature as possible modulators of NAFLD, this review pointed out only three showing a potentially relevant association with disease progression. Both chemerin and AFABP were suggested to associate with hepatocyte ballooning degeneration in NAFLD patients, reported by at least two independent study groups. In case of chemerin, its hepatic expression was found to be associated with fibrosis, steatosis and inflammation as well, indicating a potential involvement of liver chemerin in the development of NASH. Although serum resistin had been most extensively studied throughout the last years, only half of the studies effectively reported significant associations with liver histology in NAFLD patients with grade of steatosis as the strongest associated parameter, followed by inflammation. Furthermore, contribution of SAT or VAT to serum resistin levels or NAFLD progression could not be derived from literature. Finally, contribution of RBP4, visfatin, vaspin and apelin to NAFLD progression could not be concluded from literature search, as results were mainly inconsistent or studies reported no significant findings. Although the pathogenesis of NAFLD remains unclear, it has been suggested that adipokines may promote hepatocellular damage, inflammation, fibrosis and progressive liver disease. Together with other recognized cytokines produced partially by inflammatory cells like macrophages infiltrating adipose tissue, they could play a role in pathogenesis of IR and NAFLD through complex and interactive paracrine and endocrine mechanisms (56, 57). Each adipokine, however, may have its specific molecular effects that will be discussed shortly in the following section and is summarized in Fig. 2.

Increased levels of adipocyte-derived chemerin have been reported in patients with type 2 diabetes, an increase that parallels to the worsening of glucose tolerance status and associates with IR (58-60). Binding of chemerin to its chemokine-like receptor (CMKLR)-1 results in inflammatory effects by promoting the recruitment of cells of the innate immune system, i.e. macrophages and natural killer cells, to tissue injury sites (61). This could explain the results concerning a potential role of liver chemerin in the development of NASH, a well-known state of chronic inflammation. An elevated liver expression has also been demonstrated in NASH patients as well as NASH mice models compared with respective controls by Krautbauer *et al.*, of which primary human hepatocytes were indicated as the major source of hepatic chemerin (62). Chemerin was also found to regulate adipocyte differentiation, lipid homeostasis and insulin sensitivity (60, 63, 64). In human skeletal muscle cells, chemerin was found to induce IR by inhibiting the insulin signaling pathway at the level of insulin receptor substrate-1 (IRS-1),

Akt and glycogen synthase kinase 3 β (GSK3 β). Furthermore, chemerin activated nuclear factor- κ B (NF κ B), a known inflammatory transcription factor (65). These effects on molecular level might similarly occur in hepatocytes, contributing to hepatic IR and inflammation that leads to NAFLD progression.

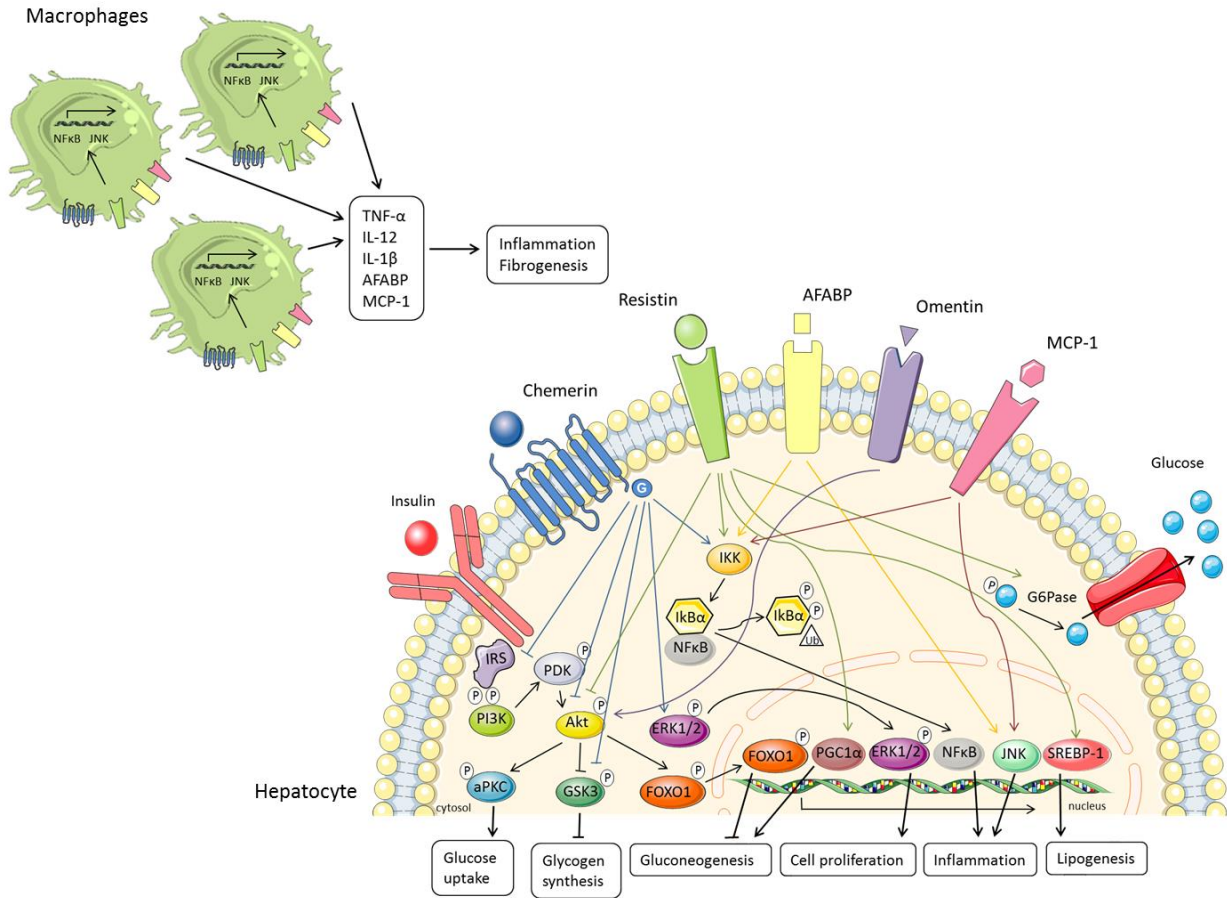


Figure 2 Suggested adipokine-induced molecular regulation of signaling pathways in hepatocytes. In normal physiological conditions, insulin binding to its receptor induces a phosphorylation cascade. The phosphorylation of the insulin receptor substrates (IRS) and phosphatidylinositol 3-kinase (PI3K) followed by phosphorylation of 3-phosphoinositide-dependent protein kinase (PDK), results in the phosphorylation and activation of Akt. Akt then phosphorylates numerous targets to promote glucose uptake, glycogen synthesis and inhibit hepatic glucose production (gluconeogenesis). Both chemerin and resistin are suggested to inhibit Akt phosphorylation and its downstream targets, interfering with insulin signaling and increasing glucose levels. Resistin has been found to activate peroxisome proliferator-activated receptor- γ co-activator 1 α (PGC1 α), leading to the transcriptional activation of gluconeogenic target genes, and to promote glucose-6-phosphatase to hydrolyse glucose-6-phosphate into free glucose leading to an increased glucose output. Furthermore, it has been reported that resistin induces de novo lipogenesis by activating sterol regulatory element-binding protein-1 (SREBP-1), potentially promoting hepatic steatosis. Omentin is the only adipokine who is suggested to perform insulin-sensitizing effects by stimulating Akt phosphorylation. Chemerin, resistin, adipocyte-fatty-acid-binding protein (AFABP) and monocyte chemoattractant protein-1 (MCP-1) are generally proposed to induce an inflammatory response by activating the nuclear factor- κ B (NF κ B) and c-Jun N-terminal kinase (JNK) pathway. Activation of NF κ B

occurs primarily via activation of I κ B kinase (IKK), leading to the release of its repressor I κ B. Finally, these pro-inflammatory adipokines were also found to promote the recruitment and activation of macrophages, resulting in the release of inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-12 (IL-12), IL-1 β , AFABP and MCP-1, which in turn induce inflammation and even hepatic fibrogenesis.

Although human data are still controversial, resistin is an adipokine that is predominantly secreted by adipocytes, inflammatory cells and hepatic stellate cells (HSC), which is believed to cause hepatic IR and bind immune cells (66-68). The liver has been suggested to be the major target site of resistin, with hyperresistinemia leading to increased glucose production and hepatic IR (69, 70). In rodent models, resistin has been reported to influence hepatic glucose and lipid metabolism and to play a major role in hepatic IR in response to a high-fat diet (71). On the basis of these animal studies, resistin has been proposed to represent a link among obesity, IR and NAFLD (66, 69, 72). In addition, resistin-deficient mice on a high-fat diet showed an ameliorated lipid profile and hepatic steatosis, suggesting a role of resistin in the induction of hepatic steatosis (73). Recently, Song *et al.* reported that resistin disrupted the phosphorylation of GSK3 β in primary cultures of rat hepatocytes under high insulin and glucose levels, leading to lowered glycogen synthesis and hepatic insulin action (74). Furthermore, resistin is proposed to induce the secretion of inflammatory cytokines TNF- α and IL-12 from macrophages via an NF κ B-dependent cascade (75).

Increased levels of AFABP, a fatty-acid-binding chaperone that is mainly expressed by mature adipocytes and activated macrophages, have been associated with parameters of adiposity, IR and metabolic syndrome (76, 77). Similar to the results described in this review, studies have indicated an association of elevated serum AFABP levels with ultrasound-diagnosed NAFLD in both metabolically healthy (78) and type 2 diabetic subjects (79). Moreover, hepatic expression of AFABP has been shown to be elevated in chemically and diet-induced obese mice with NAFLD as well as in insulin resistant subjects with NAFLD (80, 81). Yoon *et al.* demonstrated a high expression of AFABP mRNA and protein in visceral fat and a higher ratio of visceral/liver for AFABP protein expression in patients with NASH versus non-NASH patients (82). In relation to NAFLD and metabolic syndrome, mice lacking AFABP were even protected against hepatic steatosis, IR and dyslipidemia in both genetic and diet-induced obesity (83, 84). Furthermore, the pharmacological inhibition of AFABP has led to an alleviation of both acute liver injury and NASH in mice (80). Notwithstanding the principal source of AFABP within the context of NAFLD is still contradictory, this adipokine is a key mediator of inflammatory response in macrophages. Its expression has been shown to be elevated in hepatic Kupffer cells in the presence of NAFLD, suggesting

that it may contribute to liver injury by recruiting a cluster of pro-inflammatory cytokines, including IL-1 β , IL-12 and TNF- α (85).

Although not included in this systematic review because they were reported only once, two additional adipokines have been described in literature as potential modulators of NAFLD. Omentin is a VAT-specific anti-inflammatory secretory protein that is synthesized by visceral stromal vascular cells, of which low serum levels have been reported in patients with obesity, IR and type 2 diabetes (86, 87). It is generally considered that omentin negatively associates with obesity and IR because of its insulin-sensitizing effects, enhancing insulin-mediated glucose uptake and Akt phosphorylation in human adipocytes (88-90). Although these findings might predict a negative relation between omentin and NAFLD, Yilmaz *et al.* reported a positive association of elevated serum omentin levels with hepatocyte ballooning degeneration in patients with NAFLD (27). Because no additional studies were found that could confirm results, the possibility that higher omentin levels in these NAFLD patients merely result from a compensatory counter-regulatory mechanism to the increased IR could not be excluded. Secondly, the study group of Haukeland *et al.* analyzed serum levels of the inflammatory adipokine monocyte chemoattractant protein-1 (MCP-1) in healthy controls as well as biopsy-proven patients with NAFLD and NASH. They found that NAFLD and NASH significantly predicted higher MCP-1 levels independent of age, sex, BMI and metabolic syndrome and indicated the potential importance of MCP-1 for the persistent inflammation in NAFLD (91). These findings corroborate those by Kirovski *et al.*, reporting elevated serum MCP-1 in patients with ultrasound-diagnosed NAFLD and indicating elevated MCP-1 mRNA expression in liver and VAT of diet-induced steatotic mice (92). Two additional studies even reported elevated hepatic MCP-1 expression in patients with NAFLD but did not perform associations with liver histopathologic severity (93, 94). Animal models and human studies have reported that the expression and circulating levels of MCP-1 increase with obesity and associate with adiposity and IR (95-98). MCP-1 is known to play a pivotal role in the development of inflammatory responses as well as the recruitment of immune cells to sites of inflammation (99). Its role in inflammatory and fibrogenic processes has been demonstrated by inactivating MCP-1 or its receptor in experimental murine models of chronic liver injury. This resulted in a blockade of macrophage infiltration in the liver and inhibited HSC activation, which led to the suppression of hepatic inflammation and fibrosis (100, 101). Furthermore, an increased MCP-1 expression in adipose tissue has been suggested to contribute to macrophage infiltration, IR and hepatic steatosis in genetically as well as diet-induced obese mice (102).

In general, NAFLD has been proposed to develop according to a 'multihit hypothesis', with a dysregulated hepatic fatty-acid metabolism leading to simple steatosis as an initial insult (first hit). IR is suggested to play a central role in this first hit, contributing to an imbalance between the promotion of hepatic fat accumulation (fatty-acid influx and *de novo* lipogenesis) and its prevention (fatty-acid efflux and oxidation). Consequently, hepatocytes would become more susceptible to secondary insults, multiple hits like adipokines, inflammatory markers, oxidative stress and mitochondrial dysfunction, which lead to the progression of NAFLD to NASH (103, 104). Alterations in adipokine levels are thus suggested to be involved in this 'multihit' process, and their imbalance may play an important role in both the development of NAFLD and the progression to NASH (104). In the setting of liver injury, adipokines are suggested to induce increased reactive oxygen species (ROS) levels as well as activating the NF κ B cascade, provoking HSC and Kupffer cells to produce inflammatory cytokines such as TNF- α and IL-6. Cytokines, in turn, promote collagen production, the progression of fibrosis, and inhibitory phosphorylation of IRS-1/2 that attenuates insulin signaling, leading to the development of NAFLD (Fig. 2) (105).

Although liver biopsy continues to be the gold standard in diagnosing NAFLD, there is a high need for less invasive diagnostic techniques because of the risk on comorbidities related to liver biopsy (106). This review cannot answer the question whether serum levels of these newly described adipokines are involved in NAFLD pathogenesis and whether they could be used as a non-invasive marker of NAFLD. Nevertheless, these results strengthen the potential role of several adipokines although future prospective studies are needed, evaluating their predictive and therapeutic potential within the clinical setting of NAFLD.

This review has certain limitations, with the most crucial being the cross-sectional design of all included studies. This leads to inconclusive results concerning the potential role of studied adipokines in NAFLD progression, thereby not being able to indicate a cause-effect relationship. Up to date, no study has prospectively followed up adipokine levels in NAFLD patients, evaluating them when NAFLD progresses to NASH. Another limitation was the major heterogeneity observed across study results. Most importantly, the usage of different ELISA resulted in wide ranging adipokine amounts across different studies. Resistin levels, e.g. ranged from ± 0.9 ng/ml in one study (37) to ± 26 ng/ml in two other studies (36, 39). This leads to difficulties in comparing study results and even reduces the reliability of the results. Consequently, as a defined 'normal range' for adipokine serum concentrations does not exist yet, their potential usage as a disease biomarker is even more questionable. An important notification

concerning the inconsistent results of liver fibrosis is that the progression of liver fibrosis may occur over a longer period of time in NASH. Serum adipokine levels, however, are known to fluctuate over time, depending on the metabolic environment. Therefore, cross-sectional measurements of adipokines may variably correlate with the severity of liver fibrosis, depending on the time of determination (107). A final limitation is the heterogeneity across patient groups, reflecting the different study populations compared in studies or the lack of adjustment for several factors that may influence adipokine levels such as obesity or adiposity, IR, age or gender.

In conclusion, clinical studies suggest that chemerin, resistin and AFABP are potentially involved in NAFLD pathogenesis and/or progression. Based on literature, most adipokines are suggested to be involved in the inflammatory response that develops within the context of NAFLD, at either hepatic or systemic level, and/or hepatic IR. However, data regarding the impact of these and other newly described adipokines on disease progression is still scarce and often confined to small patient numbers. There is a high need for longitudinal studies on larger and homogenous patient groups compared with carefully matched healthy controls, together with the need of a standardized assay to determine adipokine levels in serum. Once it has been clearly elucidated that adipokines are key players in the pathogenesis and progression of NAFLD, the pharmacologic manipulation of adipokines could be a feasible option in future therapeutics, and the use of adipokines as part of a non-invasive diagnostic tool for NAFLD could be of interest.

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3.2. Reduced expression of chemerin in visceral adipose tissue associates with hepatic steatosis in patients with obesity

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Abstract

OBJECTIVE: We aimed to evaluate whether circulating levels and/or visceral adipose tissue (VAT) expression of recently described adipokines associate with histopathological severity of non-alcoholic fatty liver disease (NAFLD), independently from obesity and insulin resistance.

METHODS: Serum levels of adiponectin, omentin, chemerin, monocyte chemoattractant protein-1 and secreted frizzled-related protein 4 were measured using enzyme-linked immunosorbent assay (ELISA) in 81 patients with obesity and NAFLD and 18 lean control subjects. Expression in VAT was measured using real-time PCR and histopathological grading was scored using the NAFLD activity score (NAS).

RESULTS: When NAFLD patients were subdivided into groups with simple steatosis, borderline NASH and NASH, adiponectin serum levels and omentin expression were lower in NASH versus simple steatosis patients. Serum adiponectin was generally lower with higher histopathological grading. Chemerin VAT expression was negatively associated with NAS ($r=-0.331$, $P=0.022$) and steatosis score ($r=-0.335$, $P=0.020$) independent of age, BMI and HOMA-IR. In addition, adjusting for chemerin VAT expression in a multivariate model explained part of the association between NAS and HOMA-IR.

CONCLUSIONS: These findings suggest that lower VAT expression of chemerin in patients with obesity may be involved in the pathophysiology of hepatic steatosis, potentially by modulating the link between insulin resistance and NAFLD.

Introduction

The growing epidemic of obesity has led to a simultaneously increased prevalence of non-alcoholic fatty liver disease (NAFLD), often referred to as the hepatic manifestation of metabolic comorbidity [1]. NAFLD is a chronic liver disease that encompasses a broad histopathological spectrum ranging from simple steatosis (SS) to non-alcoholic steatohepatitis (NASH), and is associated with a higher risk of developing cirrhosis and hepatocellular carcinoma [2]. To better address this growing health problem, more insight in the pathophysiology is needed.

Recent data indicate that adipose tissue-derived secretory factors (adipokines) could play a role in the development of NAFLD and influence disease progression [3]. Many adipokines have already been described and several studies suggested that they play a role in whole body energy homeostasis and/or inflammatory responses [4], thus possibly influencing metabolic abnormalities in peripheral tissues in persons with obesity. Elevated chemerin levels have been described frequently in patients with metabolic syndrome and have been associated with body mass index (BMI), serum glucose, triglycerides (TG), high-density lipoprotein (HDL) cholesterol levels, and blood pressure [5]. Chemerin is also known to be involved in inflammation by inducing chemotaxis and to induce insulin resistance in skeletal muscle cells [6, 7]. In contrast, adiponectin and omentin have been found to display anti-inflammatory, insulin-sensitizing and cardioprotective properties [8, 9]. Monocyte chemoattractant protein-1 (MCP-1), an adipokine of which higher levels are described in obesity and insulin resistance, has also been suggested to play a role in hepatic inflammatory and fibrogenic processes [10, 11]. Finally, secreted frizzled-related protein 4 (SFRP4), a modulator of the Wnt signaling pathway, has also been described as an adipokine [12] and several Wnt pathway components are associated with lipid and glucose metabolism as well as inflammation [13]. Overall, hepatic insulin resistance, inflammation and modulation of the activity of Wnt signaling pathway have been reported repeatedly in NAFLD patients, suggesting a common link that potentially contributes to obesity-related NASH [1, 14, 15]. In this study, we assessed serum levels as well as visceral adipose tissue (VAT) expression of chemerin, adiponectin, omentin, MCP-1 and SFRP4 in patients with biopsy-proven NAFLD and lean controls. We hypothesized that a disturbed signature of these adipokines may contribute to the progression of SS to an inflammatory and insulin resistant NASH status. Therefore, we aimed to evaluate whether these adipokines associate with histopathological disease severity independently from known insulin resistance in obesity.

Subjects and methods

Study design and subjects

Ninety Caucasian patients with obesity (57 males and 33 females; mean BMI 41 kg/m²), aged between 20 and 68, were recruited from the NASH and Hepobster cohort for evaluation in this study. All patients were scheduled for gastric banding or gastric bypass surgery and met the criteria for bariatric surgery of the International Federation for the Surgery of Obesity: BMI ≥40 kg/m² or BMI ≥35 kg/m² in conjunction with obesity-induced co-morbid conditions. Twenty men with obesity had type 2 diabetes. Liver and VAT biopsies were obtained during surgery and patients with other causes of liver disease, e.g. hepatitis B or C, viral or autoimmune hepatitis, Wilson disease or any drug-induced liver disease, or evidence of excessive alcohol consumption (≥20 g per day) were excluded. Additional exclusion criteria were primary hypogonadism, abnormal thyroid function, malignancies or carcinoma, serum total cholesterol >300 mg/dl and/or TG >450 mg/dl. None of the subjects used steroids, insulin or thiazolidinediones, and oral glucose lowering-medication were discontinued prior to surgery. For comparison, 18 Caucasian control men (mean BMI 24 kg/m²; mean age 44±12 years) from the Hepobster cohort were recruited. These men underwent elective abdominal surgery for adhaesiolysis, hernia diaphragmatica, intestinal resection or Nissen fundoplication and VAT biopsies were obtained during surgery. Similar exclusion criteria as mentioned above were applied. In addition, all control subjects had overall good health without medication and with normal liver function tests (chronic elevation of transaminase levels indicates liver disease, i.e. >1.5 times the upper normal value for ≥3 months). The study protocol was approved by the Ethics Committee of Ghent University Hospital and conducted according to the principles of the Declaration of Helsinki. All participants gave their written informed consent.

Anthropometry and biochemical assays

Anthropometric measurements were performed during a pre-operative examination. Body weight was measured to an accuracy of 0.1 kg in light indoor clothing without shoes, whereas height was measured using a wall-mounted stadiometer. Blood samples were collected after overnight fasting, prior to surgery, and were centrifuged, fractionated and stored at -80°C until analysis. Triglyceride and glucose levels were determined colorimetrically (Roche Diagnostics, Mannheim, Germany) and insulin levels were measured using electrochemoluminescent immunoassay (Modular immunoassay, Roche Diagnostics). Homeostasis model of the assessment for insulin resistance (HOMA-IR) was calculated with the following formula: (fasting glucose [mmol/L] x fasting insulin [μU/mL])/22.5]. Aspartate

aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyltransferase (GGT) and C-reactive protein (CRP) were routinely measured using a conventional automated analyser.

Measurement of adipokine levels

Serum levels of adipokines were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits, according to manufacturer's instructions. Chemerin, MCP-1 and adiponectin were determined with kits provided by R&D Systems (Minneapolis, MN, USA). The respective intra- and interassay coefficients of variation (CV) were 2.8% and 6.4% for chemerin, 4.9% and 4.6% for MCP-1, and 4.7% and 4.9% for adiponectin. Omentin serum levels were assessed using the Human Omentin-1 ELISA kit from BioVendor (Brno, Czech Republic). Intra- and interassay CV were 3.7% and 4.6%, respectively, for omentin. Circulating levels of SFRP4 were measured using the USCN Life Science ELISA kit (Hoelzel Diagnostika, Cologne, Germany). Intra- and interassay CV for the SFRP4 were <10% and <12%, respectively.

For gene expression analysis, VAT biopsies were collected from the gastrosplenic or gastrocolic ligaments at the end of surgery from 63 male subjects of the Hepobster cohort (13 controls and 50 subjects with obesity), immediately frozen and stored at -80°C until further use. RNA was isolated from 100 mg of the frozen fat samples with the Tripure Isolation Reagent kit (Roche Diagnostics) according to manufacturer's instructions. Contaminating genomic DNA was removed with RNase-free DNase incubation (Qiagen, Hilden, Germany), followed by a clean-up step with the RNeasy Mini Kit (Qiagen). cDNA synthesis was carried out using the Go Script Reversed Transcription System (Promega, Mannheim, Germany) according to manufacturer's instructions. Expression of MCP-1 and chemerin was quantified by real-time PCR using the following QuantiTect primer assays: Hs_CCL2_1_SG (MCP-1, *CCL2* gene; Cat#QT00212730), Hs_RARRES2_1_SG (chemerin, *RARRES2* gene; Cat#QT00091945) (Qiagen). Primers to measure omentin (*INTL1* gene) and SFRP4 expression were TCAGCTTCCTGCTGTTTCTCATA and GGAGACGAAGAACAGGTCCATT for omentin and CACCCATCCCTCGAACTCAA and TGTGTGGACACTGGCAAGAAG for SFRP4 (Eurogentec, Köln, Germany). Real-time PCR analysis was carried out on a StepOne Plus system (Applied Biosystems) with GoTaq qPCR Master Mix (Promega). Gene expression levels were calculated from the obtained threshold cycle (Ct) values after normalization for the expression of stable reference genes, *UBE2D2*, *YWHAZ*, (Eurogentec) and *RPS18* (QuantiTect Primer Assay, Cat#QT02323251, Qiagen), using Qbase Plus software (version 2.6; Biogazelle, Ghent, Belgium).

Hepatic histopathological analysis

Liver biopsies were obtained from all subjects with obesity at the end of surgery, each measuring 5 by 5 mm, and were taken from the lateral edge of the left liver lobe (segment 3) using bipolar forceps. These were immediately fixated in formalin (buffered 4% paraformaldehyde solution; Klinipath, Belgium) at room temperature for microscopic analysis. Formalin fixed liver biopsies were routinely processed and stained with hematoxylin-eosin and Masson trichrome. An experienced pathologist (M.P.) established the histological diagnosis of NAFLD according to the scoring system of Kleiner *et al.* [16], blinded to characteristics of participants. At least 6 complete portal tracts in liver specimen were required for adequate histological evaluation. Steatosis was judged as the percentage of hepatocytes containing fat droplets, with a minimum of 5%. NAFLD activity score (NAS) is defined as the unweighted sum of scores for steatosis intensity (0-3; <5%, 5-33%, 33-66% or >66%), lobular inflammation (0-3; no, <2, 2-4 or >4 inflammatory foci), and hepatocellular ballooning (0-2; no, few or many ballooning cells), ranging from 0 to 8 (Figure 1). Scores of 0-2 were considered as SS and scores of >5 were considered as NASH. Intermediate scores of 3 or 4 were considered as borderline NASH [16]. Stage of fibrosis was scored using a 4-point scale.

Statistics

Statistical analysis was performed using IBM SPSS Statistics (version 21.0). Data distribution of continuous variables was evaluated with the Kolmogorov-Smirnov test. Normally distributed variables were expressed as mean +/- SD, whereas non-Gaussian distributed variables were described as median (interquartile range). Adipokines were compared among the different subject groups, i.e. lean subjects, subjects with obesity but without NAFLD and subjects with obesity and NAFLD. Furthermore, among NAFLD patients, adipokines were compared between patients with low, moderate or high level of histopathological severity, i.e. degree of steatosis, hepatocyte ballooning, lobular inflammation and fibrosis. For these comparison analyses among the different study groups and histological parameters, ANOVA and Tukey HSD post hoc test or Kruskal-Wallis and Mann-Whitney *U* test were applied. Categorical variables were analyzed with the Chi-square test and Fisher's test. Multivariate analysis was performed to identify independent factors associated with disease severity in NAFLD patients. Our findings were adjusted for potential confounders such as age, BMI and insulin resistance, using multivariate linear regression analysis (ENTER model).

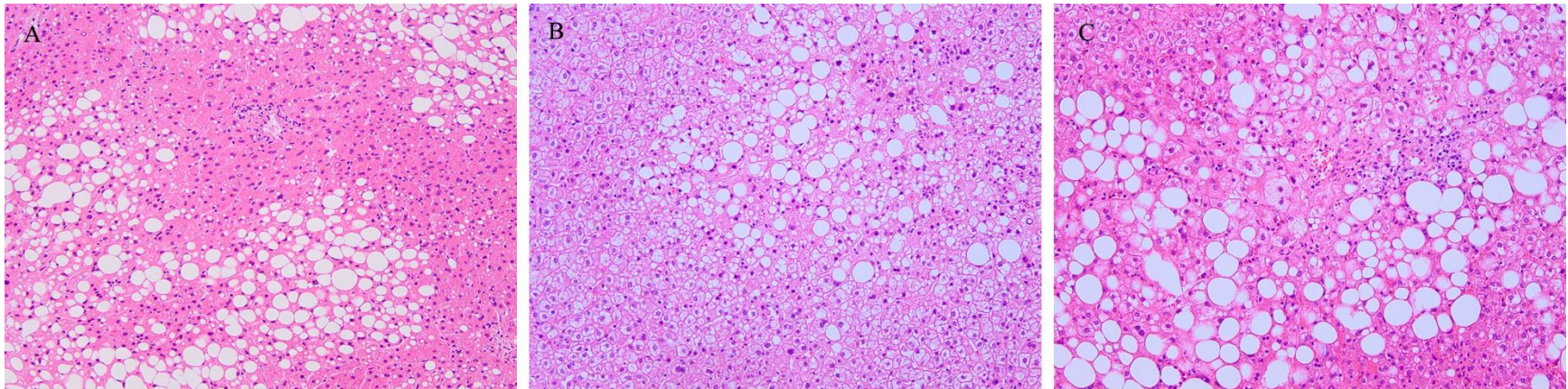


Figure 1 Three different examples of histopathological scoring according to non-alcoholic fatty liver disease (NAFLD) activity score (NAS). 1A is an example of a low NAS (steatosis, grade 2; ballooning, grade 0; lobular inflammation, grade 0; NAS 2) diagnosed as simple steatosis. 1B is an example of an intermediate NAS (steatosis, grade 2; ballooning, grade 1; lobular inflammation, grade 1; NAS 4) diagnosed as mild steatohepatitis or borderline NASH. 1C is an example of a high NAS (steatosis, grade 2; ballooning, grade 2; lobular inflammation, grade 1; NAS 5) diagnosed as definite steatohepatitis or NASH. (10X, Hematoxylin and Eosin).

Results

General characteristics, adipokine serum levels and VAT expression in controls and NAFLD patients

A total of 108 subjects were included in the study cohort (75 males and 33 females), including 18 normal-weight controls, 9 patients with obesity but without NAFLD and 81 patients with obesity and biopsy-proven NAFLD. NAFLD patients had higher BMI, insulin levels, HOMA-IR, ALT, GGT and CRP levels compared to normal-weight controls, while there were no differences in TG and AST levels. 25% of NAFLD patients had type 2 diabetes, whereas none of the controls or obese patients without NAFLD had diabetes (Table 1).

Serum adiponectin levels were lowest in NAFLD patients compared to normal-weight controls and obese patients without NAFLD ($P=0.004$ and 0.020 , respectively), whereas serum MCP-1 levels were lowest in the obese without NAFLD group versus controls and NAFLD patients ($P<0.05$). Chemerin serum levels were higher in NAFLD patients compared to normal-weight controls ($P=0.020$). Circulating omentin and SFRP4 levels were not different among the groups. With regard to adipokine VAT expression, omentin and SFRP4 were higher ($P=0.043$ and <0.001 , respectively) and chemerin tended to be lower in NAFLD patients compared to normal-weight controls ($P=0.053$). MCP-1 expression was similar between control and NAFLD patients (Table 1).

General characteristics and adipokine levels according to disease severity in NAFLD patients

Potential differences in circulating adipokine levels and VAT expression were examined in relation to disease severity. From the 81 patients with obesity diagnosed with NAFLD, 32 subjects had SS, 24 subjects borderline NASH and 25 subjects confirmed NASH. General characteristics as well as adipokine levels among groups are listed in Table 2. There were no differences in gender, age, BMI, fasting glucose and insulin, TG, ALT and CRP levels among the groups. HOMA-IR, AST and GGT levels were higher in both borderline and confirmed NASH patients as compared to those with SS ($P=0.033$, 0.023 and 0.002 , respectively). There were no clinical or biochemical differences between borderline NASH and NASH patients.

Serum levels of adiponectin were lower in NASH patients compared to patients with SS ($P=0.030$). The other serum adipokines were not different among the three NAFLD groups. Similarly, there were no differences in VAT expression of adipokines among the groups, except for lower omentin expression in patients with NASH versus patients with SS ($P=0.043$).

Table 1 General characteristics of healthy controls, obese patients without NAFLD and obese NAFLD patients

Parameter	Healthy controls (N=18)	Obese w/o NAFLD (N=9)	NAFLD patients (N=81)	P value
General characteristics				
M/F	18/0	2/7***#	55/26**	< 0.001
T2D	0	0	20 (25%)*	0.017
Age, years	44 ± 12	45 ± 9	45 ± 10	0.995
BMI, kg/m ²	24 [22-26]	36 [34-41]***#	41 [38-44]***	< 0.001
Glucose, mmol/L	5.44 [4.63-5.84]	5.11 [4.78-5.86]	5.33 [4.78-6.31]	0.766
Insulin, pmol/L	25.7 [21.1-40.4]	57.1 [35.8-111.1]**	92.9 [57.5-150.9]***	< 0.001
Triglycerides, mg/dl	156.0 [111.5-204.9]	104.0 [70.5-139.5]*###	185.0 [144.0-241.0]	< 0.001
HOMA-IR	0.87 [0.60-1.47]	1.79 [1.04-3.89]**	3.21 [1.92-6.10]***	< 0.001
AST, IU/L	22.0 [17.8-33.0]	20.0 [15.5-22.0]#	28.0 [20.0-40.0]	0.023
ALT, IU/L	11.3 [8.5-29.5]	21.0 [9.0-26.5]	25.0 [14.5-36.5]*	0.036
GGT, U/l	22.2 [16.0-29.2]	15.0 [10.5-31.0]#	30.0 [19.5-50.5]*	0.009
CRP, mg/L	1.05 [0.60-2.20]	0.50 [0.15-5.30]	3.00 [1.10-5.35]*	0.046
Serum adipokine levels				
Omentin, ng/ml	398.2 [336.0-490.3]	394.9 [263.4-534.8]	391.6 [307.1-480.7]	0.894
Adiponectin, mg/ml	6.15 [5.30-10.89]	9.45 [4.10-12.54]#	4.28 [2.94-7.11]**	0.002
Chemerin, ng/ml	164.7 [112.1-211.0]	177.5 [155.2-235.4]	200.3 [146.6-256.1]*	0.063
MCP-1, pg/ml	288.6 [239.5-354.0]	210.1 [192.0-234.8]*###	299.5 [252.4-395.6]	0.010
SFRP4, pg/ml	4569.0 ± 2833.8	4255.6 ± 2351.4	4650.8 ± 1805.0	0.709
Adipokine VAT expression, AU				
N	15		48	
Omentin	1.42 [0.07-13.98]		6.17 [1.98-22.28]*	0.043
Chemerin	1.09 [0.81-1.46]		0.87 [0.62-1.07]	0.053
MCP-1	1.91 [0.20-5.05]		0.58 [0.36-1.59]	0.488
SFRP4	1.01 [0.67-1.23]		2.86 [1.75-4.32]***	< 0.001

Data are presented as median [interquartile range] and were analyzed using Mann-Whitney U test and Chi-square test for categorical variables. * vs healthy controls; # vs NAFLD patients. * P < 0.05; ** P < 0.01; *** P < 0.001. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, gamma-glutamyltransferase; M/F, male-to-female ratio; NAFLD, non-alcoholic fatty liver disease; T2D, type 2 diabetes; w/o, without.

Table 2 Clinical and biochemical characteristics of obese NAFLD patients with simple steatosis, borderline NASH and NASH

Parameter	SS (N=32)	Borderline NASH (N=24)	NASH (N=25)	P
Histological grading according to NAS				
NAS	1-2	3-4	5-8	< 0.001
Steatosis grade (0/1/2/3)	0/26/6/0	0/8/15/1	0/0/5/20	< 0.001
Lobular inflammation (0/1/2/3)	27/5/0/0	4/20/0/0	2/16/6/1	< 0.001
Hepatocellular ballooning (0/1/2)	31/1/0	2/21/1	0/7/18	< 0.001
Fibrosis (0/1/2/3/4)	23/8/1/0/0	6/11/7/0/0	2/12/6/3/1	< 0.001
Clinical and biochemical characteristics				
M/F	19/13	17/7	19/6	0.384
T2D	4 (12.5%)	10 (42%)	6 (24%)	0.043
Age, years	43 ± 11	47 ± 11	46 ± 10	0.360
BMI, kg/m ²	42 [39-46]	39 [36-44]	42 [37-43]	0.302
Glucose, mmol/L	5.25 [4.69-5.67]	5.39 [4.74-7.21]	5.56 [4.97-6.58]	0.376
Insulin, pmol/L	78.0 [47.9-119.9]	107.6 [76.7-158.8]*	103.7 [57.1-229.2]	0.095
Triglycerides, mg/dl	178.5 [118.8-215.2]	208.5 [161.8-258.5]*	174.0 [143.5-288.5]	0.129
HOMA-IR	2.41 [1.70-4.08]	4.00 [2.20-7.00]*	3.50 [1.97-8.19]*	0.033
AST, IU/L	24.5 [17.0-33.5]	25.5 [20.5-40.0]	34.0 [25.0-55.7]**	0.023
ALT, IU/L	21.2 [15.3-34.0]	29.5 [11.5-35.8]	28.0 [10.0-45.4]	0.607
GGT, U/l	23.2 [16.0-34.8]	32.0 [20.0-54.5]*	41.5 [28.5-66.5]**	0.002
CRP, mg/L	2.30 [1.10-5.60]	2.10 [0.70-5.18]	4.10 [1.65-5.48]	0.436
Serum adipokine levels				
Omentin, ng/ml	363.4 [301.8-492.5]	430.3 [313.5-519.7]	353.3 [293.5-437.9]	0.234
Adiponectin, mg/ml	5.08 [3.56-8.36]	3.99 [2.83-6.39]	4.18 [2.37-4.75]*	0.063
Chemerin, ng/ml	203.0 [154.0-271.5]	195.9 [147.6-255.7]	202.4 [139.9-232.2]	0.759
MCP-1, pg/ml	282.7 [239.9-396.9]	319.9 [274.2-408.5]	300.7 [263.3-381.3]	0.531
SFRP4, pg/ml	4725.7 ± 1414.3	4273.4 ± 1754.7	4939.9 ± 2167.4	0.673
Adipokine VAT expression, AU (N=48)				
N	16	16	16	
Omentin	16.78 [3.59-36.15]	5.28 [1.59-26.11]	5.87 [1.63-10.86]*	0.166
Chemerin	0.88 [0.65-1.25]	0.97 [0.66-1.07]	0.69 [0.56-0.92]	0.260
MCP-1	0.52 [0.32-2.18]	0.54 [0.32-1.35]	0.66 [0.40-1.43]	0.896
SFRP4	3.78 [1.85-4.59]	2.54 [1.68-3.96]	2.99 [2.07-4.38]	0.484

* vs SS. Data are presented as number of cases and median [interquartile range] and were analyzed using Kruskal Wallis and Mann-Whitney U test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, gamma-glutamyltransferase; M/F, male-to-female ratio; MCP-1,

monocyte chemoattractant protein-1; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; Sfrp4, secreted frizzled-related protein 4; T2D, type 2 diabetes; VAT, visceral adipose tissue.

Associations between adipokines and histopathological grading.

We next examined whether adipokine levels and VAT expression were related to the various histopathological states. As shown in Figure 2, serum adiponectin levels ($r=-0.308$, $P=0.005$; Figure 2A), and chemerin VAT-expression ($r=-0.331$, $P=0.022$; Figure 2B) negatively associated with NAS. The other adipokines were examined similarly and showed no significant relations with NAS.

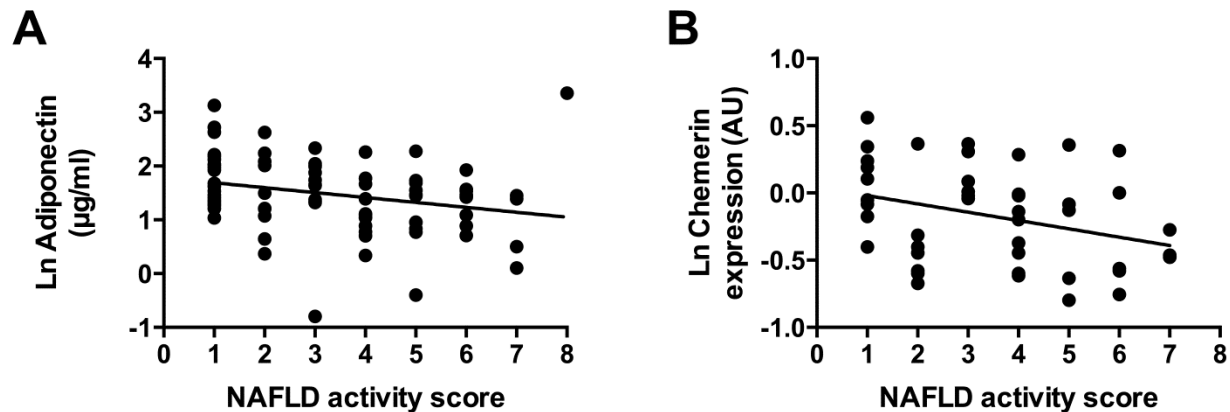


Figure 2 The inverse relationship between disease severity, presented as the non-alcoholic fatty liver disease (NAFLD) activity score (NAS), and ln-transformed serum adiponectin (A; N = 81) as well as visceral adipose tissue expression of chemerin (B; N = 63) in biopsy-proven NAFLD patients.

When considering the individual histopathological parameters, it was found that serum adiponectin levels and chemerin VAT expression were lower in patients with moderate or severe steatosis (>33%) versus patients with mild steatosis (<33%) ($P=0.001$ and 0.007 , respectively; Figure 3 and Figure 4). Furthermore, serum adiponectin was lower in patients with higher grades of hepatocyte ballooning, lobular inflammation and fibrosis (all $P<0.05$; Figure 4). There were no differences in levels of other adipokines, both serum levels and VAT expression, according to histopathological grading.

Finally, we investigated whether serum adiponectin and chemerin VAT expression function as independent predictors of NAS in addition to known confounders such as HOMA-IR and BMI. Among these NAFLD patients, BMI was similar in patients with different grades of histopathological parameters and did not correlate with overall NAS. In contrast, HOMA-IR was higher in patients with higher grades of steatosis, hepatocyte ballooning and lobular inflammation (all $P<0.05$) and was positively associated with NAS ($r=0.285$, $P=0.010$).

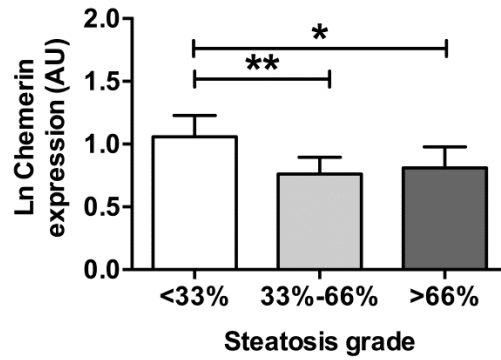


Figure 3 Chemerin VAT expression was inversely associated with steatosis grade (P=0.007; Kruskal Wallis test). Expression was highest in patients with < 33% of steatosis and was lower in patients with moderate (33-66%) to severe (> 66%) steatosis grade. * P < 0.05; ** P < 0.01.

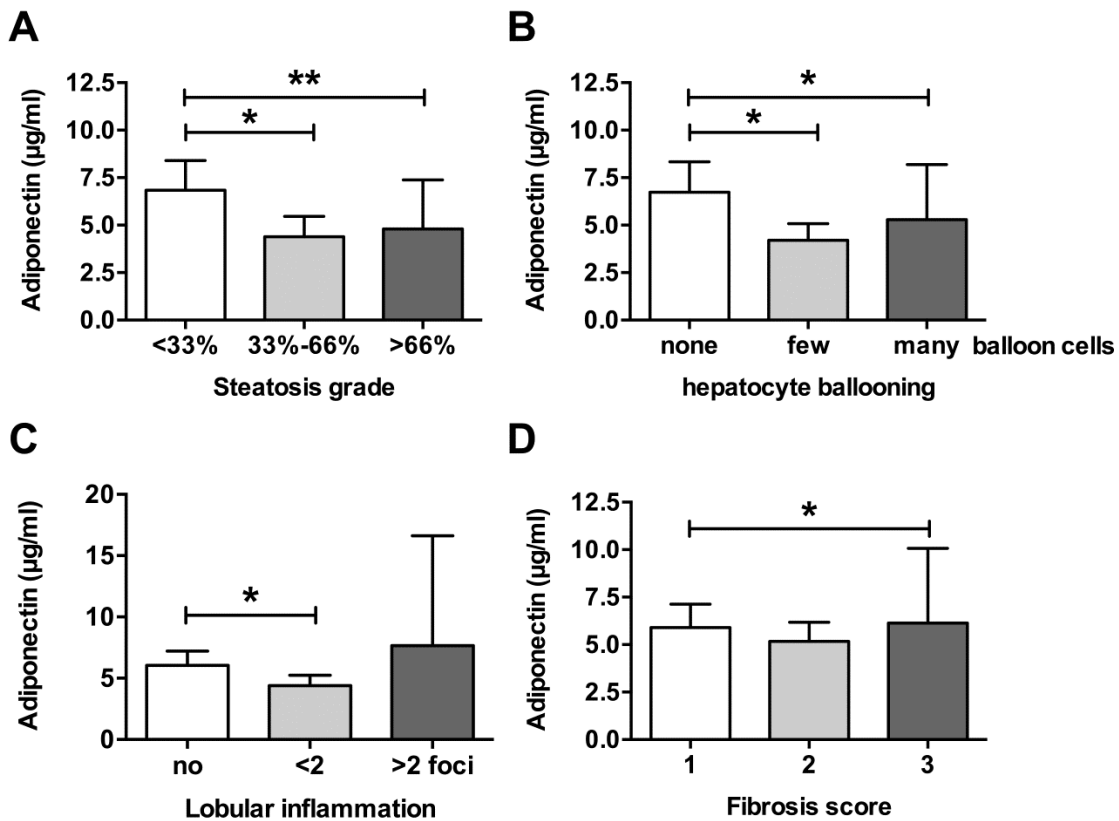


Figure 4 Adiponectin serum levels among the histopathological parameters in NAFLD patients. Serum adiponectin was inversely associated with the grade of steatosis (A), hepatocyte ballooning (B), lobular inflammation (C), and fibrosis (D) (P=0.001, P=0.033, P=0.027 and P=0.042 respectively; Kruskal Wallis test among groups), with lower levels in patients with higher grades. * P < 0.05; ** P < 0.01.

Multivariate linear regression analysis was performed to identify independent predictors of NAS in NAFLD patients (Table 3). Whereas the association between HOMA-IR and NAS was independent of sex, age and BMI (model 1), it lost significance after correcting for chemerin VAT. Chemerin VAT expression remained negatively associated with NAS independent of age, BMI and HOMA-IR in male NAFLD patients (model 2), whereas adiponectin serum levels were no longer associated with NAS after correcting for sex, age, BMI and HOMA-IR (model 3). In a final model, adjusting for adiponectin levels made the relation between chemerin VAT expression and NAS borderline significant ($\beta=-0.284$, $P=0.072$; model 4).

Table 3 Multivariate linear regression model with NAS as dependent variable in biopsy-proven NAFLD patients

	Model 1	Model 2	Model 3	Model 4
	F 3.028; R² 0.139	F 3.209; R² 0.234	F 2.742; R² 0.156	F 2.678; R² 0.246
Independent variables	β (\pmSE); P value	β (\pmSE); P value	β (\pmSE); P value	β (\pmSE); P value
Sex	0.244 (\pm 0.476); 0.035	Constant	0.209 (\pm 0.511); 0.090	Constant
Age	0.205 (\pm 0.951); 0.085	0.103 (\pm 1.172); 0.505	0.223 (\pm 0.951); 0.062	0.114 (\pm 1.182); 0.465
HOMA-IR	0.275 (\pm 0.265); 0.020	0.244 (\pm 0.286); 0.088	0.210 (\pm 0.302); 0.116	0.185 (\pm 0.323); 0.248
BMI	-0.056 (\pm 1.484); 0.643	-0.211 (\pm 2.062); 0.150	-0.047 (\pm 1.477); 0.693	-0.202 (\pm 2.077); 0.170
Chemerin VAT expression		-0.317 (\pm 0.797); 0.038		-0.284 (\pm 0.830); 0.072
Serum adiponectin levels			-0.135 (\pm 0.379); 0.313	-0.128 (\pm 0.454); 0.422

The independent variables in the different models were: Model 1: sex, age, HOMA-IR and BMI; Model 2: sex was constant, age, HOMA-IR, BMI and chemerin visceral adipose tissue (VAT) expression; Model 3: sex, age, HOMA-IR, BMI and serum adiponectin levels; Model 4: sex was constant, age, HOMA-IR, BMI, chemerin VAT expression and serum adiponectin levels. Data are presented as β coefficients with standard error (SE) of the predicted coefficients (B) and F and R² values are given per model. Because VAT was only sampled from male patients with NAFLD, sex was constant in Model 2 and Model 4. HOMA-IR, homeostasis model of the assessment for insulin resistance; NAS, non-alcoholic fatty liver disease (NAFLD) activity score.

Discussion

In a group of biopsy-proven NAFLD patients with obesity, we found lower adiponectin and higher chemerin serum levels as compared to healthy control subjects. Adiponectin, but not chemerin serum levels were also associated with disease severity and grade of steatosis. In contrast to the serum levels, chemerin VAT expression appeared inversely associated with NAFLD severity, as expression was significantly lower in patients with higher degrees of steatosis and overall NAS. This finding persisted after adjustment for age, BMI and HOMA-IR. Further, our results suggest that the well-established link between insulin resistance and NAFLD/NASH might in part be dependent on VAT expression of chemerin.

NAFLD develops when surplus fat accumulates in the liver, which might be both resulting from and contributing to insulin resistance. Indeed, insulin resistance is known to result in an increased hepatic *de novo* lipogenesis, together with an increased delivery of lipids to the liver [17]. The prevalence and severity of NAFLD have been strongly related to BMI, waist circumference, hyperinsulinemia, hypertriglyceridemia, impaired glucose tolerance and type 2 diabetes, which are all metabolic parameters with the common underlying factor insulin resistance [18, 19]. Although NAFLD severity did not associate with BMI in this study, HOMA-IR was associated with steatosis, hepatocyte ballooning and lobular inflammation score and with overall NAS independent of sex, age and BMI

Our study also showed an inverse association between serum adiponectin levels and grade of steatosis, lobular inflammation, hepatocyte ballooning, fibrosis and overall NAS, corroborating previous findings [20]. Adiponectin has an anti-steatotic effect on hepatocytes by increasing fatty acid oxidation and decreasing gluconeogenesis, fatty acid influx and *de novo* lipogenesis [21]. Additionally, anti-inflammatory and anti-fibrotic effects of hepatic adiponectin have been reported [22]. Recently, a meta-analysis confirmed lower adiponectin levels in NASH/NAFLD compared to controls [23]. The few prospective studies with paired liver biopsies however, reported conflicting results and could not always associate adiponectin with NAFLD progression [24, 25]. In our multivariate analysis, the association between NAS and adiponectin was lost when HOMA-IR was added to the model, suggesting that insulin resistance might mediate the relation between NAFLD and adiponectin. Since adiponectin is highly associated with insulin resistance, these findings suggest that low circulating levels of adiponectin may contribute to insulin resistance, which in turn may influence NAFLD.

Chemerin has been described as a marker of insulin resistance, with both higher serum levels and adipose tissue expression in patients with impaired glucose tolerance or type 2 diabetes [26, 27].

Chemerin has been reported to interfere with insulin signaling in skeletal muscle cells and to be implicated in the regulation of inflammation, suggesting a potential involvement in NAFLD [6, 7]. Indeed, several studies have reported elevated serum or hepatic expression levels of chemerin in NAFLD and/or NASH patients and found positive associations with NAS or hepatic inflammation [28, 29]. In this study, serum chemerin levels were higher in NAFLD patients with obesity compared to controls; however, they were similar among the three NAFLD groups and were not related to histopathological severity. In contrast, chemerin VAT expression had a tendency to be lower in patients with NAFLD versus controls and was lower in patients with higher grades of steatosis compared to lower steatosis grades. Chemerin expression was moderately negatively associated with NAS, independent of confounding factors. Moreover, the association between HOMA-IR and NAS lost significance after correcting for chemerin VAT expression, suggesting that chemerin may at least partly influence the link between HOMA-IR and NAFLD severity. Recently, Wolfs *et al.* similarly reported a negative association between chemerin VAT expression and histopathological parameters of NAFLD patients. In their study, chemerin expression was negatively associated with steatosis, lobular and portal inflammation, independent of obesity, HOMA-IR and type 2 diabetes [30]. Experimental studies have reported inconsistent results, indicating both pro- and anti-inflammatory properties of chemerin. It has been described as a chemoattractant of innate immune cells such as Kupffer cells, which in turn are believed to be key players in the pathophysiology of NAFLD [31]. However, while a mouse model with diminished chemerin action showed no effects on insulin resistance and NAFLD compared to wild type [32], the administration of recombinant chemerin attenuated the inflammatory response in another model [33]. Although the associations were moderate, the anti-inflammatory effects of chemerin and its negative association with NAS suggest that this lower VAT expression of chemerin in patients with obesity may be involved in the pathophysiology of NAFLD. Importantly, none of the adipokine serum levels were associated with their respective VAT expression. As the majority of blood flow ($\pm 80\%$) to the liver is delivered via the portal vein, which is closely connected to VAT, it is likely that systemic serum concentrations of adipokines do not accurately reflect hepatic delivery of adipokines. Furthermore, secretion of adipokines is different between subcutaneous adipose tissue (SAT) and VAT, of which SAT is suggested to contribute more to systemic levels. Indeed, Alfadda *et al.* recently highlighted the differential expression of chemerin between SAT and VAT in subjects with obesity, with higher expression in SAT [34].

Other adipokines neither associated with histological parameters nor with NAFLD severity in our cohort. VAT expression of omentin and SFRP4 were significantly higher in subjects with obesity versus controls, without differences in serum levels. This counteracts with the description of omentin in literature as an

anti-inflammatory and insulin-sensitizing adipokine, of which low serum levels have been reported in patients with obesity, insulin resistance and type 2 diabetes [35]. NAFLD and higher degree of hepatocyte ballooning in biopsy-proven NAFLD patients have previously been positively associated with serum omentin levels [36]. Higher levels of SFRP4 have been linked to type 2 diabetes and obesity [37] and could be related to NAFLD because a mouse model with the hepatocyte-specific knockout of the canonical Wnt pathway was more sensitive to develop NASH and fibrosis compared to wild type [38]. However, we investigated SFRP4 levels in human NAFLD for the first time and could not confirm this. Finally, although serum MCP-1 levels were lower in the small group of patients with obesity but without NAFLD, serum levels nor VAT expression were associated with histopathological severity. This contrasts with experimental studies suggesting involvement of MCP-1 in hepatic inflammation [10, 11] and reports of higher MCP-1 levels in NAFLD patients [39].

This study has some limitations. Firstly, the cross-sectional design does not allow to indicate a causative link or to address the mechanisms behind the observed associations. These results are thus merely suggestive and need further investigation in larger-scaled prospective studies. The use of paired biopsies could allow investigating changes of certain adipokines when NAFLD progresses or regresses over time. Next, the relatively small sample size among study groups limits the generalizability of our results and conclusions. Thirdly, patients with obesity, type 2 diabetes and NAFLD are closely linked because of the common causative factor insulin resistance, which makes it difficult to distinguish metabolic confounding factors. Furthermore, it is known that VAT, and not SAT, is strongly associated with NAFLD severity [40]. In this cohort, adipose tissue distribution was not assessed, which might potentially explain the lack of association between BMI and NAFLD. Finally, although liver biopsy is recognized to be the golden standard to diagnose NAFLD, the chance of sampling error remains inevitable.

In summary, we found that in patients with obesity and NAFLD, disease severity and the degree of hepatic steatosis inversely associate with chemerin VAT expression independent of insulin resistance. These findings suggest a potential role of lowered chemerin VAT expression in the pathophysiology of hepatic steatosis. Moreover, chemerin VAT expression even appears to modulate the relation between NAFLD severity and insulin resistance. More knowledge on the determinants of chemerin VAT expression might help to elucidate the relation between both consequences of obesity.

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IV. DISTURBED SEX STEROID PROFILE IN OBESE SUBJECTS

4.1. Determinants of testosterone levels in human male obesity

4.1. Determinants of testosterone levels in human male obesity

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Abstract

Testosterone (T) levels are decreased in obese men, but the underlying causes are incompletely understood. Our objective was to explore the relation between low (free) T levels and male obesity, by evaluating metabolic parameters, subcutaneous adipose tissue (SAT) aromatase expression and parameters of the hypothalamic-pituitary-gonadal axis. We recruited 57 morbidly obese men (33 had type 2 diabetes (DM2)) and 25 normal-weight men undergoing abdominal surgery. Fourteen obese men also attended a follow-up, 2 years after gastric bypass surgery (GBS). Circulating T levels were quantified by LC-MS/MS, whereas free T levels were measured using serum equilibrium dialysis and SHBG, LH and FSH by immunoassay. SAT biopsies were used to determine adipocyte cell size and aromatase expression by real-time PCR. Total and free T levels were decreased in obese males vs. controls, with a further decrease in obese men with DM2 versus obese men without DM2. There were no differences in aromatase expression among the study groups and sex steroids did not correlate with aromatase expression. Pearson analysis revealed an inverse association between (free) T and SAT cell size, triglycerides and HOMA-IR. Multivariate analysis confirmed the inverse association between (free) T and SAT cell size ($\beta=-0.321$, $P=0.037$ and $\beta=-0.441$, $P=0.011$; respectively), independent of age, triglycerides, HOMA-IR, obesity or diabetes. T levels were normalized 2 years after GBS. These data suggest SAT cell size rather than SAT aromatase expression or parameters of the hypothalamic-pituitary-gonadal axis is related to low T in male obesity, which points to adipose cell size-related metabolic changes as a major trigger in decreased T levels.

Introduction

The relationship between decreased testosterone (T) levels and male obesity is incompletely understood. In general, the synthesis of T by Leydig cells in the testis is stimulated by luteinizing hormone (LH) together with follicle-stimulating hormone (FSH). Estradiol (E_2) is known to be an important down-regulator of serum T levels by inhibiting the release of these gonadotropins from the pituitary gland [1,2]. Some studies suggest that an increased activity of aromatase, the enzyme converting T into E_2 , in adipose tissue may contribute to elevated E_2 levels in obese men [3,4]. However, data on E_2 levels in obese men are inconsistent, as some studies reported increased E_2 levels while others reported no changes or even lower levels [5-7]. Alternatively, abdominal obesity itself may contribute to the decline in circulating T levels [8,9]. Obesity leads to an expansion of fat (hypertrophy of adipocytes), which is associated with a deteriorated metabolic profile, including glucose intolerance, dyslipidemia, hypertension and inflammation [10,11]. Prospective studies have shown that both adiposity as well as presence of the metabolic syndrome are predictive of future low T levels and could accelerate the age-related decline of T [12,13]. Consistently, weight loss has led to increased T levels in obese men [14,15]. Although the underlying mechanisms are still unclear, these findings suggest that hypertrophy of the adipocytes and its related metabolic changes may associate with the decline in T levels in obese men.

This cross-sectional study aimed to explore potential determinants of (free) T levels in a cohort of morbidly obese men with and without type 2 diabetes as well as men with normal body weight. Specifically, associations with adipose tissue aromatase expression levels, subcutaneous adipose tissue (SAT) cell size, insulin resistance and triglyceride (TG) levels (marker of adiposity), were examined. Furthermore, the effects of weight loss following gastric bypass surgery (GBS) on (free) T levels and potential determinants thereof were examined in morbidly obese men at a follow-up examination 2 years after GBS.

Research design and methods

Subjects

The study cohort consisted of twenty-five normal-weight and fifty-seven morbidly obese men. Thirty-three of the morbidly obese men had type 2 diabetes according to the American Diabetes Association (ADA) criteria [16]. All men were scheduled for abdominal surgery. The obese men underwent GBS, and normal-weight men had surgery for adhaesiolysis, rupture of the stomach, intestinal resection, stomach closing or Nissen fundoplication. Obesity was defined as BMI > 30 kg/m². Although having abdominal surgery, the normal-weight men had an overall good health. Participants with primary hypogonadism, abnormal thyroid function, hepatitis or malignancies, serum total cholesterol > 300 mg/dl and/or serum TG > 450 mg/dl were excluded. None of the subjects used steroids, and oral glucose lowering-medication were discontinued prior to surgery. A subgroup of thirty-six patients that underwent GBS was invited for a follow-up examination, when two years had passed since surgery. Fourteen of them were willing to participate (39 %). Subjects attending follow-up were not using sex steroids or other androgen-related drugs and three out of fourteen subjects were using metformin medication. The study was approved by the institutional ethics committee and participants gave their written informed consent, which was validated by the Ethical Review Board of Ghent University Hospital and conducted according to the principles of the Declaration of Helsinki (Registration no B67020084018).

At baseline and during follow-up, clinical and anthropometric parameters were assessed as described previously [17]. The fat percentage of body weight (fat %) was estimated by bio-impedance (Bodystat 1500, Bodystat, Ltd, Isle of Man, UK).

Hormonal and biochemical assays

Blood samples were collected from the patients after overnight fasting, prior to surgery. Serum samples were centrifuged, fractionated and stored at -80°C until analysis. Fasting TG, glucose and insulin levels were measured using standard laboratory assays (modular immunoassay, Roche Diagnostics, Mannheim, Germany). HOMA-IR was calculated with the following formula: (fasting glucose [mmol/L] x fasting insulin [μ U/mL])/22.5 [18]. Total T and E₂ were measured with liquid chromatography tandem mass spectrometry (LC-MS/MS). Serum T levels were analyzed using a Waters C-18 Acquity Ultra-Performance Liquid Chromatography (UPLC) column (Waters Corporation, Milford, MA), with a limit of quantification (LOQ) of 0.087 nmol/L (CV<20%, n≥6). Intra- and inter-assay coefficients of variation (CV) were 9.1% at 0.48 nmol/L and 7.3% at 1.35 nmol/L, respectively. For determination of E₂ levels, 2D-LC-

MS/MS was performed on a AB Sciex 5500 triple-quadrupole mass spectrometer (AB Sciex, Toronto Canada) as described by Fiers T et al [19]. LOQ (CV<20%, n≥6) could be ascertained at 1.1 pmol/L for E₂ and intra- and inter-assay CV were 3.7% at 69.4 pmol/L and 4.0% at 77.4 pmol/L, respectively. Free T levels were measured using a validated equilibrium dialysis method, as described previously by Vermeulen et al. [20]. Free E₂ levels were calculated from total E₂, sex hormone-binding globulin (SHBG) and albumin concentrations as described elsewhere [21]. Commercial immunoassays were used to determine SHBG (Orion Diagnostica, Espoo, Finland), LH and FSH (Elecsys LH and FSH immunoassay; Roche Diagnostics).

Adipose tissue processing

SAT biopsies were obtained at the end of the surgical intervention and stored at -80°C until further analysis, or fixated in formol (buffered 4% paraformaldehyde solution; Klinipath, Belgium) at room temperature for microscopic analysis. Fixation, dehydration, cleaning and paraffin impregnation (Tissue Tek Vip, Sakura, USA) of these samples was performed, followed by embedding with a TBS 88 Paraffin Embedding System (Mediate, USA). By means of a Tissue Tek Prisma (Sakura), Haematoxylin-eosin staining and film coverslipping of 3µm slides was completed. Digital photographs of the paraffin slides were taken with an AxioCam ERc 5s camera and Axioskop 20 light microscope (Zeiss, Jena, Germany) at 20x magnification, with a total of 6 photographs per slide (Figure 1). The surface area of by average 143 adipocytes per slide was then measured using the ZEN 2011 software (Zeiss) by indicating the margins of the cell membrane of all complete adipocytes. As adipocytes were assumed to be spheres, as many as possible complete imaged adipocytes were measured in order to calculate the median surface area as expressed in µm² per study patient, followed by calculation of the median SAT cell size per study group. SAT cell size assessment was blinded to grouping and was determined in 50 subjects from the cross-sectional study cohort.

Aromatase expression was determined in the frozen SAT samples of 36 subjects using real-time polymerase chain reaction (real-time PCR). First, RNA was isolated out of 100 mg of the frozen fat biopsies with the Tripure Isolation Reagent kit (Roche Diagnostics) according to the manufacturer's instructions. Contaminating genomic DNA was removed with the RNase-Free DNase Set (Qiagen, Hilden, Germany), followed by a clean-up step with the RNeasy Mini Kit (Qiagen). cDNA synthesis was carried out using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen, Ghent, Belgium) according to the manufacturer's instructions. Aromatase expression levels were quantified by real-time PCR using a Quantitect CYP19A1 primer assay (Qiagen) and SYBR Green PCR Master Mix (Applied

Biosystems, Foster City, CA, USA) on a StepOne Plus system (Applied Biosystems). The obtained threshold cycle (Ct) values were normalized for the expression of the stable reference gene RPS18 (Quantitect RPS18 primer assay; Qiagen) using the Δ Ct-method.



Figure 1 Digital photographs of the subcutaneous adipose tissue (SAT) paraffin slides from a control, obese without and obese subject with type 2 diabetes, respectively. Photographs were taken with an AxioCam ERc 5s camera placed on an Axioskop 20 light microscope at 20x magnification. Mean surface area of adipocytes was measured using the ZEN 2011 software by indicating margins of all complete adipocytes imaged on the slides, expressed in μm^2 . Presented images were randomly selected. Scale bar represents 100 μm .

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics (version 20.0). Data distribution was evaluated with the Kolmogorov-Smirnov test. Variables displaying a normal distribution were expressed as mean \pm S.D, whereas non-Gaussian distributed variables were described as median (interquartile range). ANOVA and Tukey HSD *post hoc* test were used for the comparison of variables among non-obese men, morbidly obese men and morbidly obese men with type 2 diabetes of the cross-sectional study. Non-Gaussian distributed variables were tested using Kruskal-Wallis and Mann-Whitney U test. Pearson correlation coefficients were calculated for the whole study samples as well as for the subgroups (normal-weight men, obese men with and obese men without type 2 diabetes), and were adjusted for age. If not normally distributed, the variables underwent a logarithmic transformation prior to analysis. Multivariate linear regression analysis was used to identify independent factors associated with T levels in men, using a model containing significant variables from univariate analysis. Regression analysis was corrected for grouping by adding obesity and type 2 diabetes as independent binary variables (yes/no) to the model. Prior to regression analysis, variables were standardized in order to retrieve standard error (SE) and 95 % confidence intervals (95 % CI) on β coefficients. Effects after bariatric surgery were analysed using a paired Student t test or Wilcoxon matched-pairs signed-ranks

test in case of non-parametric data distribution. P -values < 0.05 (two-tailed) were considered as statistically significant.

Results

Subject characteristics

Characteristics of the study participants are listed in Table 1. Briefly, obese men without type 2 diabetes were younger as compared to the normal-weight men and obese men with type 2 diabetes (41 [32-49] versus 49 [43-64] versus 54 [50-61] years, respectively; (median[Q1-Q3]) $P = 0.001$). Apart from a higher BMI, the obese men were insulin resistant and had an increased fasting glucose, fasting insulin and fasting TG levels versus normal-weight men. In obese men with type 2 diabetes, BMI, HOMA-IR, and fasting insulin as well as fasting glucose levels were even higher as compared to obese men without type 2 diabetes. Finally, although SAT cell size was similar between obese men with or without type 2 diabetes, it almost doubled versus normal-weight men (Table 1).

Obese men with type 2 diabetes had lowest T levels versus obese men without type 2 diabetes and normal-weight men, respectively (7.17, 10.81 and 16.36 nmol/L, respectively; $P < 0.001$). Similarly, free T levels were lowest in obese men with type 2 diabetes versus obese men without type 2 diabetes and normal-weight men, respectively (164.1, 239.5 and 295.0 pmol/L, respectively; $P < 0.001$). There were no differences in circulating E_2 and LH levels and SAT aromatase expression among the groups. Finally, levels of FSH and SHBG were 35% and 43% lower in obese men, irrespective of type 2 diabetes, whereas LH levels were similar among the groups (Table 1).

Associations with testosterone levels

To identify potential mechanisms underlying the decrease in circulating (free) T levels in obese men, we performed a univariate correlation analysis. SAT aromatase expression showed no associations with sex steroids or with metabolic parameters neither in the whole study sample nor when analysing subgroups (latter data not shown). In contrast, T was strongly negative associated with TG levels ($r = -0.390$, $P < 0.001$), HOMA-IR ($r = -0.444$, $P < 0.001$) and SAT cell size ($r = -0.619$, $P < 0.001$), in the whole study cohort. Similarly, free T levels were also negatively associated with TG levels ($r = -0.242$, $P = 0.033$), HOMA-IR ($r = -0.286$, $P = 0.013$) and SAT cell size ($r = -0.599$, $P < 0.001$) (Table 2). All correlation analyses were adjusted for age. An additional BMI adjustment retained the inverse association between T levels and TG levels ($r = -0.235$, $P = 0.041$), as well as between free T levels and SAT cell size ($r = -0.340$, $P = 0.020$). Furthermore, subgroup analyses revealed that the inverse correlation between total T levels and TG ($r = -0.506$, $P = 0.016$), as well as between free T levels and SAT cell size ($r = -0.719$, $P = 0.019$) (Fig. 2) remained significant in obese men without type 2 diabetes.

Table 1 Clinical and laboratory data of the study cohort, including control, obese and obese subjects with DM 2

Parameter	Controls (N=25)	Obese (N=24)	Obese + DM2 (N=33)	P	P _{control vs obese}	P _{control vs obese+DM2}	P _{obese vs obese+DM2}
Clinical and biochemical characteristics							
Age, years	49 [43-64]	41 [32-49]	54 [50-61]	0.001	0.015	0.350	<0.001
BMI, kg/m ²	24 ± 4	41 ± 6	44 ± 6	<0.001	<0.001	<0.001	0.051
Glucose, mmol/L	4.72 [4.08-5.30]	5.33 [4.77-5.61]	6.99 [6.30-9.10]	<0.001	0.014	<0.001	<0.001
Insulin, pmol/L	35.9 [22.3-64.6]	114.8 [61.8-179.4]	197.9 [122.2-278.0]	<0.001	<0.001	<0.001	0.003
Triglycerides, mmol/L	1.20 [0.81-2.27]	2.10 [1.30-3.18]	1.76 [1.34-2.66]	0.015	0.012	0.011	0.588
HOMA-IR	0.64 [0.40-1.15]	1.99 [1.07-3.19]	4.02 [2.56-4.90]	<0.001	<0.001	<0.001	0.001
SAT cell size ^a , μm ²	3346 ± 1494	6370 ± 1009	5765 ± 1192	<0.001	<0.001	<0.001	0.381
Sex steroids							
SAT aromatase ^b , AU	94.0 [60.0-148.0]	137.0 [70.0-226.0]	92.5 [55.3-244.0]	0.546	0.237	0.702	0.547
Testosterone, nmol/L	16.36 ± 6.74	10.81 ± 5.09	7.17 ± 2.90	<0.001	0.001	<0.001	0.024
Estradiol, pmol/L	63.9 [45.1-88.6]	87.1 [57.8-103.1]	67.5 [52.5-96.0]	0.180	0.060	0.460	0.247
Free testosterone, pmol/L	295.0 ± 138.7	239.5 ± 109.0	164.1 ± 69.9	<0.001	0.174	<0.001	0.029
Free estradiol, pmol/L	1.17 [0.77-1.67]	1.61 [1.13-2.15]	1.32 [1.07-1.90]	0.076	0.024	0.165	0.310
SHBG, nmol/L	41.0 [32.3-49.0]	22.4 [18.2-29.3]	24.0 [16.4-27.9]	<0.001	<0.001	<0.001	0.771
LH, IU/L	5.0 [3.6-8.0]	4.0 [3.0-5.1]	4.6 [2.8-6.8]	0.347	0.143	0.714	0.298
FSH, IU/L	7.2 [4.3-17.3]	4.2 [2.9-5.6]	5.1 [3.6-10.5]	0.024	0.010	0.173	0.076

BMI, SAT cell size, T and Free T were analysed by one-way anova and Tukey HSD post hoc test. Non-Gaussian distributed variables were tested using Kruskal Wallis test and Mann-Whitney U test. SAT subcutaneous adipose tissue; DM2 type 2 diabetes.

^a SAT cell size was determined in N = 16 controls; N = 12 obese men without DM2 and N = 22 obese men with DM2

^b SAT aromatase expression was determined in N = 11 controls; N = 11 obese men without DM2 and N = 14 obese men with DM2

Table 2 Associations between sex steroids, aromatase and metabolic parameters

Parameter	Testosterone	P value	Free testosterone	P value	Ratio T / E ₂	P value	N
SAT aromatase	-0.095	0.600	0.027	0.879	-0.343	0.047	36
LH	0.187	0.104	0.061	0.597	0.112	0.326	82
FSH	0.216	0.064	0.088	0.452	0.173	0.136	82
SHBG	0.694	< 0.001	0.383	0.001	0.604	< 0.001	82
E ₂	0.211	0.064	0.214	0.056	-0.308	0.005	82
Free E ₂	0.054	0.637	0.162	0.151	-0.463	< 0.001	82
TG	-0.390	< 0.001	-0.242	0.033	-0.388	< 0.001	82
HOMA-IR	-0.444	< 0.001	-0.286	0.013	-0.606	< 0.001	82
SAT cell size	-0.619	< 0.001	-0.599	< 0.001	-0.648	< 0.001	50

Data are Pearson correlation coefficients (r) adjusted for age. In case of non-gaussian distribution, variables were log-transformed. LH luteinizing hormone; E₂ estradiol; TG triglycerides; HOMA-IR homeostasis model of assessment for insulin resistance; SAT subcutaneous adipose tissue.

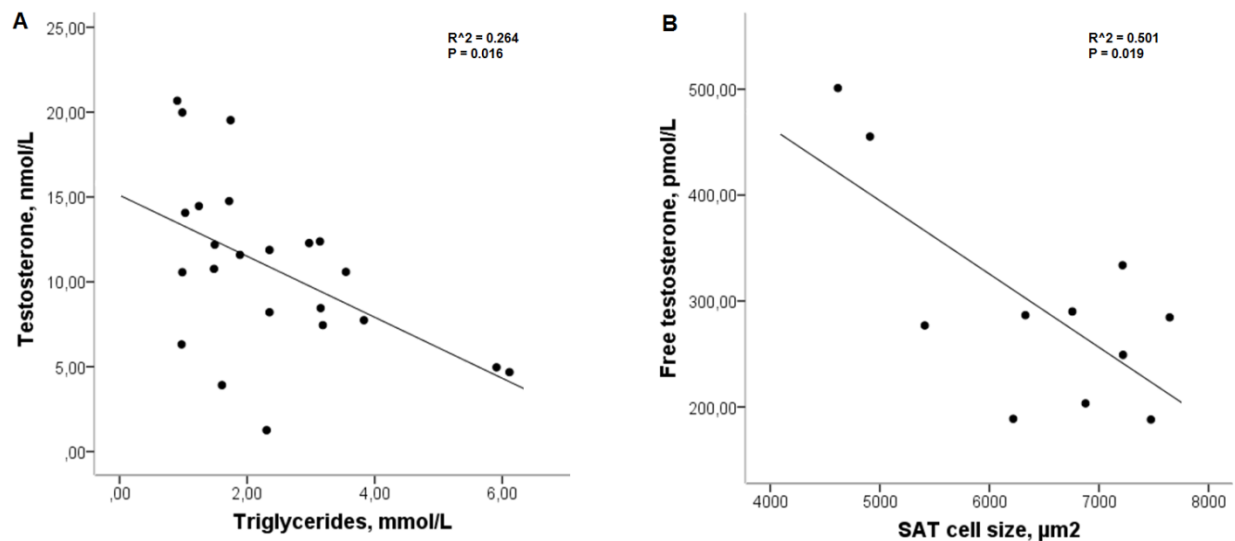


Figure 2 The relationship between triglycerides and serum testosterone levels (n=23; A) as well as inverse associations between free testosterone and subcutaneous adipose tissue (SAT) cell size (n=11; B) in obese men without type 2 diabetes.

Finally, when T to E₂ ratio (ratio T / E₂) was considered, similar results were found. Apart from the obvious relations to E₂, Ratio T / E₂ was negatively associated with TG (r = -0.388, P < 0.001), HOMA-IR (r = -0.606, P < 0.001) and SAT cell size (r = -0.648, P < 0.001). Furthermore, there was a negative association with SAT aromatase, though this association was less strong (r = -0.343; P = 0.047) (Table 2).

To further substantiate the above findings, multivariate linear regression analysis was applied. Parameters showing statistical significance in univariate analysis were entered as variables of interest, after controlling for age and grouping (control vs. obesity/diabetes). Regression analysis confirmed associations between T levels and SAT cell size ($\beta = -0.324$, $P = 0.040$) in a model which contained age, grouping, TG levels and HOMA-IR. An identical model with free T levels and T / E₂ ratio (the latter model contained also SAT aromatase considering its statistical significance in univariate analysis) dependent variable showed similar results ($\beta = -0.446$, $P = 0.013$ and $\beta = -0.391$, $P = 0.051$; respectively) (Table 3).

Table 3 Multivariate linear regression model of variables significantly associated with total and free testosterone levels as well as testosterone to estradiol ratio (ratio T / E₂) as dependent variables. All β coefficients were standardized. N = 50

Variable	β	SE (β)	95 % CI	P value
<i>Total testosterone</i>				
Obesity	-0.153	0.194	-0.546; 0.240	0.435
DM 2	-0.617	0.220	-1.061; -0.173	0.008
Age	-0.171	0.118	-0.410; 0.068	0.157
Triglycerides	-0.054	0.106	-0.269; 0.160	0.610
HOMA-IR	-0.015	0.168	-0.354; 0.324	0.929
SAT cell size	-0.324	0.153	-0.633; -0.015	0.040
<i>Free testosterone</i>				
Obesity	0.049	0.218	-0.391; 0.489	0.822
DM 2	-0.239	0.246	-0.735; 0.258	0.337
Age	-0.472	0.132	-0.740; -0.205	0.001
Triglycerides	0.043	0.119	-0.197; 0.282	0.722
HOMA-IR	-0.077	0.188	-0.457; 0.302	0.683
SAT cell size	-0.446	0.171	-0.791; -0.100	0.013
<i>Ratio T / E₂</i>				
Obesity	-0.183	0.274	-0.772; 0.357	0.455
DM 2	-0.519	0.323	-1.271; 0.061	0.073
Age	0.089	0.211	-0.325; 0.543	0.610
Triglycerides	-0.158	0.143	-0.437; 0.153	0.330
HOMA-IR	0.116	0.302	-0.493; 0.750	0.673
SAT cell size	-0.391	0.218	-0.897; 0.002	0.051
SAT aromatase	-0.167	0.184	-0.565; 0.192	0.319

Obesity and DM 2 are binary variables (yes/no). DM 2 type 2 diabetes; HOMA-IR homeostasis model of assessment for insulin resistance; SAT subcutaneous adipose tissue; SE standard error; CI confidence interval

Characteristics and sex steroid levels of study participants after GBS

Fourteen men agreed to undergo a follow-up investigation 2 years after GBS. Table 4 displays their characteristics and sex steroid levels at baseline and 2 years after GBS. Metabolic characteristics of subjects who participated in the prospective study changed with a significant improvement of BMI (45 kg/m² to 34 kg/m²), fat mass (45 to 36 %), TG (borderline; 1.55 to 1.09 mmol/L), glucose (6.47 to 5.35 mmol/L) and insulin (122.0 to 61.1 pmol/L) concentrations and insulin sensitivity (HOMA-IR 3.3 to 1.1). Mean total T levels increased from 8.99 to 14.62 nmol/L (P = 0.004), SHBG levels increased from 27.9 to 52.4 nmol/L (P < 0.001) and FSH levels increased from 5.7 to 7.4 IU/L (P < 0.001). No significant differences could be established for free T or E₂ before and after bariatric surgery. There were no associations of Δ (free) T levels with any of the improved parameters (latter data not shown).

Table 4 Subject characteristics and sex steroid levels before and after bariatric surgery

	Pre-bariatric surgery (N=14)	Post-bariatric surgery (N=14)	P value
Clinical and biochemical characteristics			
Age, years	51 ± 12	53 ± 12	< 0.001
BMI, kg/m ²	45 ± 8	34 ± 8	< 0.001
Fat, %	45 ± 9	36 ± 12	0.009
Triglycerides, mmol/L	1.55 ± 0.79	1.09 ± 0.46	0.067
Glucose, mmol/L	6.47 ± 1.69	5.35 ± 0.74	0.051
Insulin, pmol/L	122.0 [99.7-211.7]	61.1 [49.9-121.3]	0.001
HOMA-IR	3.3 [2.1-4.2]	1.1 [0.9-2.3]	0.002
Sex steroids			
Testosterone, nmol/L	8.99 ± 4.70	14.62 ± 6.65	0.004
Free testosterone, pmol/L	189.6 ± 110.5	229.0 ± 116.8	0.157
Estradiol, pmol/L	70.4 ± 32.1	68.8 ± 25.9	0.848
Free estradiol, pmol/L	1.33 ± 0.52	1.11 ± 0.44	0.117
SHBG, nmol/L	27.9 [17.0-36.0]	52.4 [39.6-59.1]	< 0.001
LH, IU/L	5.2 [3.0-9.3]	5.8 [2.7-12.6]	0.832
FSH, IU/L	5.7 [5.0-12.0]	7.4 [5.7-18.4]	< 0.001

Data are means ± SD or median (1st -3rd quartile) in case of non-Gaussian distribution. P values were determined using a paired Student *t* test. Non-Gaussian distributed variables were log-transformed.

Discussion

The present study showed a link between enlarged SAT cell size and low T levels in male obesity, and could not establish a predominant role for adipose tissue aromatase expression and parameters of the hypothalamic-pituitary-gonadal (HPG) axis. SAT cell size was independent inversely associated with both total and free T levels after multivariate regression analysis corrected for age, grouping (control vs. obesity/diabetes), HOMA-IR and TG levels. Overall, the findings suggest that low T in male obesity might be related to enlargement of SAT cell size and its related metabolic changes.

In response to an ongoing energy supply adipose tissue is known to expand, due to enlargement of adipocytes [22]. Previous hypotheses suggested that low T levels in obese men may result from an up-regulated aromatase activity in the expanded adipose tissue, followed by elevated E_2 levels which suppress the HPG axis [3,4]. Studies however, which examined the role of aromatase and/or elevated E_2 levels in obesity, reported conflicting results [23-25]. In males, this is one of the few studies evaluating aromatase expression in adipose tissue. Differences in SAT aromatase expression could not be established between obese men (with or without type 2 diabetes) versus normal-weight men. Furthermore, we could not determine significant results concerning visceral adipose tissue (VAT) aromatase expression (data not shown), though SAT aromatase expression only was used for remaining analysis considering the generally known higher expression of aromatase in subcutaneous versus visceral adipocytes [26]. It has previously been suggested that SAT aromatase expression was associated with generalized obesity as described by BMI, though Wake DJ et al. could not find any association between SAT aromatase and abdominal obesity with which obese men are mostly characterized with [24]. Some studies have reported data that counteracts with the increased aromatase activity-hypothesis. Firstly, Dhindsa S et al. reported lower E_2 levels, measured with LC-MS/MS, excluding down-regulation by elevated E_2 of the HPG axis as the main cause for low T levels in men with type 2 diabetes [7]. Similar findings have been reported by Tajar A et al., who used LC-MS/MS to determine E_2 as well [27]. Finally, obese males have been described to have lower SHBG levels, which also counteracts with the increased aromatase activity-hypothesis resulting in elevated E_2 levels since E_2 is known to normally stimulate SHBG production [28].

In the present study we found, apart from an inverse correlation between (free) T and SAT cell size in univariate analyses, an inverse association of (free) T levels with TG and insulin sensitivity (HOMA-IR). Inverse associations between T levels and TG are consistent with findings of previous studies [29,30]. Elevated circulating TG levels have been suggested to be an indicator of metabolic derangement,

associated with both glucose intolerance and increased amounts of VAT [31-33]. The inverse link between sex steroids and lipids has been described previously, suggesting a protective role of T and E₂ for the cardiovascular system [34]. Pitteloud et al. reported an inverse association between human chorionic gonadotropin (HCG)-induced T secretion by Leydig cells and insulin sensitivity (measured by hyperinsulinemic euglycemic clamp) among men with various degrees of glucose tolerance. Thus, Leydig cell function is suggested to be altered in insulin resistant men, resulting in decreased T secretion [35]. Another study reported a diminished T response to HCG in obese men, which correlated with baseline leptin levels [36]. Furthermore, Sertoli cell function was also suggested to be impaired in obese insulin resistant men, since two Sertoli cell markers inhibin B and anti-Müllerian hormone were found to be lower in obese vs. control men [37]. The inverse correlation between (free) T and adipocyte cell size, HOMA-IR as well as circulating TG in this study also supports the findings of Pitteloud et al. [35]. On multivariate regression analysis however, SAT cell size remained the only metabolic parameter that was independently related to (free) T. Adipocyte cell size has been recognized for many years as an important parameter in the pathogenesis of metabolic derangement. Enlarged subcutaneous adipocytes have been shown to predict DM 2 in a prospective cohort, independent from clamp-measured insulin sensitivity [38,39]. It has been shown that insulin-sensitive, though severely obese individuals had a smaller adipocyte size compared to an equally obese but insulin-resistant group matched for age, sex and body fat, suggesting mechanisms beyond obesity *per se* determine the pathological metabolic consequences in obesity [40]. Furthermore, adipocyte cell size has been associated with low grade systemic inflammation and macrophage accumulation in adipose tissue [41,22]. Our data emphasizes the importance of enlarged SAT cell size and related metabolic changes as a potential determinant of low T levels in obese men. However, these findings derive from a cross-sectional study, limiting conclusive statements on causality.

Our results suggest a direct negative impact of adipocytes, but not IR, on free and total T levels in obese men. The influence of adipokines, which are regulatory products of adipose tissue, on reproductive hormones and function has been repeatedly reported [42-44]. For instance, high adiponectin levels (typical of normal weight) have recently been found to regulate Leydig cell steroidogenesis and T secretion, through the transcriptional regulation of steroidogenic genes [44]. Expanded adipose tissue is known to have a different secretion pattern of adipokines, leading to the release of more pro-inflammatory and less anti-inflammatory adipokines in case of obesity. A potential explanation of low (free) T levels could thus be a pro-inflammatory status in testes secondary to a deregulated adipokine secretion pattern, as enlarged adipocytes have been related to a systemic inflammatory state [41,45].

Some inflammatory mediators such as the adipokines TNF- α and leptin have been shown to negatively influence HCG-induced T secretion directly from Leydig cells as well as to disturb Sertoli cell function [35,42,43]. Especially leptin has been indicated as an important mediator in the development of reduced androgens in male obesity, as both influences on the HPG axis as well as direct testicular effects were reported by *in vitro* and *in vivo* studies [36,46-48]. A recent study reported the suppression of HCG-induced T secretion in primary Leydig cells after addition of chemerin, a novel adipokine of which elevated levels have been associated with both obesity and diabetes in humans [49]. In addition, inflammatory mediators have also been shown to suppress the HPG axis [50,51]. The latter could explain the assumed disturbances in HPG axis, since primary testes injury normally is accompanied by an up-regulated gonadotropin release which cannot be demonstrated in our study cohort. Vermeulen et al. as well as Giagulli et al. have indicated these alterations in feedback regulation of gonadotropins by determining LH pulse amplitudes in obese men [4,52].

Finally, bariatric surgery normalized BMI, TG, HOMA-IR, T, SHBG and FSH values in the prospective part of our study. Our findings are consistent with previously reported data, which found improved BMI and T levels in men after bariatric surgery together with a restored sexual function and fertility. In accordance with previous studies, we found no changes in LH levels before and after surgery. Despite the use of the dialysis method, we found no changes in free T levels after GBS, in contrast to studies that used less precise methods such as calculated free T from total T [15,53,54]. This may suggest that a change in SHBG is mainly responsible for the increase in T, instead of recovery of the initial causative factors. Another explanation is the limited number of patients willing to participate in the follow-up part of the study, which could affect detection of small changes. Longitudinal studies on a larger scale are needed to confirm these results. Recently, when bariatric surgery in hypogonadal subjects was compared to eugonadal men however, increase in (free) T levels after surgery had only been observed in hypogonadal men in combination with a more pronounced reduction in waist circumference (a marker of abdominal adiposity of these men). Furthermore, this study showed lower E₂ levels at baseline in hypogonadal vs. eugonadal obese men, also minimizing its role as a determinant of T levels in obese men [54].

The present study has the benefit of LC-MS/MS methods to determine T levels compared to previous commercial radioimmunoassay kits, leading to more reliable results, though larger studies are needed to confirm the findings. Another limitation of this study is the lack of information on adipose tissue aromatase activity in addition to the expression analysis, because SAT samples were frozen or fixated.

In conclusion, low T levels in obese men inversely associate with SAT cell size, HOMA-IR and TG levels and not with adipose tissue aromatase expression, suggesting obesity-related metabolic disturbances to be more important for explaining the T levels in obese men. Further research should be directed at primary T secretion failure of the testis.

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V. SUMMARY OF CONTRIBUTIONS AND GENERAL DISCUSSION

A complex interplay exists between the increasing epidemics of obesity, IR, NAFLD and CVD. Adipose tissue dysfunction has been repeatedly put forward as the initial insult of metabolic disease, as set out in the introduction of this thesis. Unraveling the molecular mechanisms involved in the pathophysiology of dysfunctional adipose tissue and its related conditions, may lead to the development of effective therapeutic or even preventive strategies. Interestingly, a profound sexual dimorphism exists with regard to adipose tissue distribution and a disturbed sex steroid profile is common in obese subjects, suggesting a potential link between sex steroid metabolism and adiposity. The main aim of this thesis was to enhance knowledge on the potential role of dysfunctional adipose tissue of obese subjects in the development of NAFLD and the impaired sex steroid profile in men. To this end we investigated SAT and VAT biopsies derived from obese patients with or without biopsy-proven NAFLD, to determine adipocyte cell size and expression of adipokines and the aromatase enzyme.

1. Main findings and general discussion

1.1. NAFLD in obesity

In chapter 3.2., we describe NAFLD prevalence and histological characteristics in two cohorts of obese patients that were scheduled for bariatric surgery, i.e. patients of the Hepobster and NASH study. In total, we recruited 90 obese patients, of whom a liver biopsy was obtained irrespective of a priori suspected liver disease. This is a major advantage compared to many other studies investigating biopsy-proven NAFLD patients, in which patients are mostly highly selected because of a suspected presence of NAFLD (for instance because of elevated liver function parameters). Notwithstanding our study cohort was randomly composed, 81 out of 90 obese patients were diagnosed to have NAFLD according to NAS. This confirms the very high prevalence (80 - 90%) of NAFLD amongst the high-risk obese population as described in literature [1]. Firstly, we analyzed anthropometrics, metabolic and liver function parameters in this cohort.

In literature, both the prevalence and the severity of NAFLD have been strongly related to BMI, WC, hyperinsulinemia, hypertriglyceridemia, impaired glucose tolerance and type 2 diabetes, which are all metabolic parameters with the common underlying factor IR [2, 3]. NAFLD severity did not associate with BMI in our study population, but HOMA-IR was associated with grade of steatosis, hepatocyte ballooning and lobular inflammation and with overall NAS independent of age and BMI. Further

evidence of the link between obesity, its metabolic comorbidities and NAFLD derives from prospective studies, in which weight loss in obese patients has been associated with improvement of histological features of NAFLD or even resolution of NASH. Indeed after lifestyle intervention, the magnitude of weight loss was associated with changes in histological features of NAFLD. After bariatric surgery-induced weight loss, up to 85 % of NAFLD patients achieved NASH resolution after one year [4]. These results suggest that the probability that NASH (and other comorbidities) resolves depends on the amount and extent of weight loss. For example, compared to lifestyle intervention gastric bypass surgery is known to result in a greater loss of VAT and more improvement of glucose metabolism and insulin sensitivity, whereas loss of SAT throughout the human body is comparable between both interventions [5]. This could explain at least partly our finding that although hepatic steatosis is clearly linked with obesity, once NAFLD is present its severity was predominantly associated with HOMA-IR instead of BMI. Indeed, BMI is not always a good representative measurement of obesity, as metabolically healthy obese patients ($BMI > 30 \text{ kg/m}^2$) have been described with higher insulin sensitivity and lower liver fat content [6].

In clinical practice, abnormal liver function tests, described as mildly raised transaminase (ALT > AST) and/or GGT levels, are often used as markers for evaluating liver disease and the severity of hepatic injury. However, ALT has often been wrongly assumed as a surrogate marker of significant liver disease, because $\pm 80\%$ of patients with liver disease still have normal ALT levels ($< 40 \text{ IU/L}$ in males and $< 31 \text{ IU/L}$ in females) [7]. Furthermore, ALT levels even may tend to decrease and AST levels increase as fibrosis progresses to cirrhosis and ALT was not found to correlate with histopathological disease severity in patients with NAFLD [8, 9]. We could confirm those findings for ALT in our study cohort. AST and GGT but not ALT levels were higher in obese NAFLD patients compared to healthy controls and obese patients without NAFLD. Both AST and GGT were also related to NAFLD severity, as levels were higher with higher grades of histopathological severity. Notwithstanding AST and GGT were related with liver histology in this study, conflicting results in literature suggest that the presence of NAFLD, NASH and/or fibrosis cannot be excluded in obese patients with liver function tests within the normal range [8, 10, 11]. Moreover, patients with elevated liver function tests occasionally show normal liver histology without any evidence of progressive liver disease [12]. Taken together, these findings question the use of liver function tests as a screening tool for NAFLD in obese patients and highlight the need for new biomarkers that allow identifying subjects at risk.

1.2. Associations between adipokines and liver histology in NAFLD

In general, NAFLD has been proposed to develop according a “multihit hypothesis”, with a dysregulated hepatic fatty acid metabolism and IR leading to SS as an initial insult (first hit). Consequently, hepatocytes would become more susceptible to secondary insults (second hit), like imbalances in adipokine exposure, inflammation and oxidative stress, which then can lead to the progression of NAFLD to NASH [13, 14]. Alterations in adipokine levels are suggested to be involved in this “multihit” process, and their imbalance may play an important role in both the development of NAFLD and further progression to NASH [14].

In chapter III, we investigated the associations between adipokines and histopathological severity in biopsy-proven NAFLD patients. Firstly, we performed a systematic review to recapitulate current knowledge on recently described adipokines and their relation with liver histology in NAFLD patients (chapter 3.1.). Thirty-one cross-sectional studies were included in this review, in which seven different adipokines were investigated, i.e. chemerin, resistin, RBP4, visfatin, AFABP, vaspin and apelin. Of these seven novel adipokines, our review pointed out that only three displayed a potentially relevant association with disease severity. Both chemerin and AFABP associated with hepatocyte ballooning degeneration, whereas the hepatic expression of chemerin was found to be associated with fibrosis, steatosis and inflammation. Although serum resistin had been most extensively studied during recent years, only half of the studies effectively reported significant associations with liver histology in NAFLD patients with grade of steatosis as the strongest associated parameter, followed by inflammation. Studies investigating visfatin, RBP4, vaspin and apelin were too divergent or reported no significant associations, lacking evidence of their potential involvement in NAFLD and excluding them from further analysis in this thesis. Based on experimental data, most of the NAFLD-associated adipokines are suggested to be involved in the inflammatory response that develops within the context of NAFLD, either at hepatic or at systemic level, and/or to contribute to hepatic IR [15]. For example, both chemerin and resistin are suggested to inhibit Akt phosphorylation and its downstream targets, thereby interfering with insulin signaling and increasing glucose levels [16, 17]. Resistin has been found to activate PGC1 α , leading to the transcriptional activation of gluconeogenic target genes, and to promote glucose-6-phosphatase to hydrolyse glucose-6-phosphate into free glucose leading to an increased glucose output [18]. Furthermore, it has been reported that resistin induces *de novo* lipogenesis by activating SREBP-1, potentially promoting hepatic steatosis [19]. Omentin is the only adipokine who is suggested to perform insulin-sensitizing effects by stimulating Akt phosphorylation [20]. Chemerin,

resistin, AFABP and MCP-1 are generally proposed to induce an inflammatory response by activating the NFκB and JNK pathways [16, 21-23].

Altogether, data regarding the impact of recently described adipokines on disease progression is still scarce and often confined to small patient numbers. There is a high need for longitudinal studies on larger and homogenous patient groups compared with carefully matched healthy controls. Furthermore, a major heterogeneity was observed across study results. Most importantly, the usage of different ELISA assays resulted in wide ranging adipokine serum levels across different studies, which highlights the urgent need of standardized assays to determine adipokine levels in serum.

At least in part because of the close anatomical proximity between VAT and the liver and the direct drainage of VAT through the portal vein, VAT has been proposed as a major contributor to NAFLD and IR, independent of overall BMI [24-27]. Adipokine expression analysis in VAT might therefore be an interesting tool to investigate potential contributing factors in the pathophysiology of NAFLD. In chapter 3.2., we aimed to evaluate whether circulating levels and/or VAT expression of adiponectin, omentin, chemerin, MCP-1 and SFRP4 associate with the histopathological disease severity in obese patients with biopsy-proven NAFLD, independently from obesity and IR. Adiponectin serum levels were lower, whereas omentin and SFRP4 VAT expression were higher in NAFLD patients compared to controls. When NAFLD patients were subdivided into groups with SS, borderline NASH and NASH, adiponectin serum levels and omentin expression were lower in NASH patients versus patients with SS. However, only serum adiponectin was negatively associated with NAS, with lower levels in patients with higher grades of steatosis, hepatocyte ballooning, lobular inflammation and fibrosis. This is in line with previous findings [28, 29]. Adiponectin has an anti-steatotic effect on hepatocytes by increasing FA oxidation and decreasing gluconeogenesis, FFA influx and *de novo* lipogenesis [30]. Additionally, anti-inflammatory and anti-fibrotic effects of hepatic adiponectin have been reported [31]. Importantly, the association between NAS and adiponectin was lost when HOMA-IR was added to the multivariate regression model, suggesting that IR might mediate the relation between NAFLD and adiponectin. Since adiponectin is highly associated with IR, these findings suggest that low circulating levels of adiponectin may contribute to IR, which in turn may influence NAFLD. Other adipokines, except for chemerin (see further section 1.3.), neither associated with histological parameters nor with NAFLD severity in our cohort.

1.3. Potential role of chemerin in NAFLD

Chemerin is an adipokine of which elevated levels in patients with metabolic dysfunction have been associated with BMI, serum glucose, TG, HDL cholesterol levels, and blood pressure [32]. As described previously (section 1.2.), chemerin is also known to be involved in chemotaxis and to induce IR in skeletal muscle cells [16, 33]. The relative contribution of tissues or cells to systemic levels of chemerin has not been definitively established, but evidence is supporting that adipose tissue and liver are the major, although potentially not exclusive, sites of chemerin production and secretion. Chemerin and its receptors are highly expressed in the liver, suggesting that the liver is an important target for chemerin signaling [34, 35].

In our cohort of biopsy-proven NAFLD patients (chapter 3.2.), serum chemerin levels were higher compared to healthy controls but were not different among the three groups of NAFLD patients and were not related to disease severity. Chemerin VAT expression in male NAFLD patients however, was reduced and inversely associated with overall NAS and hepatic steatosis independent of age, BMI and HOMA-IR. Moreover, the association between HOMA-IR and NAS lost significance after correcting for chemerin VAT expression, suggesting that chemerin may at least partly influence the link between HOMA-IR and NAFLD severity. Recently, Wolfs *et al.* similarly reported a negative association between chemerin VAT expression and histopathological parameters of biopsy-proven NAFLD patients. In their study, chemerin expression was negatively associated with steatosis, lobular and portal inflammation, independent of obesity, HOMA-IR and type 2 diabetes [36]. Experimental studies have reported more inconsistent results. While a mouse model with diminished chemerin function showed no differences in IR and NAFLD compared to wild type mice [37], anti-inflammatory effects of chemerin have been showed both *in vivo* and *in vitro* [38, 39]. In conjunction with the negative association between VAT chemerin expression and NAS, these results suggest that lower VAT expression of chemerin in obese patients may be involved in the pathophysiology of NAFLD progression.

The contribution of hepatic chemerin expression to NAFLD has been investigated by other groups, yielding conflicting results. Deng *et al.* reported lower hepatic chemerin expression in three different mouse models of NAFLD and human liver samples of NAFLD patients compared with controls, which was independent of obesity and IR [40]. In contrast, Döcke *et al.* reported higher hepatic chemerin expression in patients with NASH versus borderline or no NASH. They found a positive association between hepatic chemerin expression and grade of steatosis, lobular inflammation, hepatocellular ballooning and even fibrosis, all were independent of sex, age, BMI, alcohol consumption and oral

antidiabetics [41]. We did not measure hepatic expression of adipokines in our study cohort, but it would be interesting in future studies to measure both hepatic and VAT expression of chemerin and compare the results with hepatic protein measurements to determine the contribution of both sites. This could be of importance for the development of specifically targeted therapeutic strategies, once more evidence has proven the exact role of chemerin in NAFLD pathophysiology.

1.4. Low testosterone levels and adipose tissue dysfunction in obese men

As discussed, the relationship between decreased T levels and male obesity is still incompletely understood. In chapter IV, we aimed to explore potential determinants of TT and FT levels in a cohort of morbidly obese men with and without type 2 diabetes as compared to men with normal body weight. More specifically, we evaluated the potential influence of adipose tissue dysfunction, metabolic parameters and SAT aromatase expression on parameters of the HPG axis. Differences in *in situ* aromatase expression, both in abdominal SAT and VAT, could not be found between obese men (with or without type 2 diabetes) and normal-weight men. This corroborates other findings rejecting the increased aromatase activity hypothesis to explain low T levels in obese men. For instance, lower E_2 levels have been reported repeatedly in obese men with or without type 2, arguing against downregulation of the HPG axis by elevated E_2 as a main cause for low T levels in those men [42, 43]. Furthermore, a recent RCT with low-dose administration of aromatase inhibition compared to placebo in obese men, showed no beneficial effects on clinical endpoints such as body composition, glucose and lipid metabolism [44].

In our study population however, abdominal SAT cell size was inversely associated with both TT and FT levels after multivariate regression analysis corrected for age, group (control versus obesity/diabetes), HOMA-IR, and TG levels. Adipocyte cell size has been recognized for many years as an important marker in the pathogenesis of metabolic derangement. Enlarged subcutaneous adipocytes have been shown to predict type 2 diabetes in a prospective cohort, independent from clamp-measured insulin sensitivity [45, 46]. It has been shown that severely obese though insulin-sensitive individuals had a smaller adipocyte size compared to an equally obese but insulin-resistant group matched for age, sex and body fat, again suggesting mechanisms beyond obesity *per se* determine the pathological metabolic consequences in obesity [47]. Furthermore, adipocyte cell size has been associated with low grade systemic inflammation and macrophage accumulation in adipose tissue [48, 49]. Our data emphasize the importance of enlarged SAT cell size and related metabolic changes as a potential determinant of low T

levels in obese men. Two years after bariatric surgery, we observed lower BMI, TG and HOMA-IR, and higher T, SHBG and FSH values in part of our study population. However, we found no changes in FT and LH levels, suggesting that mostly changes in SHBG are responsible for the increase in TT whereas the HPG axis has still not fully recovered.

1.5. A potential role for adipokines in the disturbed sex steroids profile?

As previously described (section 1.4.) our results suggest a direct negative impact of adipocyte cell size, but not IR, on TT and FT levels in obese men. This highlights the potential role of adipose tissue dysfunction in the disturbed sex steroid profile of obese men, for instance via an impaired adipokine secretion pattern. Indeed, the influence of adipokines on reproductive hormones and function has been repeatedly reported [50-52]. A potential explanation of low (free) T levels could thus be a pro-inflammatory status in testes secondary to a deregulated adipokine secretion pattern, as enlarged adipocytes have been related to a systemic inflammatory state [48, 53]. Some inflammatory mediators such as the adipokines TNF- α and leptin have been shown to negatively influence HCG-induced T secretion from Leydig cells as well as to disturb Sertoli cell function [50, 51, 54]. Interestingly, a recent study reported the suppression of HCG-induced T secretion in primary Leydig cells after the addition of chemerin, suggesting a potential role for chemerin in regulation male reproduction [55]. Larger human studies are needed to confirm those findings, which may ultimately help in the production process of therapeutics that could combat against the high prevalence of obese subjects with impaired sex steroid levels and the associated discomforts.

2. Clinical relevance

Presence of NAFLD is mostly asymptomatic and is often diagnosed by incident because of abnormal liver function tests or steatosis on imaging. As mentioned before, the assessment of NAFLD diagnosis by liver biopsy is the most reliable approach but is too invasive to implicate in clinical practice. Other currently available diagnosing measurements like liver function parameters (serum transferase levels) and imaging techniques (CT and MRI) do not reliably assess hepatic steatosis, steatohepatitis and fibrosis in NAFLD patients [7]. Therefore, there has been major interest in developing predictive non-invasive biomarkers for identifying NAFLD and NASH patients, although firstly a more thorough knowledge on the pathophysiology is needed.

It is generally known that presence of NAFLD may exacerbate metabolic status. Indeed, metabolites of FA and TG such as DAG and ceramide accumulate in hepatic steatosis, which in turn may impair insulin signaling by inhibiting insulin signal transduction and increase hepatic glucose production [56]. Furthermore, FA can induce inflammatory pathways by activating NF κ B, JNK and oxidative as well as ER stress [57, 58]. However, recent data has revealed that in certain conditions NAFLD is not accompanied by these adverse events and is characterized by a metabolically benign state, preventing progression of disease severity. One example potentially explaining this is an elevated hepatic TG synthesis, allowing storage of lipids in their least toxic form [59]. Mechanisms on how these compensatory events occur in certain subjects are of utmost importance to identify people at risk of developing disease progression to NASH (with or without fibrosis), cirrhosis or even HCC.

Adipokines are known to be involved in the regulation of lipid metabolism, inflammation, glucose homeostasis and insulin signaling, which makes them interesting therapeutic targets for metabolic diseases including NAFLD [60, 61]. Some mouse models have already shown beneficial effects of administrated adipokines. For example, the administration of adiponectin to a mouse model of NAFLD and alcoholic fatty liver disease, attenuated hepatic steatosis and inflammation by enhancing hepatic FA oxidation and decreasing FA synthesis and cytokine production [62]. Although the specific modulation of adipokines has not yet been investigated in human clinical trials, evidence suggests that some commonly used therapeutic agents for obesity act at least in part via the activation or inhibition of adipokines. Pioglitazone for example, exerts its insulin-sensitizing effects partly through the up-regulation of adiponectin [63]. In this thesis we found an inverse association between VAT expression of chemerin as well as serum adiponectin levels on one hand and NAFLD severity on the other hand.

However, further investigation, comprising both experimental and human trials on a larger scale, is needed to prove if therapies directed at chemerin would beneficiate in patients with NAFLD.

3. Limitations and perspectives

The findings in this thesis have provided additional evidence on the high impact of adipose tissue dysfunction in obesity, suggesting a role in the development of NAFLD and the impaired sex steroid profile of obese men. However, the work presented in this thesis has some limitations. Firstly, we were unable to draw causative conclusions because of the cross-sectional design of our clinical trials.

Secondly, it is important to recognize that beside adipose tissue-derived adipokines, other tissues such as skeletal muscle and liver can release similar proteins with direct effects on several targets such as insulin signaling and inflammation. As an example, it is suggested that a fatty liver may secrete higher levels of certain factors, called hepatokines. One example is fetuin-A, which has been shown to inhibit insulin signaling and to stimulate inflammatory cytokine expression in immune cells [64, 65]. As these products of skeletal muscle (named myokines) and liver cells are also secreted into systemic circulation, one must take into account that measuring adipokines in serum does not necessarily reflect the adipose tissue-derived adipokines. Furthermore, as the majority of blood flow ($\pm 80\%$) to the liver is delivered via the portal vein that drains VAT directly, it is likely that systemic serum concentrations of adipokines do not accurately reflect the hepatic delivery of adipokines. For instance, IL-6 concentrations in the portal vein of morbidly obese individuals were found to be 50% higher than their peripheral artery [66]. Furthermore, secretion of adipokines is suggested to be different between SAT and VAT, at least partly clarifying divergent results between serum levels and VAT expression as observed in our and other studies [27, 67].

Both NAFLD and type 2 diabetes are multifactorial diseases, of which the etiology is complex and which are closely linked to each other. Although both conditions are clearly driven by obesity, not every obese subject develops NAFLD and/or type 2 diabetes and some of them even remain metabolically healthy [68]. Because of these complex multifactorial origins, many studies investigating the etiology of NAFLD and type 2 diabetes, including those within the scope of this thesis, are often restricted to studying specific aspects of the disease [69]. This allows us to identify the potential contribution of single factors and give insight into specific mechanisms involved, but interpreting those results should keep in mind the whole context of the complex disease that may involve the interplay of several mechanisms.

Although our study population did not allow us to explore this, it is generally known that gender is associated with the prevalence of NAFLD. While at first it was believed that NAFLD was more common in women, evidence from population-based studies now shows that male gender is associated with a higher prevalence of NAFLD [70]. Further, several cross-sectional population-based studies showed a

direct relationship between low TT levels and NAFLD independent of confounding factors such as BMI, VAT insulin resistance and dyslipidemia [71, 72]. In contrast, others reported inverse associations between SHBG but not TT or FT levels and NAFLD severity in patients with type 2 diabetes [73, 74]. Monosaccharide-induced *de novo* lipogenesis inhibited hepatic SHBG expression both *in vitro* and *in vivo* in human hepatocytes, potentially explaining the link between low SHBG and TT levels and NAFLD [75]. However, Seo *et al.* reported after a median follow-up of four years that serum TT levels did not independently associate with the development or regression of NAFLD, whereas obesity and metabolic parameters (i.e. BMI, WC, glucose, HDL cholesterol, TG, systolic blood pressure) did [76]. Furthermore, E₂ levels were suggested to be lower in men with NAFLD versus no NAFLD, which may promote oxidative stress and lipid peroxidation in the liver [77]. It is important to notice that the above mentioned studies measured TT levels using radioimmunoassays, whereas presence of liver fat content and NAFLD was determined by imaging. The LC-MS/MS method is currently the state of the art technique for measuring T and E₂ and should be encouraged in future research to overcome the lack of both specificity and sensitivity of the still widely used radioimmunoassays. The potential link between sex steroids and NAFLD should be further investigated in larger-scaled studies using LC-MS/MS methods in patients with biopsy-proven NAFLD diagnosis. To this end, we plan to expand the Hepobster study by measuring sex steroid levels in serum samples of biopsy-proven NAFLD patients using LC-MS/MS, both at the time of bariatric surgery as well as during a follow-up period.

Up till now, we and others were not able to identify any adipokine of which circulating levels are clearly linked with NAFLD severity and that could be a potential biomarker. Therefore, larger-scaled studies are needed to examine adipokines prospectively, evaluating them when NAFLD progresses or resolves via paired liver biopsies. Furthermore, as a defined “normal range” for adipokine serum concentrations has not yet been established and the range of measurement assays used across different studies is very divergent, their potential usage as a disease biomarker is still questionable. However, once it has been clearly elucidated that adipokines are key players in the pathogenesis and progression of NAFLD as well as in the impaired sex steroid profile of obese subjects, the pharmacologic manipulation of adipokines could be a feasible option in future therapeutics of obesity and the use of adipokines as part of a noninvasive diagnostic tool for NAFLD could be of interest.

In order to further elucidate the potential role of adipokines in the pathophysiology of NAFLD, the inclusion of patients in the Hepobster study cohort will be continued to maximize statistical power on additional analyses. Furthermore, a follow-up study is currently being set-up in collaboration with the

department of Gastroenterology and Hepatology and the department of Pathology, both at Ghent University Hospital. While the baseline study protocol will be similar to the Hepobster study, patients will additionally be investigated at several timepoints after bariatric surgery. This allows to examine the progression of NAFLD on liver biopsy and the progression of adipose tissue (dys)function and metabolic parameters simultaneously. Adipokine levels will be measured in serum and their expression will be assessed in VAT, SAT and liver.

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Marlies Bekaert was born on August 14th, 1988. After finishing her high-school education in 2006, she started her studies in Biomedical Sciences at the Ghent University and obtained a Master degree in 2011 (with distinction). In September 2011, she started as a PhD student at the Department of Endocrinology in Ghent University Hospital to perform research on the involvement of adipose tissue in pathophysiology of metabolic comorbidities in obesity. From November 2015 onwards, she started working at the clinical Research Center of Ghent University Hospital, Bimetra, as an advisor in pediatric clinical trials.

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Awards

Ipsen “Perspectives in Endocrinology” 2014 poster award, 3th winner; Brussels, Belgium

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