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VASCULAR ENDOTHELIAL GROWTH FACTOR B - ROLE IN METABOLISM, LIPOTOXICITY AND DISEASE

Annika Mehlem

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VASCULAR ENDOTHELIAL GROWTH FACTOR B - ROLE IN METABOLISM, LIPOTOXICITY AND DISEASE

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Vascular Endothelial Growth Factor B (VEGF-B) är ett tillväxtprotein som styr mängden fett som transporteras genom blodkärlsväggen till celler, till exempel muskelceller. Genom att genmanipulera möss kan mängden VEGF-B minskas, vilket reducerar den mängd fett som transporteras igenom blodkärlsväggen och in i cellen.

I **delarbete I**, undersöker vi hur VEGF-B regleras. Vi kan visa, både genom analyser i celler och i möss, att mängden VEGF-B styrs av ett protein känt som peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). PGC-1α reglerar också antalet mitokondrier som finns i cellen. I mitokondrierna används de fetter som cellen tar upp för att utvinna energi. Eftersom PGC-1α också reglerar mängden VEGF-B, koordineras antalet mitokondrier med mängden fett som transporteras in i cellen, och därmed undviks sjuklig fettansamling.

Patienter med Typ 2 Diabetes (T2D) har mycket högre mängder fett i centrala organ såsom hjärta, muskler och lever, jämfört med friska individer. I **delarbete II** ville vi undersöka om det felplacerade fettet kunde vara orsaken till att man utvecklar T2D. Vi kan i flera olika experimentella djurmodeller av T2D visa att om vi minskar mängden VEGF-B, genetiskt eller genom att använda en läkemedelskandidat, så reduceras även mängden fett i de centrala organen. Detta leder till att de djurmodeller som har mindre VEGF-B har en förbättrad sjukdomsutveckling. Därför skulle en läkemedelskandidat mot VEGF-B kunna erbjuda en lovande behandling för patienter med T2D.

Diabetes är kopplat till ett flertal följdsjukdomar och diabetisk njursjukdom, även kallat diabetisk nefropati (DN), är en av dessa. I patienter med DN, har man kunnat observera höga mängder fett i njurarna. Vi ville därför i **delarbete III** studera om man genom att minska mängden fett i njurarna, genom att reducera mängden VEGF-B, kunde hindra eller förbättra sjukdomsutvecklingen. I flera musmodeller av DN kan vi visa att fett ansamlas i njurarna. Om man minskar mängden VEGF-B är fettansamlingen i njurarna kraftigt reducerat och dessa möss har även en mildare sjukdomsutveckling samt en bättre njurfunktion. Vidare visar vi även att patienter med DN har högre mängder VEGF-B i njurarna än friska individer. Att reducera mängden fett i njurarna, via VEGF-B antagonism, skulle därför kunna vara en möjlig behandling mot DN.

Slutligen, i **delarbete IV**, har vi optimerat en metod som gör att man kan mäta mängden fett som finns i vävnaden. Denna metod har möjliggjort en noggrann kvantifiering av hur mycket fett som finns inlagrat, och har därför varit ovärderlig för samtliga delarbeten som diskuterats ovan.

I denna avhandling föreslår vi sammanfattningsvis att en ökad inlagring av fett i centrala organ som muskel, hjärta och njure kan leda till T2D och DN. Genom att minska mängden VEGF-B, och därigenom fettansamlingen, kan vi bromsa utvecklingen av båda sjukdomarna. Därför anser vi att reduktion av mängden fett via minskad VEGF-B signallering, skulle kunna vara en ny lovande behandlingsmetod för patienter med T2D och DN.

ABSTRACT

Vascular Endothelial Growth Factor B (VEGF-B) was previously shown to control lipid uptake from the bloodstream via the endothelium into tissue cells, and when ablating *Vegfb,* intra-tissue lipid accumulation was reduced. However, very little is known regarding the metabolic role of VEGF-B in physiologic, or pathophysiologic conditions.

In **paper I**, we characterized the upstream regulatory mechanism controlling VEGF-B expression. We showed *in vitro* and *in vivo* that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a major regulator of mitochondrial biogenesis, controls the expression of VEGF-B. The tight regulation of VEGF-B via PGC-1α enables the coordination of lipid uptake with mitochondrial biogenesis and β-oxidation, and hence the prevention of lipotoxicity.

Lipotoxicity and insulin resistance are suggested as key pathologies in type 2 diabetes (T2D). In **paper II** we analysed the effects of reduced VEGF-B signalling on lipotoxicty and disease progression in different rodent models of T2D. VEGF-B signalling was reduced by either genetic, or pharmaceutical means, and this reduced lipotoxicity, increased glucose uptake into peripheral tissues, improved dyslipidaemia and enhanced sensitivity to insulin, in rodent models of T2D. Therefore, targeting VEGF-B signalling is a promising therapeutic method for the treatment of T2D.

Lipotoxicity has also lately been attributed a larger role in the pathogenesis of diabetic nephropathy (DN), a comorbidity of both type 1 diabetes (T1D) and T2D. Therefore, **in paper III**, we investigated the effects of reducing VEGF-B signalling in mouse models of DN. We showed that renal lipotoxicity was an important element of DN in these models, and by reducing VEGF-B signalling renal lipotoxicity was ameliorated. Also, renal function, morphology and the filtration capacity were enhanced. Furthermore, VEGF-B signalling was present and activated in patients with DN in comparison to healthy individuals. Thus, targeting VEGF-B signalling represents a novel therapeutic approach for DN.

Finally, **in paper IV**, a protocol for detecting and imaging of intra-tissue neutral lipids is presented. This protocol enables the exact quantification of neutral lipids and was crucial for all papers discussed above.

To conclude, our data show that lipotoxicity is a major driving force for the development and progression of T2D and DN. Hence, VEGF-B could be a novel target for the treatment of both T2D and DN.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals

I. PGC-1α coordinates mitochondrial respiratory capacity and muscular fatty acid uptake via regulation of VEGF-B
Annika Mehlem, Isolde Palombo, Xun Wang, Carolina E Hagberg, Ulf Eriksson and Annelie Falkevall
Submitted manuscript

II. Targeting VEGF-B as a novel treatment for insulin resistance and type 2 diabetes
Hagberg CE*, **Mehlem A***, Falkevall A, Muhl L, Fam BC, Ortsäter H, Scotney P, Nyqvist D, Samén E, Lu L, Stone-Elander S, Proietto J, Andrikopoulos S, Sjöholm A, Nash A, Eriksson U.
Nature. 2012 **490**:426-30
**These authors contributed equally to this work*

Reducing VEGF-B signalling ameliorates renal lipotoxicity and protects against diabetic nephropathy
Annelie Falkevall*, **Annika Mehlem***, Isolde Palombo, Benjamin Heller-Sahlgren, Lwaki Ebarasi, Liqun He, Jimmy Ytterberg, Jaakko Patrakka, Pierre Scotney, Andrew Nash and Ulf Eriksson
Submitted manuscript
**These authors contributed equally to this work*

III. Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease.
Mehlem A, Hagberg CE, Muhl L, Eriksson U, Falkevall A.
Nature Protocols. 2013 **6**:1149-54

Additional publications not discussed within this thesis

Gpr116 Receptor Regulates Distinctive Functions in Pneumocytes and Vascular Endothelium.
Niaudet C, Hofmann JJ, Mäe MA, Jung B, Gaengel K, Vanlandewijck M, Ekvärn E, Salvado MD, **Mehlem A**, Al Sayegh S, He L, Lebouvier T, Castro-Freire M, Katayama K, Hultenby K, Moessinger C, Tannenberg P, Cunha S, Pietras K, Laviña B, Hong J, Berg T, Betsholtz C.
PLoS One. 2015 **9**:e0137949

Endothelial fatty acid transport: role of vascular endothelial growth factor B.
Hagberg C*, **Mehlem A***, Falkevall A, Muhl L, Eriksson U.
Physiology (Bethesda). 2013 **2**:125-34 Review.

EGF-R regulates MMP function in fibroblasts through MAPK and AP-1 pathways.
Kajanne R, Miettinen P, **Mehlem A**, Leivonen SK, Birrer M, Foschi M, Kähäri VM, Leppä S.
J Cell Physiol. 2007 **2**:489-97.

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LIST OF ABBREVIATIONS

AGEs	Advanced Glycation End-products
AS160/ TBC1D4	TBC1 domain family, member 4
AT	Adipose Tissue
CD2AP	CD2-associated protein
DN	Diabetic Nephropathy
EC(s)	Endothelial Cell(s)
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ESRD	End-Stage Renal Disease
ESRRα	Estrogen-related Receptor alpha
FATP3/4	Solute carrier family 27 (fatty acid transporter), member ¾
GBM	Glomerular Basement Membrane
GFB	Glomerular Filtration Barrier
GLUT4	Glucose Transporter 4
GME	Glomerular Mesangial Expansion
HFD	High-Fat Diet
IMCL	Intramyocellular Lipid
IRS	Insulin Receptor Substrate
LD(s)	Lipid Droplet(s)
(LC)FA(s)	(Long Chain) Fatty Acid(s)
MAPK	Mitogen-activated protein kinases
NRF1	Nuclear Respiratory Factor 1
NRP11	Neurophilin 1
ORO	Oil-Red O
PDK1	3-phosphoinositide dependent protein kinase-1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PlGF	Placental Growth Factor
PPARg/a	Peroxisome Proliferator-activated Receptor gamma/alpha
Ppargc1a / PGC-1α	Peroxisome Proliferator-activated Receptor γ Coactivator 1α
RAAS	Renin-Angiotensin-Aldosterone System
ROS	Reactive Oxygen Species
STZ	Streptozotocin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TG(s)	Triglyceride(s)
TGF- β	Transforming Growth Factor β
TNF-α	Tumour Necrosis Factor alpha
UPR	Unfolded Protein Response
VEGF(R)	Vascular Endothelial Growth Factor (Receptors)
WT	Wild-Type

1 INTRODUCTION

Diabetes mellitus is a disease characterized by elevated blood sugar levels, referred to as hyperglycaemia. There are two major types of diabetes, type 1 and type 2 diabetes (T1D and T2D). Today, 387 million individuals are estimated to live with diabetes, although the prevalence is expected to increase with an additional 205 million diabetic individuals by 2035[1]. Diabetes is no longer a Western disease as, 77% of diabetic individuals today live in low- or middle-income countries[1]. Both types of diabetes increase the risk of long-term complications such as, cardiovascular disease, stroke, diabetic nephropathy (DN), diabetic retinopathy, diabetic foot ulcers, cancer and cognitive defects. Therefore, to find new therapeutic targets to treat these complications is of vital importance. In this thesis, we show that vascular endothelial growth factor B (VEGF-B) could be a target for the treatment of diabetes and DN. By reducing the signalling of VEGF-B, ectopic uptake of lipids into tissues was decreased. We also elucidate the regulation of VEGF-B expression via peroxisome proliferator-activated receptor γ coactivator 1α (*Ppargc1a* / PGC-1α). This introductory part aims to give a general overview of T2D, T1D and DN and the underlying mechanisms of these diseases. Also, a brief summary of the current research regarding VEGF-B and PGC-1α will be presented.

1.1 DIABETES

1.1.1 Type 2 Diabetes

Ninety per cent of all subjects with diabetes suffer from the insulin-independent T2D. Major risk factors of the disease include genetic predisposition, obesity, high caloric diets, systemic hyperlipidaemia and physical inactivity. T2D is characterized by a state called insulin resistance, during which the cells of the body are not sensitive to insulin. Insulin signalling has numerous actions, all promoting the storage of dietary nutrients (Fig. 1a). In the insulin sensitive state, dietary glucose promotes insulin secretion from the pancreatic β-cells. In skeletal muscle, insulin increases glucose uptake via binding to the insulin receptor, which subsequently translocates glucose transporter 4 (GLUT4) to the plasma membrane (Fig. 2). In the liver, insulin promotes glycogen synthesis and *de novo* lipogenesis while gluconeogenesis is inhibited. In the adipose tissue (AT), insulin supresses lipolysis and promotes lipogenesis (Fig. 1a) [2].

In the insulin resistant state, many key actions of insulin are reversed with large consequences for whole body metabolic homeostasis (Fig. 1b). Insulin resistance reduces muscular glucose uptake and instead glucose accumulates in the bloodstream[3]. Hyperglycaemia is counteracted by an increase in the secretion of insulin from the pancreatic β-cells [4,5] and consequently islet hyperplasia develops [4,6]. In parallel, islet hyperplasia causes an increase in the production of glucagon from the pancreatic α-cells. In the liver, this together with hepatic insulin resistance, results in elevated glucose production (gluconeogenesis) contributing to hyperglycaemia[6]. If this state is left untreated, β-cell exhaustion and apoptosis will develop and ultimately insulin production will be lost. Insulin resistance in the adipocytes results in lack of insulin-mediated inhibition of lipolysis, thus fatty acids (FAs) are released and accumulate in peripheral tissues[7,8]. Atypical lipid accumulation is also promoted by increased *de novo* lipogenesis in the liver and by upregulation of muscular lipid uptake[9,10].

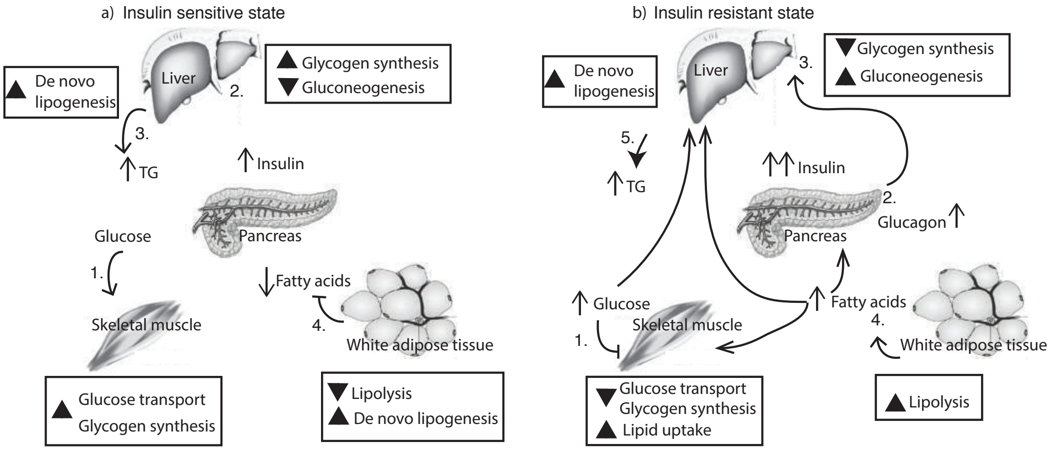
a) Insulin sensitive state
De novo lipogenesis
Liver
2.
Glycogen synthesis
Gluconeogenesis
3.
↑TG
↑Insulin
Glucose
Pancreas
1.
↓Fatty acids
Skeletal muscle
4.
White adipose tissue
Glucose transport
Glycogen synthesis
Lipolysis
De novo lipogenesis
b) Insulin resistant state
De novo lipogenesis
Liver
3.
Glycogen synthesis
Gluconeogenesis
5.
↑TG
↑↑Insulin
2.
Glucagon↑
Pancreas
↑Glucose
Skeletal muscle
↑Fatty acids
4.
1.
White adipose tissue
Glucose transport
Glycogen synthesis
Lipid uptake
Lipolysis

Fig 1. Schematic overview of key events in insulin sensitive and insulin resistant states
a)*: In the insulin sensitive fed state, dietary glucose promotes insulin secretion that 1. increases muscular glucose uptake, 2. promotes glycogen synthesis and inhibits gluconeogenesis, 3. increases hepatic de novo lipogenesis, 4. supresses lipolysis and promotes lipogenesis in AT.* ***b)*** *In the insulin resistant fed state 1. muscular glucose uptake is decreased 2.elevating both insulin and glucagon secretion. 3. Hepatic gluconeogenesis is elevated and glycogen synthesis is reduced and 4. lipolysis is increased in AT. GLUT4, glucose transporter 4, TG, triglycerides, AT; adipose tissue. Derived from* [11-14]

Hence, as diabetes proceeds, glucose, lipid species, glucagon and insulin accumulate in the bloodstream. However, the exact order in which these events occur and their interconnected importance is still debated.

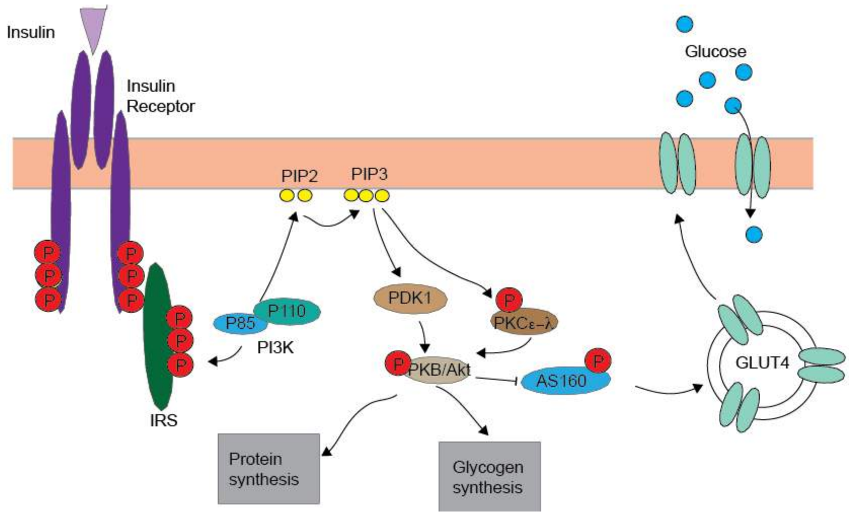
Insulin
Insulin Receptor
Glucose
PIP2
PIP3
P
P
P
P
P
P
P
P
P
P110
P85
PI3K
PDK1
P
PKCε-λ
P
PKB/Akt
AS160
P
GLUT4
IRS
Protein synthesis
Glycogen synthesis

Fig 2. Schematic overview of insulin signalling and glucose transport
Insulin binds to the insulin receptor, leading to phosphorylation of IRS, that in turn recruits PI3K. The P110 subunit is activated, producing the lipid second messenger PIP3 from PIP2. PIP3 phosphorylates PH-domain containing proteins, PDK1, PKCξ-λ and PKB. PKB phosphorylation inhibits AS160, which relieves its inhibitory effect on GLUT4, triggering translocation to the plasma membrane. PKB phosphorylation also activates pathways involved in protein and glycogen synthesis.
MAPK; Mitogen-activated protein kinases, IRS; Insulin Receptor Substrate, PI3K; Phosphatidylinositol-4,5-bisphosphate 3-kinase PIP3; Phosphatidylinositol (3,4,5)-trisphosphate, PIP2; Phosphatidylinositol 4,5-bisphosphate, PDK1; 3-phosphoinositide dependent protein kinase-1, PKC; Protein kinase C, PKB; Protein kinase B, AS160/ TBC1D4; TBC1 domain family, member 4.

Traditionally, it has been assumed that insulin resistance primarily, and only, develops in cells with established functions in glucose handling, such as myocytes, adipocytes and hepatocytes[4]. However, data has shown that insulin resistance also develops in other cell types such as podocytes[15] and endothelial cells (ECs)[7]. Insulin resistance in ECs even precedes that in myocytes[16,17,7] which was supported by a study, where IRS2 was ablated specifically in ECs in mice. These mice displayed reduced muscular insulin delivery, capillary recruitment and insulin-induced endothelial nitric oxide synthase (eNOS) phosphorylation[18].

1.1.1.1 Mechanisms of insulin resistance

Several different mechanisms underlying insulin resistance in T2D have been suggested, the two best studied are hyperglycaemia (glucotoxicity) and lipotoxicity.

1.1.1.1.1 Glucotoxicity

Hyperglycaemia impairs both the action of and the secretion of insulin, and has been regarded as the major pathology causing T2D. Today, most of the T2D drugs on the market act to reduce glucose levels by different molecular mechanisms. A vast amount of studies have been published regarding how the insulin signalling pathway is affected by hyperglycaemia, often focusing on how insulin release from the pancreatic islets is altered.

Chronic elevation of plasma glucose levels increased the generation of reactive oxygen species (ROS) that impaired both insulin secretion and action[19]. In line with this, levels of markers for oxidative stress were increased in pancreatic islets from T2D subjects compared to islets from non-diabetic subjects[20]. Moreover, the intracellular concentration of these oxidative stress markers was inversely correlated to glucose-stimulated insulin release from β-cells[20,21]. Glucotoxicity could also induce the activation of the unfolded protein response (UPR), also termed endoplasmic reticulum (ER) stress in pancreatic β-cells and in hepatocytes[22,23]. ER stress have been shown to contribute to the development of hepatic insulin resistance via activation of enzymes involved in gluconeogenesis, lipogenesis and kinases involved in the UPR pathway[10]. Furthermore, hyperglycaemia induced ER-stress in islets from T2D subjects, but not in islets from non-diabetic subjects[22]. Finally, chronic hyperglycaemia affected insulin secretion and insulin resistance by increasing flux through the hexose biosynthetic pathway[24] thus inactivating Akt2 mediated GLUT4 translocation[25,26]. However, a direct causal relationship between hexose biosynthetic pathway and insulin resistance has not yet been established[27].

Large cohort studies have been conducted to explore whether intensive management of blood glucose levels using anti-diabetic agents, could reduce the risk for diabetes-related deaths and comorbidities. The ACCORD consortium study included 10,000 patients with T2D with an elevated risk for cardiovascular disease, and patients were randomly assigned to intensive therapy (HbA_{1c} ≤6.0%), or standard therapy (HbA_{1c} 7.0–7.9%). Surprisingly in the intensive group in which blood glucose levels were normalized, no significant difference in the primary end points (nonfatal myocardial infarction, nonfatal stroke, or death from cardiovascular causes) were detected. All-cause mortality was even 22% higher, in the intensive therapy group[28]. In a more recent ACCORD trial with a similar setup, an increased risk of cardiovascular events and mortality was found in younger participants in the intensive therapy group, whereas no effect was found in older participants[29].

Taken together, even though the T2D agents available today are efficient in lowering blood glucose levels, even to normoglycaemic levels, they do not prevent diabetes-related complications. This implies that other mechanisms, perhaps more significant than glucotoxicity, may contribute to diabetes and its complications.

1.1.1.1.2 Lipotoxicity

Considering that T2D typically develops in association with weight gain, prolonged physical inactivity, and/or systemic hyperlipidaemia, it is seemingly intuitive that insulin resistance might be driven by an excess of lipids. Indeed, research has provided a strong causative relationship between insulin resistance, organ dysfunction and atypical storage of neutral lipids in tissues such as liver, heart, pancreas, and skeletal muscle[6,30-32]. Lipids stored outside the adipose tissue are the most dangerous ones[33,34] and individuals with more abdominal obesity are more susceptible to metabolic syndrome[35] due to lipid overflow to other organs. High intramyocellular lipid (IMCL) content in both humans and rodents were associated with insulin resistance[36,37] and was a stronger predictor of insulin resistance than circulating FAs[38]. Infusing lipids into cardiac and skeletal muscle in healthy subjects induced insulin resistance[39,40]. In line with this, weight loss decreased IMCL content together with an improved insulin sensitivity[41], and reversely, lipid infusion in combination with a high-fat diet (HFD) increased IMCL content and impaired insulin sensitivity in healthy subjects[42].

Why do lipids accumulate in the peripheral tissues in diabetic patients? A common observation from diabetic rodent models is that atypical lipid accumulation and insulin resistance are linked to the absence of functional adipocytes[34,43]. Restriction of the adipocyte expansion capacity in *db/db* mice prevented obesity but instead increased cardiac and liver lipid accumulation rendering the animals diabetic[34]. In contrast, when overexpressing adiponectin in diabetic mice, thus increasing adipocyte number, the mice became overtly obese but still maintained normoglycaemia[43]. Also, lipodystrophy patients characterized by progressive loss of AT, generally display metabolic disorders such as insulin resistance, T2D and hyperlipidaemia[44]. Studies have indicated that subcutaneous AT is the largest and least metabolically harmful storage site of excess fat[45]. Atypical fat accumulation may therefore be due to a limited ability of this tissue to recruit new adipose cells and thus retain a bulk of the lipids that have been ingested[46].

Several different lipid species have been shown to affect insulin signalling and/or insulin mediated glucose uptake in multiple organs (Fig. 2 and 3)[32]. Diacylglycerols from lipid droplets (LDs) activated PKCθ that altered the phosphorylation pattern of the IRS-1, leading to decreased insulin receptor signalling[47]. Ceramides, synthesised from esterification of FAs and sphingosines, can inactivate Akt2, consequently inhibiting the translocation of GLUT4 vesicles to the cell surface[47-49]. Impaired Akt2 activity also decreased insulin-mediated glycogen synthesis, which further contributed to the development of hyperglycaemia[50]. Obesity caused an inflammation in AT that may develop into a low-grade chronic inflammation in the whole body[51]. Increased expression of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα) have been detected in the AT in rodents and diabetic subjects with insulin resistance[52,53]. Increased TNFα signalling in AT promoted lipolysis via decreased expression of proteins stabilizing LD[54,55] causing lipid overload in peripheral tissues. Furthermore, elevated circulating levels of TNFα impaired insulin signalling via inactivation of IRS-1[56]. ER stress activated enzymes involved in *e.g.* lipogenesis[10] and altered cellular lipid balance in muscle and AT via accumulation of lipid intermediates, thus interfering with insulin signalling[23]. Finally, muscular mitochondrial content was lower in obese and T2D subjects[57]. Decreased β-oxidation would increase lipid accumulation and fuel the viscous cycle of lipotoxicty and impaired insulin signalling, but whether or not mitochondria have a causal role in insulin resistance is still debated.

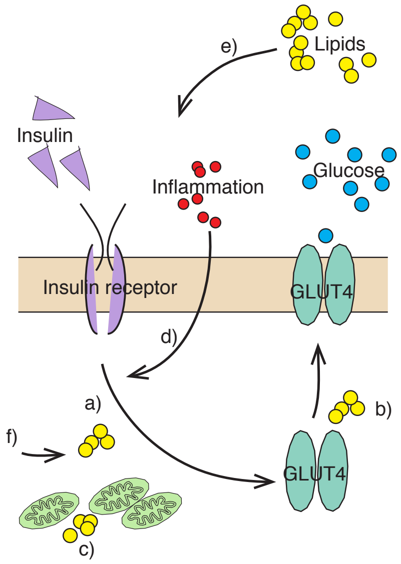

Fig 3. Overview of the mechanisms behind lipid-induced insulin resistance
Lipid-induced insulin resistance can occur via; a) Inhibition of the insulin receptor substrate and intracellular insulin signalling b) Trapping vesicle fusion proteins preventing GLUT4 translocation c) Mitochondrial dysfunction as well as d) inflammatory responses and e) lipid spill-over from adipose tissue. f) ER-stress activates lipogenesis, contributing to high IMCL levels.

Taken together, several lipid-mediated mechanisms in various organs have been shown to contribute to the pathogenesis of insulin resistance and T2D (Fig 3).

1.1.2 Type 1 Diabetes

T1D stems from a deficiency of insulin production caused by a destruction of β cells as a result of an autoimmune attack (advanced type 1 diabetes) and usually manifests before 10 years of age. However, there is a notable heterogeneity of the disease and several other variants of insulin deficiency occur in patients with *e.g.* pancreatitis and some monogenic forms of diabetes[58]. Traditionally, T1D and T2D have been considered as two different diseases, however today the borderline between these two is not as clear. Experimental evidences from both human subjects and experimental animal models suggest, that insulin resistance may also be present in T1D[59]. For unclear reasons, there is a 4% annual increase in the prevalence of T1D in European children[60,61] but similar trends are observed worldwide[62,63]. Children with both T1D and T2D, are increasingly more often observed in the clinic[64] as the rise in childhood obesity has resulted in elevated prevalence of T2D also in children[65,66].

Despite adequate or even higher insulin availability, supplied by insulin therapy, lower whole-body insulin sensitivity was detected in T1D patients. Other hallmarks of T2D have been found in T1D patients such as reduced insulin-stimulated glucose clearance[59] and abnormal mitochondrial function[67]. At the molecular level, lower expression levels of the insulin receptor and GLUT4 were found in lean[68] and obese T1D patients[69]. Thus, insulin resistance does not only occur in T2D but also in T1D subjects and the overall metabolic consequences seem to be similar, although more studies on this topic are required.

May lipotoxicity be the underlying mechanism of insulin resistance in T1D subjects as well, even though obesity is not a predictor, or a trait of the disease? Abnormal lipid accumulation in the liver[70] heart and muscle[59] has been observed in T1D subjects. Also, high IMCL content was associated with the development of insulin resistance in these patients[59]. Furthermore, T1D patients displayed adverse changes in HDL/chylomicron metabolism in response to multiple high-fat meals[71]. Additionally, insulin resistance was detected in AT in T1D patients[69] indicating an impaired insulin-mediated suppression of lipolysis[72]. T1D mice lacking perilipin 5 (Plin5-KO), an essential component that protects LDs from attack by lipases, did not exhibit excessive ROS generation or heart malfunction in contrast to T1D control mice. This was contributed to lower cardiac levels of diacylglycerol and ceramides detected in Plin5-KO compared to wild-type (WT) mice[73]. Hence, lipids may accumulate in liver and muscle also in T1D patients.

1.2 DIABETIC NEPHROPATHY

Currently, DN is the leading cause of chronic kidney disease and one of the major mechanisms underlying diabetes-related deaths. This section will cover the structure and function of the kidney, followed by a discussion of the pathological alterations occurring in DN.

1.2.1 Kidney anatomy and function

The kidney filters the blood by excreting waste products to the urine and allowing molecules that are to be re-used to re-enter into the bloodstream. Proper secretion and reabsorption ensures metabolic homeostasis and normal blood pressure. Blood that is to be filtered enters the kidney via the renal artery, and is then directed to smaller arterioles in the cortex, ultimately entering the nephrons, which are the filtration units of the kidney. The nephrons are composed of the renal corpuscle where the initial filtering occurs and the renal tubule specialized for reabsorptions and secretion. After passing through the renal tubule, the filtrated urine continues to the collecting duct system consisting of a series of tubules and ducts that finally connect the nephrons to the ureter (Fig 4)[74,75].

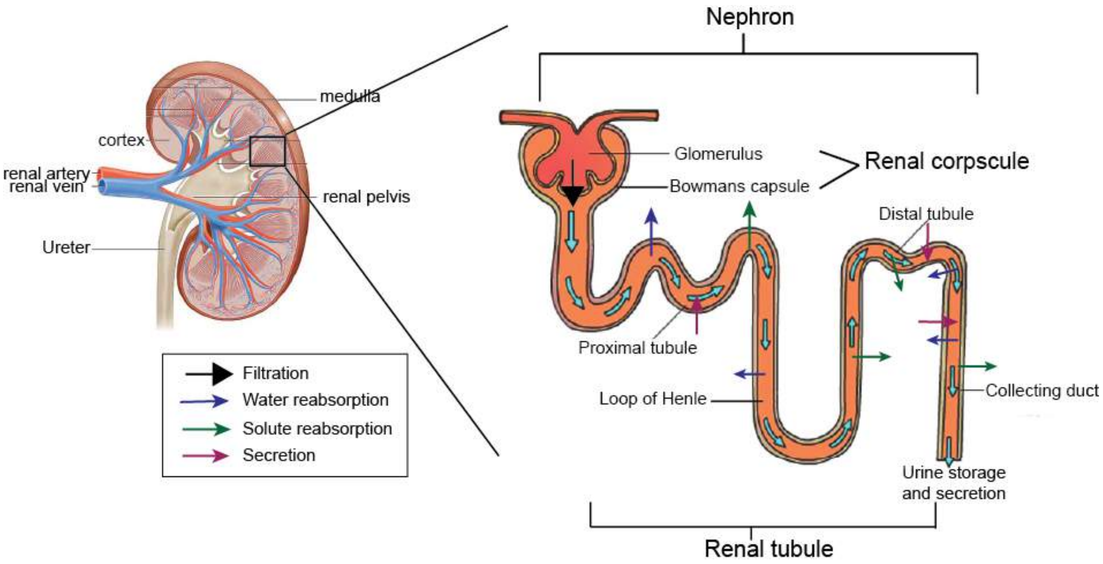
Nephron
medulla
cortex
renal artery
renal vein
renal pelvis
Ureter
Filtration
Water reabsorption
Solute reabsorption
Secretion
Glomerulus
Bowmans capsule
Renal corpscule
Distal tubule
Proximal tubule
Loop of Henle
Collecting duct
Urine storage and secretion
Renal tubule

Fig 4. Schematic overview of the kidney and the morphology of the nephron
The outer and inner part of the kidney are called the cortex and the medulla. Derived from [76]

1.2.1.1 Renal corpuscle

Renal corpuscles are situated in the beginning of the nephron and act as the initial filtering component. The renal corpuscle consists of the glomerulus and the surrounding Bowmans capsule. Both the nephron and Bowmans capsule contain several different cell types, which all in synchrony maintain a proper filtration barrier (Fig 4 and 5)[74,75].

Blood enters the glomerular capillaries via the afferent arteriole where an initial filtration occurs. This capillary network is referred to as the glomerular filtration barrier (GFB). Blood that has been filtered exits the glomeruli via the efferent arteriole and after returning from the inner medulla returns to the renal vein and the general circulation. A hydrostatic pressure created by the afferent and efferent arteriole exists in the glomerular capillaries allowing the ultrafiltration of metabolic waste products and other small molecules such as water, glucose, amino acids, urea and sodium. Larger molecules such as albumin, immunoglobulins and plasma transport proteins are largely retained in the blood. Molecules and waste products that have been filtered through the glomerular capillaries, depicted as the primary urine, end up in the space between the podocytes and the parietal epithelial cells that line the Bowmans capsule. Here is where the primary urine is collected before being led out to the renal tubule[74,75].

In detail, GFB is composed of ECs, glomerular basement membrane (GBM) and podocytes (Fig 5). Renal ECs are fenestrated and would thus allow for free passing of fluid, plasma solutes and proteins, but trap red blood cells[77]. However, electron microscope images have shown that negatively charged glycocalyx are bound to the luminal side of the endothelium, also covering the fenestrae[78]. Thus, macromolecules are retained in the blood by the ECs based both on charge and size[79]. The GBM consists of laminins, type IV collagen, nidogen and heparan sulfate proteoglycans, synthetized by ECs and podocytes[80]. GBM contributes to the filtration process based on size and charge but also constitutes the main structural support for the glomerular capillary wall[81]. Podocytes, that are specialized and differentiated cells, consist of a cell body, major processes and foot processes and have an essential role in maintaining the filtration barrier (Fig 5.). The podocyte foot processes interact with the neighbouring podocytes and are regulated by their actin cytoskeleton. The filtration slit constitutes the space between foot processes, and is bridged by the slit diaphragm[82]. The slit diaphragm is a structure composed of several different molecules that all have specific roles in maintaining the podocyte specific filtration barrier [82-84]. Apart from size exclusion, the exact filtration capacity of the slits is still debated.

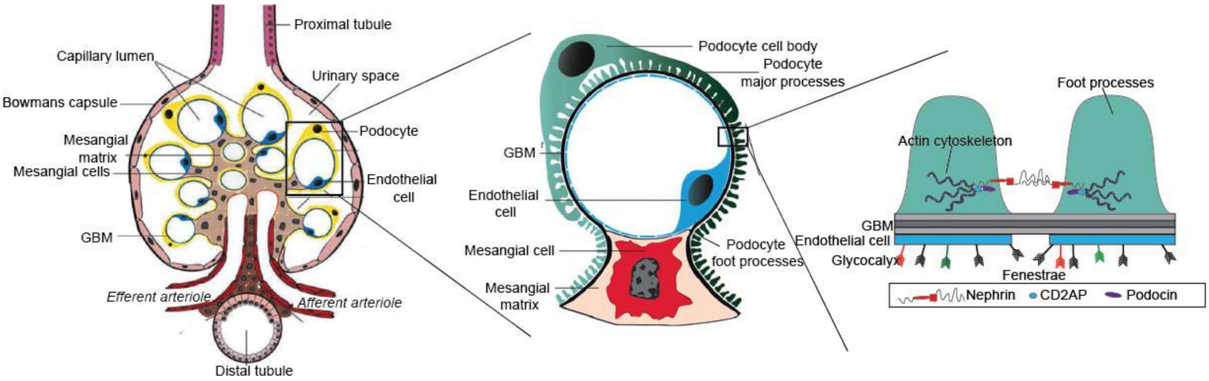
Proximal tubule
Capillary lumen
Urinary space
Bowmans capsule
Mesangial matrix
Mesangial cells
Podocyte
Endothelial cell
GBM
Efferent arteriole
Afferent arteriole
Distal tubule
Podocyte cell body
Podocyte major processes
GBM
Endothelial cell
Mesangial cell
Mesangial matrix
Podocyte foot processes
Foot processes
Actin cytoskeleton
GBM
Endothelial cell
Glycocalyx
Fenestrae
Nephrin
CD2AP
Podocin

Fig 5. Schematic overview of the renal corpuscle, renal capillaries and the glomerular filtration barrier
CD2AP ;CD2-associated protein. Derived from [76]

1.2.1.2 Renal tubule

The renal tubule (Fig. 4) is the latter part of the nephron and holds the primary urine that has been filtered through the glomerulus. The primary urine passes through the proximal convoluted tubule that constitutes of an epithelial cell layer connected by tight junctions. Here glucose and about 70% of the sodium and water are reabsorbed from the primary urine. Next in the loop of Henle, a U-shaped tubule, sodium and the remaining water are reabsorbed.

Finally, in the distal convoluted tubule, sodium is reabsorbed through coupled secretion of protons and potassium ATP-dependent ion channels[74,75].

1.2.2 Pathology of diabetic nephropathy

DN is caused by impaired filtration capacity of the kidney, and as a consequence larger molecules, like proteins, will leak into the urine[85]. Both genetic and environmental factors contribute to the development of DN. The disease slowly progresses over several years, and during the later stages there is a subsequent decline in the glomerular filtration rate that may ultimately lead to end-stage renal disease (ESRD)[86]. With the global increase in the prevalence of diabetes, the number of renal replacement therapy patients is also rapidly rising[87,88].

The major histopathological characteristic of DN is the presence of glomerular lesions referred to as glomerusclerosis[89]. A clinical indication of an injury to the GFB is the presence of albuminuria[85], however among the first histological signs of DN is thickening of the GBM and glomerular mesangial expansion (GME)[89]. GME leads to the development of glomerusclerosis, which may be focal, diffuse, segmental or global[90]. Also, an increase in the overall size of the glomerulus is detected, probably due to GME as well as a compensatory hyperfiltration[89]. Leakage in the glomeruli increases the pressure on the tubule to reabsorb solutes, which in turn activates the renin-angiotensin-aldosterone system (RAAS). RAAS activation increases both blood pressure[91] and the hydrostatic pressure in the glomeruli that may cause thickening of both the afferent and efferent arteriole. This morphology is referred to as arteriolar hyalinosis. In advanced nephropathy, tubular atrophy and interstitial fibrosis are also observed[92], which can be a compensatory mechanisms for hypertension and the increased volume of the glomerular filtrate. Injury to the proximal convoluted tubule is further enhanced by an increase in sodium reabsorption and oxidative stress, which further contributes to hypertension[93].

Several different animal models recapitulate relatively well the phenotypic characteristics of DN, most studies include the T2D model *db/db* and the T1D STZ-injected mouse models (Fig. 6). However, they do not recapitulate all the morphologies detected in DN patients and mentioned above, and often consist of complicated genetic mutations, or toxic agents. Therefore, much research is focused on finding novel animal models[94].

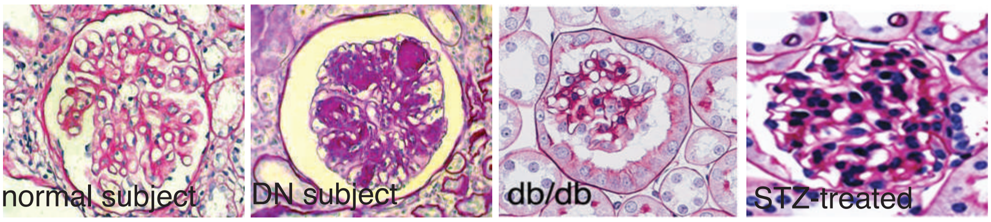

Fig 6. General glomerular morphology in health and disease
Periodic acid–Schiff staining of glomeruli from normal or DN subjects, T2D db/db and STZ-treated T1D mouse models. The animal models recapitulate well the general morphology of DN with GME and thickening of Bowmans capsule basement membrane. However, the Kimmelstiel-Wilson Nodules are not detected in any animal models. STZ; Streptozotocin. Modified from [76,95,96]

It is still debated in which cell type the primary injury of DN occurs. Even though the tubule is affected, most studies indicate that injury to GFB is the causative factor for disease development. Specifically, mutations in genes coding for slit diaphragm proteins such as nephrin, CD2AP and podocin (Fig. 5), cause nephropathies[97-99] showing the importance of, at least, the slit diaphragm and podocytes in maintaining functional GFB. In line with this, podocyte number correlated inversely with albuminuria[100] and reduced podocyte numbers were reported in both T1D[101] and T2D[102]. Furthermore, podocyte detachment[103] and apoptosis[104] contributed to reduced GFB capacity in DN patients. Additionally, widening of podocyte foot processes has been observed in renal biopsies of diabetic patients with increased albumin excretion[105,106].

1.2.2.1 Mechanisms underlying DN

The molecular mechanisms behind the abnormal glomerular alterations in DN are not understood. Homeostasis of the GFB depends on the integrity of podocytes, ECs and GBM and therefore, one could argue that any factor injuring, or changing the behaviour of any of these cell types may cause renal damage. The major causative factors for DN include hyperglycaemia and lipotoxicity[84].

Over the years hyperglycaemia has predominantly been suggested to contribute to the pathogenesis of DN. Hyperglycaemia triggered the synthesis of advanced glycation end-products (AGEs) and induced oxidative stress[107]. Furthermore, hyperglycaemia promoted the synthesis of angiotensin II of the RAAS system[91], that affects normal podocyte function and afferent arteriolar tone[108,109]. High levels of angiotensin II caused hypertension and induced cell damage, leading to proteinuria and initiating glomerulosclerosis[110]. Hyperglycaemia also activated transforming growth factor β (TGF-β), a key factor that drives the activation of fibroblasts, and thus renal fibrosis[108]. Also, activated TGF-β diminished the expression of nephrin on the slit diaphragm[111].

Today, interventions against DN focus largely on targeting hyperglycaemia, blood pressure and lifestyle changes. However, the prevalence of DN has increased in parallel with diabetes, despite higher usage of glucose-lowering agents[112]. Large cohort studies on T2D patients have been conducted to explore whether intensive management of blood glucose levels using anti-diabetic agents, could reduce the risk for DN and ESRD. Despite intensively controlled blood glucose levels, albuminuria was only slightly improved and serum creatinine levels were unaffected[113,114]. Furthermore, there was little or no effect on the incidence of ESRD and no change in death from renal disease despite intensive blood glucose management[113,114].

Lipotoxicity has therefore lately been considered as a major underlying factor of the pathogenesis of DN. Already the original article on glomerulosclerosis in subjects described lipid deposits in the glomeruli[90], and these were later confirmed by electron microscopy. Also, a high correlation between glomerular filtration rate, inflammation and lipid metabolism genes was detected in subjects with DN[115]. Lipids were shown to accumulate specifically in the podocytes[115] and podocytes treated with sera from patients with DN displayed increased cholesterol levels, which were detrimental for podocyte cell function[116]. Higher intracellular lipid accumulation was found in tubule from patients with chronic kidney disease and mice with tubulointerstitial fibrosis in comparison to healthy controls[117]. Genetically manipulated mice with tubulointerstitial fibrosis could be protected from disease development if FA metabolism was restored[117]. Furthermore, oxidised LDL is associated with the progression of DN[118]and overexpression studies of the receptor for oxidised LDL and lipoproteins in mice, increased the expression of TGF-β, vascular endothelial growth factor A (VEGF-A) together with kidney failure[119]. VEGF-A is secreted by podocytes, and its expression has been shown to be either up- or down-regulated in diabetic subjects, depending on the duration and stage of the disease[120,121]. VEGF-A seems to maintain the integrity of the GFB as both podocyte-specific excess, or deficiency, cause glomerular damage[122,123].

Interestingly, podocytes can also develop insulin resistance. Podocytes express all components of the insulin-signalling cascade and glucose uptake in podocytes was increased after insulin stimulation, mainly through translocation of GLUT4[124]. It was found that insulin resistance correlated with the development of albuminuria in both T1D and T2D subjects[125-128]. Additionally, impaired insulin sensitivity altered renal glucose cell metabolism and caused kidney damage independent of hyperglycaemia[128] and GLUT4 expression was downregulated upon the development of albuminuria[129]. Mice carrying a podocyte-specific deletion of the insulin receptor gene developed a phenotype resembling DN without any effect on blood glucose levels[15]. There have been a few studies elucidating what mechanism causes insulin resistance in podocytes, and linking lipotoxicity as the causative factor. Palmitate could block insulin-stimulated glucose uptake in human podocytes *in vitro*, suggesting a lipid-mediated inhibitory effect on insulin sensitivity[130]. Indeed, increased ceramide production in podocytes caused reduced phosphorylation of the insulin receptor and impaired translocation of GLUT4 to the cell surface[130]. Furthermore, LDL[131] and saturated FAs have also been shown to affect podocyte function an induce insulin resistance[130]. Taken together, lipotoxcicity and insulin resistance in podocytes could be important contributors to the disruption of the GFB.

1.3 ENDOTHELIAL LIPID UPTAKE

Lipids are the most energy dense nutrients, and are vital for normal cell processes and function. Reliable systems for transport of lipids to all cells of the body are therefore imperative. Nutrients are delivered to tissue cells via blood vessels, characterized by a lining of ECs, the endothelium, covering the inner surface and thus adjacent to the blood that passes through. It has for long been acknowledged that the endothelium has a barrier function[132]. However, it has been overlooked that nutrients such as lipids, going from the bloodstream into the tissue cells, also have to pass the endothelium. The preferred sort of nutrient varies between tissue and cell type and may also change upon different physiological stimuli. Therefore, mechanisms controlling nutrient uptake should exist. Furthermore, it would also demand less energy for the organism to limit nutrient uptake already at the vascular wall. Research has shown that the endothelium can act as a barrier for FA uptake[133-135]. However, it was only recently that a detailed mechanism for how myocytes can regulate lipid uptake through the endothelium was discovered, via secreting VEGF-B[136].

1.3.1 Lipid transport and lipid transporters

Organs with high metabolic activity such as heart, skeletal muscle and brown adipose tissue use lipids as their primary nutrient source. Most dietary lipids consist of long chain fatty acids (LCFAs, FAs with 12-20 carbons). FA are transported in the bloodstream either as TG rich lipoproteins during fed-states, or bound to albumin during fasting[137]. TGs are hydrolyzed at the site of peripheral tissues by lipoprotein lipase, which is anchored at the luminal side of the endothelium[138]. Research has focused on LCFA transport across the sarcolemma[139,140] since lipids were suggested to simply diffuse through the endothelium. However, secreted VEGF-B from tissue cells has been shown to signal in a paracrine fashion to the endothelium to induce the expression of two fatty acid transport proteins, FATP3 and FATP4 (Fig. 8) and consequently promote regulated lipid uptake[136].

FATP3 and 4 belong to the evolutionary conserved FATP family constituted of six mammalian 70-80 kDa large multi-transmembrane spanning proteins (FATP1-6)[141,142]. *Fatp4* is abundantly expressed, for example in myocytes, skin and in the endothelium[141,143] whereas *Fatp3* is expressed specifically in the vasculature at least in muscular tissues[136]. *Fatp4*$^{-/-}$ mice are embryonically/neonatally lethal[143] due to defects in FA absorption and/or to a disrupted epidermal barrier[143]. All FATPs have been shown to enhance cellular FA uptake *in vitro*[136,144]. However, the subcellular localization of the FATPs has been debated as well as if they simply drive cellular FA influx by intracellular acylation of FAs[145]

1.3.2 VEGF-B signalling

The VEGFs have a major role in controlling angiogenesis and lymphangiogenesis, both during embryogenesis and in the adult state[146,147] as well as during pathophysiological conditions[148]. Mammalian members include VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (Plgf)[148]. VEGFs act by binding to the tyrosine kinase receptors: VEGF receptor (VEGFR) 1, VEGFR2, VEGFR3 and the co-receptor neurophilin (NRP)-1 in an overlapping pattern (Fig. 7). VEGF-B binds to VEGFR1 and NRP1. VEGF-B is synthetized as two isoforms by alternative splicing, VEGF-B$_{167}$ and VEGF-B$_{186}$[149,150]. In adults, VEGF-B$_{167}$ is the prevalent isoform and binds to cell surface heparan sulphate proteoglycans[151]. On the contrary, VEGF-B$_{186}$ is freely diffusible and requires proteolytic cleavage before binding to NRP1[152]. However, the exact signalling mechanism of VEGF-B/NRP1/VEGFR1 is still far from understood.

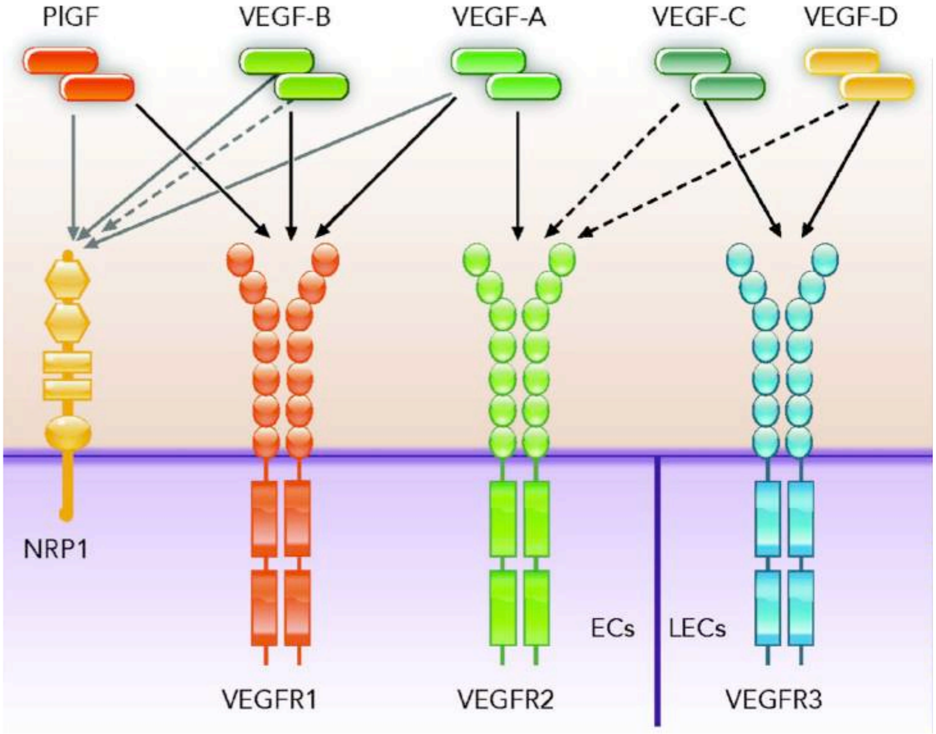

Fig 7. VEGF receptors, ligands and signalling
The VEGF ligands bind to VEGFRs in a partially overlapping fashion. VEGF-B, PlGF and VEGF-A also bind to the co-receptor NRP1 (grey arrows). Dashed lines indicate that proteolytic cleavage is needed before receptor binding. From[153]

1.3.2.1 VEGFR1 and NRP1, receptors of VEGF-B

VEGFR1 is a member of the tyrosine kinase receptor superfamily with an approximately 750-amino-acid-residue extracellular domain that binds VEGF-A, -B and PlGF[154]. The role of VEGFR1 during development has been extensively studied, during which VEGFR1 seems to primarily trap VEGF-A[155], via its higher affinity for VEGF-A[156] and thus hinder VEGF-A/VEGFR2 signalling. *Vegfr1*$^{-/-}$ as *Vegf*$^{+/-}$ embryos, die prenatally[155,157], whereas deletion of only the intracellular signalling domain of VEGFR1 (*Vegfr1 TK*$^{-/-}$ mice) resulted in healthy and fertile mice[158]. VEGFR-1 has the ability to bind tightly to its ligands but has a weak tyrosine kinase activity, generating signals weaker than VEGFR-2[159]. Therefore, it was suggested that VEGFR1 in the adult state does not have a signalling capacity *per se* but rather acts as solely a sink, trapping VEGF-A[160]. Even though the signalling capacity of VEGFR1 is not yet exactly understood, recent data has shown that upon stimulation with VEGF-A or PlGF, VEGFR-1 can initiate phosphorylation of distinct downstream proteins in monocytes[147,161]. *Vegfr1* is expressed on ECs, monocytes and macrophages[162,163] and has been shown to be a positive regulator of monocyte and macrophage migration[161,162] and to have a role in tumour progression[164].

NRP1 is a 130- to 140-kDa transmembrane glycoprotein[165] and is expressed in ECs, neural progenitors, macrophages and myocytes[166-168]. NRP1 has been shown to contain a PDZ-domain in its C-terminus, which enables the binding and signalling to downstream targets [169]. NRP1 is important for normal nervous system development and required for blood vessel patterning and normal lymphatic valve formation[170,171].

1.3.2.2 The biological function of VEGF-B

VEGF-B was for long assumed to have a similar function as its closest homologue, VEGF-A. Therefore, several studies have focused on VEGF-B in areas related to the known functions of VEGF-A. However, in contrast to the other VEGFs, VEGF-B is not upregulated by hypoxia, poorly angiogenic and does not induce vascular permeability in animals or tissues[172-174]. Although *Vegfb*$^{-/-}$ animals are healthy and fertile and present a normal life span[175], VEGF-B has been reported as a survival factor for different cell types by inhibiting apoptosis[176,177]. *Vegfb*$^{-/-}$ animals have minor cardiac abnormalities, such as smaller hearts and an increased PQ interval[175,178]. VEGF-B has been shown to induce arteriogenesis, both in rats with cardiac VEGF-B overexpression and in pigs with local adenoviral delivery of VEGF-B[174,179]. Increased VEGF-B levels have also been implicated in the development of different cancers[180]. In contrast, retarded tumour growth was detected when overexpressing VEGF-B in a mouse model for pancreatic cancer (RIP-Tag)[181]. Hence, the role of VEGF-B in angiogenesis, cell survival and tumour growth remains enigmatic.

Vegfb is expressed in tissues with high metabolic activity with enriched mitochondrial content such as heart, skeletal muscle and brown fat[136,151,152]. Mice with cardiac VEGF-B overexpression, displayed increased accumulation of ceramides, hypertrophy, mitochondria lysis and premature death[182]. When overexpressing VEGF-B by adenoviral delivery in pig and rat heart two diverging metabolic states were detected. Overexpression of VEGF-B in pig myocardium caused an upregulation of the *Fatps* as well as tissue lipid accumulation[183]. In contrast, VEGF-B overexpression in rats downregulated genes involved in FA metabolism whereas glucose uptake was increased[179]. These opposite effects may stem from receptor saturation, as techniques such as adenoviral delivery or transgenic overproduction may produce unphysiological protein levels.

Genetic deletion of *Vegfb* in mice decreased the expression of muscular *Fatps* and peripheral FA uptake, and instead the FAs were shunted to the AT[136]. The FA transport capacity was unique for VEGF-B as endothelial cells stimulated with, PlGF or VEGF-A, did not upregulate the expression of FATPs and FA transport. The FA uptake capacity was also dependent on both VEGFR1 and NRP1. *Vegfr1 TK*$^{-/-}$ and inducible EC-specific *Nrp1*$^{-/-}$ mice displayed reduced cardiac *Fatp3* and *Fatp4* expression and tissue lipid accumulation[136]. Positron emission tomography analysis of *Vegfb*$^{-/-}$ mice showed that glucose uptake to the cardiac muscle was increased[136]. These data suggest that inhibition of VEGF-B reprograms the endothelium to change substrate utilization – from lipids to glucose.

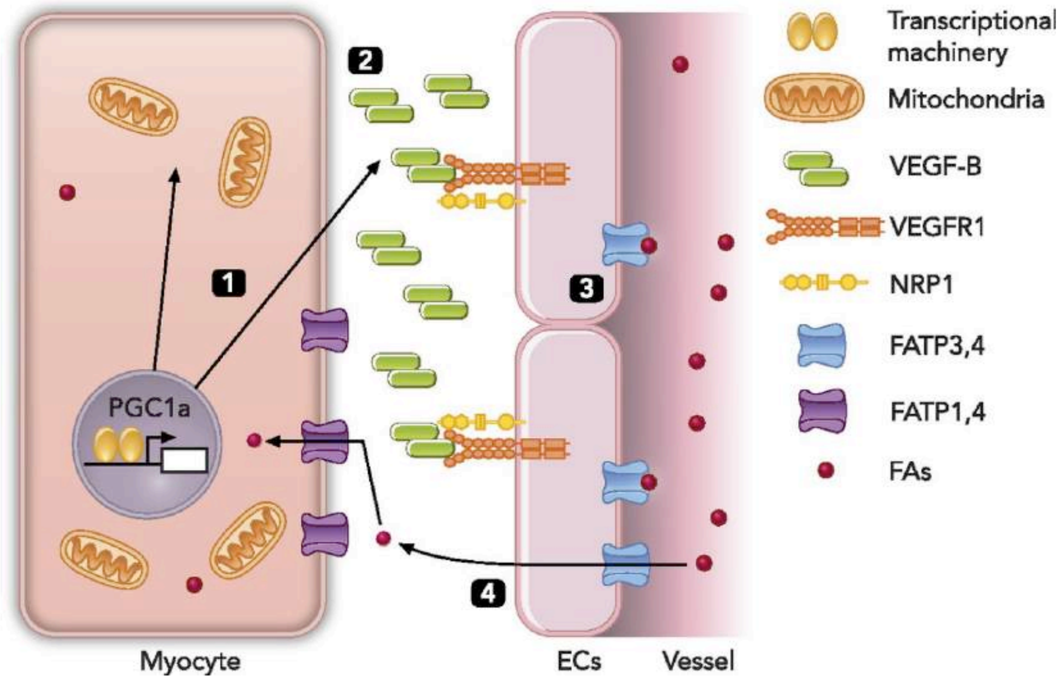

Fig 8. Schematic illustration on the role of VEGF-B in FA-transport
1: Vegfb expression is coordinated with the expression of mitochondrial proteins to co-regulate lipid uptake and β-oxidation. 2: VEGF-B, signals in a paracrine fashion to the receptors VEGFR1 and NRP1 present on endothelial cells (ECs), which 3:upregulates the expression of FATPs and induces subsequent transport of FAs across the EC layer into tissue cells (4). From [153]

There are some implications linking the VEGF-B signalling pathway to FA-transport and diabetes in humans. VEGF-B is upregulated in omental AT from obese subjects compared to lean individuals[184], suggesting an increased lipid uptake into AT. Also, serum VEGF-B levels were positively correlated to total cholesterol and triglyceride (TG) levels in T2D subjects[185]. Furthermore, serum VEGF-B levels were positively associated with polycystic ovary syndrome and insulin resistance[186]. Also, associations between the genes in the VEGF-B signalling pathway and traits coupled to lipid handling and diabetes have been detected. *Vegfr1* sequence variants have been correlated with coronary heart disease[187] and body weight[188]. Genetic variants of *Nrp1* have been linked with body weight[188] and cardiac hypertrophy[189]. A sequence variant in *Fatp4* was identified to be associated with the metabolic syndrome and insulin resistance[190]. To conclude, the biological role of VEGF-B has for long been enigmatic, with several publications showing diverging, or even opposite results. However, both human and mouse data imply that VEGF-B has an important role in endothelial FA uptake, via VEGFR1, NRP1, FATP3 and FATP4.

1.3.3 PGC-1α, master regulator of mitochondrial biogenesis

Peroxisome proliferator-activated receptor γ coactivator 1α (*Ppargc1a* / PGC-1α) is a transcriptional coactivator that exerts its functions via binding to several transcription factors, *e.g.* estrogen-related receptor alpha (ESRRa)[191], peroxisome proliferator-activated receptor gamma (PPARg)[192] and nuclear respiratory factor 1 (NRF1)[193]. PGC-1α is ubiquitously expressed in organs such as heart, skeletal muscle, kidney, liver, AT and pancreas[194]. Several different physiological stimuli can induce PGC-1α both transcriptionally and post-transcriptionally, including exercise, cold and fasting[194,195]. PGC-1α is synthesised as multiple isoforms with somewhat different expression patterns and functions, although the detailed function for each isoform is to date not clear[196-198].

Binding of PGC-1α to transcription factors induces specific signalling pathways and the biological functions that are regulated by that transcription factor. Importantly, PGC-1α is a powerful inducer of mitochondrial biogenesis by co-activating NRF1 and ERRα, and thus regulating hundreds of nuclear-encoded genes activating mitochondrial biogenesis and β-oxidation[193]. Furthermore, activation of PPARα by PGC-1α increased the expression of genes involved in FA import and β-oxidation[199,200]. PGC-1α also increased lipid anabolism *in vitro*, including intracellular FA and ceramides[201]. Also *in vivo, de novo* lipogenesis, fatty acid synthase and FATP4 expression were increased in muscle specific PGC-1α transgenic mice (muscle creatine kinase PGC-1α transgenic mice, MPGC-1α TG)[202,203]. Thus, PGC-1α can simultaneously coordinate FA import, mitochondrial biogenesis, β-oxidation and *de novo* lipogenesis depending on different extrinsic signals.

A common pathology in T2D is dysfunctional and insufficient muscular mitochondria[204]. Microarray studies of muscle biopsies from T2D patients showed decreased levels of PGC-1α as well as genes coding for mitochondrial biogenesis[57,205,206]. Also, increased muscular expression of PGC-1α, increased GLUT4-dependent glucose uptake *in vitro*[207]. These data suggested that, insulin resistance in T2D might be attributed to reduced levels of or dysfunctional PGC-1α and thus decreased mitochondrial content[57]. Studies using animal models have however not been as straightforward. PGC-1α full body knockout (*Pgc-1α*$^{-/-}$) mice had fewer mitochondria and diminished respiratory capacity, but surprisingly in response to HFD the *Pgc-1α*$^{-/-}$ mice were more insulin sensitive than WT controls[208]. Mice with a specific deletion of PGC-1α in skeletal muscle, did not develop insulin resistance, but had an altered glucose homeostasis[209]. MPGC-1α TG mice displayed reduced insulin sensitivity under HFD-feeding despite increased mitochondrial density and activity[16,202]. In rats, long term HFD-feeding increased the expression of *Ppargc1a*. Also in humans, 3 days of overfeeding caused elevated PGC-1α protein levels that returned to basal at the end of the study[210,211]. Moreover, increased PPARα and PGC-1α expression in murine insulin-resistant and in diabetic hearts have been detected[212-214]. Therefore, in the absence of exercise or during obesity, atypically high PGC-1α levels and thus an increased rate of glycogen and fat anabolism may lead to metabolic imbalance, eventually developing into diabetes.

1.3.3.1 Linking PGC-1α to angiogenesis and lipid uptake

PGC-1α is a major regulator of mitochondrial biogenesis whereas VEGFs are major regulators of angiogenesis and lipid uptake. Therefore, it would make sense to couple PGC-

1α with VEGFs to synchronize cellular and tissue growth via angiogenesis, endothelial lipid uptake and mitochondrial biogenesis.

PGC-1α has in many studies been shown to control VEGF-A expression and consequently angiogenesis. Upon starvation, PGC-1α regulated angiogenesis via increased VEGF-A expression, mediated via ERRα in myocytes[215]. Also, exercise and cold induced angiogenesis via PGC-1α and VEGF-A in muscle and AT[196]as well as deletion of PGC-1α in cardiomyocytes, lead to vascular defects[216]. Interestingly, all these angiogenic pathways were hypoxia-independent arguing that there might be a difference in how physiological and pathological angiogenesis is regulated[215,196]. In diabetes, vessels and ECs are damaged and reduced in number and factors such as AGEs, ROS, and FAs have all been suggested to contribute to the diabetic pathology[217,218]. Upregulation of PGC-1α levels were detected in ECs from diabetic patients, and PGC-1α also inhibited endothelial migration via activation of Notch signalling. Therefore, hyperglycaemia was thought to activate PGC-1α in ECs that in turn would inhibit vessel re-endothelialisation, processes involved, in for example, wound healing[219].

As discussed earlier, PGC-1α induced lipid uptake via *e.g.* CD36 and FATP4. How about PGC-1α and lipid uptake in the context of VEGF-B? The expression of VEGF-B was closely correlated to the expression of mitochondrial genes controlled by PGC-1α in a large-scale survey of the expression of nuclear-encoded mitochondrial genes[57]. Also, this co-expression between VEGF-B and mitochondrial genes was found when analysing publically available microarray data[136]. *Vegfb* expression clustered together with genes coding for oxidative phosphorylation and this co-expression was specific for *Vegfb* and not detected for *Vegfa* or *Plgf*[136]. Furthermore, in an attempt to identify novel PGC-1α targets in muscle, array analysis identified *Vegfb* as a novel myokine in MCK-PGC-1α TG[220].

Thus, these data suggest that by regulating PGC-1α levels, the tissue cell can quickly respond to changes in the environment, and as a consequence lipid uptake, mitochondrial biogenesis and β-oxidation as well as angiogenesis can be synchronized.

2 AIMS OF THIS THESIS

VEGF-B was previously shown to control lipid uptake from the endothelium into tissue cells and genetic ablation of *Vegfb* reduced intra-tissue lipid accumulation in mice. Apart from this, little is known regarding the metabolic role of VEGF-B in physiology and pathophysiology.

The aim of this thesis was to characterize the function of VEGF-B in metabolism.

The specific aims include;

- To optimize a robust protocol for visualization and quantification of neutral lipids in different tissues (Paper IV)
- To identify the upstream regulation of VEGF-B (Paper I)
- To evaluate if reducing VEGF-B signalling is beneficial for disease progression in rodent models of T2D (Paper II)
- To study whether reducing VEGF-B signalling, is beneficial for the disease progression in mouse models of DN (Paper III)
- To investigate wheather the VEGF-B signalling pathway is present and altered in human DN subjects (Paper III)

3 PAPERS AND DISCUSSION

Since the material and methods and the results included in this thesis are discussed and presented in detail within each paper, only a short summary of the rationale and the main findings are presented and discussed here below.

3.1 PAPER I; PGC-1 ALPHA COORDINATES MITOCHONDRIAL RESPIRATORY CAPACITY AND MUSCULAR FATTY ACID UPTAKE VIA REGULATION OF VEGF-B

The upstream regulatory mechanisms of controlling VEGF-B expression had not previously been characterised, and were thus unknown. It had been shown that the expression of VEGF-B clustered together with the expression of nuclear-encoded mitochondrial genes, and that VEGF-B regulated FA-transport[136]. The co-activator PGC-1α is a major regulator of mitochondrial function and energy metabolism[221]. Increased expressional levels of VEGF-B were reported in mice with a specific overexpression of PGC-1α in muscles (MPGC-1α TG mice)[220]. Therefore, we aimed to investigate whether PGC-1α could be a potential direct regulator of VEGF-B.

First, transfection analyses *in vitro* confirmed that co-expression of ESRRa and PGC-1α induced expression of *Vegfb* luciferase reporter construct containing the first kilobase of the 5´ untranslated region together with the first exon and the first intron of the *Vegfb* gene. Neither of the other transcription factors tested, NRF1 and PPARg could activate *Vegfb*. Also, we detected a putative ESRRa binding site upstream of the *Vegfb* gene. Next, we aimed to validate these data *in vivo*, using the MPGC-1α TG mice. Q-PCR analysis of MPGC-1α TG muscle revealed that expression of *Vegfb* was upregulated, and in parallel IMCL accumulation was increased, compared to WT littermate. In contrast, when *Vegfb* was deleted in MPGC-1α TG mice, IMCL levels were greatly decreased, however without affecting β-oxidation. This implies that the reduced IMCL accumulation in MPGC-1α TG/*Vegfb*$^{-/-}$ mice was due to reduced lipid uptake and that PGC-1α can control tissue lipid content via regulating VEGF-B expression.

It has previously been shown that HFD-fed MPGC-1α TG mice developed insulin resistance, which was considered paradoxical as mitochondrial biogenesis was increased in these mice[16]. We hypothesized that the insulin resistance in these mice could be due to an upregulation of VEGF-B expression and consequently increased IMCL accumulation (Fig 9a). Indeed, markedly increased IMCL accumulation was detected in HFD-fed MPGC-1α TG mice. Consistently, in HFD-fed MPGC-1α TG/*Vegfb*$^{-/-}$ mice, IMCL levels were greatly decreased and insulin sensitivity restored, and even normalized to lean control levels. These data suggested that insulin resistance in MPGC-1α TG mice is due to increased VEGF-B levels resulting in enhanced lipid uptake promoting lipotoxicity.

Taking together, we show both *in vitro* and *in vivo* that PGC-1α regulates the expression of *Vegfb* and by this mechanism lipid uptake can be coordinated with mitochondrial biogenesis and β-oxidation (Fig 9a). We also provide evidences that the paradoxical phenotype of the

MPGC-1α TG HFD-fed mice is due to increased VEGF-B expression and signalling. However, whether in humans overfeeding activates the PGC-1α/ESRRa pathway and consequently increases *Vegfb* expression, and if this is the mechanism by which muscular lipotoxicity and insulin resistance develops in progressive T2D remains to be established (Fig 9b).

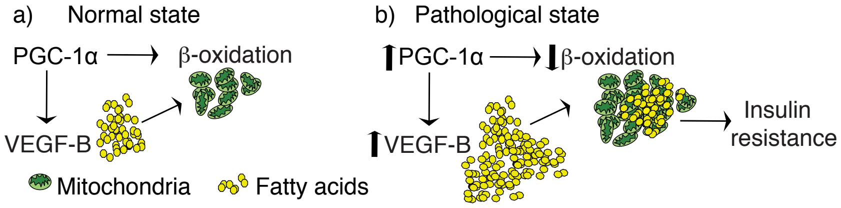

Fig. 9 Schematic illustration of PGC-1α dependent regulation of VEGF-B and FA uptake*. a: During normal physiological conditions, PGC-1α coordinates mitochondrial biogenesis and β-oxidation with FA uptake. b: In pathological states such as obesity or T2D, VEGF-B and PGC-1α levels could be upregulated, whereas mitochondrial function and β-oxidation are known to decline. This would cause a metabolic imbalance where FA uptake remains high, whereas the oxidative capacity of the tissue is reduced, resulting in tissue lipid accumulation, inducing insulin resistance and T2D (Modified from Mehlem et al under revisionin Diabetes).*

3.2 PAPER II; TARGETING VEGF-B AS A NOVEL TREATMENT FOR INSULIN RESISTANCE AND TYPE 2 DIABETES

It has previously been shown that inhibition of VEGF-B signalling in unchallenged mice, not only reduced FA uptake but also increased glucose uptake into myocytes[136]. Therefore we rationalized that one of the core pathologies causing T2D, namely lipotoxcicity, could be targeted by reducing VEGF-B signalling and potentially be beneficial for treating the disease.

To study this, we first analysed how genetic ablation of *Vegfb* in two different mouse models of obesity and T2D (*db*/*db* and HFD-fed mice) affected the development of T2D. In both animal models, *Vegfb* deficient mice showed reduced peripheral lipid deposition and improved hyperglycaemia, dyslipidaemia, glucose tolerance as well as enhanced insulin sensitivity in comparison to control animals.

Next, to rule out any possible developmental effects, and to explore the therapeutic potential of VEGF-B inhibition we used antibody administration to pharmacologically reduce VEGF-B signalling in *db*/*db* mice. Anti-VEGF-B antibody treatment reduced peripheral lipid accumulation, enhanced glucose tolerance and improved dyslipidaemia compared to control treated *db*/*db* mice. Interestingly, also pancreatic islet architecture and β-cell function were maintained by anti-VEGF-B treatment in *db*/*db* mice. Whether this improved effect on the β-cells is due to reduced metabolic pressure from lipotoxicity, glucotoxicity, both, or other unknown factors remain to be investigated.

Since *db*/*db* mice have been selected to spontaneously develop T2D, we sought to analyse the effects of anti-VEGF-B antibody treatment on T2D in another diabetic model and in a different species. To address this, HFD-fed rats were treated with an anti-VEGF-B antibody or isotype-matched control antibody, and subjected to a hyperinsulinemic-euglycaemic clamp assay, where insulin sensitivity is monitored. Inhibition of VEGF-B signalling increased insulin sensitivity and glucose uptake to skeletal muscle and heart compared to control antibody injected rats.

Recently, diverging results from our study have been found when comparing the two available different *Vegfb*$^{-/-}$ strains side by side on HFD, including the one used in paper II[178]. Dijkstra *et al* could not detect any differences between WT and *Vegfb*$^{-/-}$ mice in glucose tolerance or dyslipidaemia after HFD-feeding. However, Dijkstra *et al* analysed plasma lipids after 18h of fasting, whereas we analysed postprandial levels. Importantly, Dijkstra *et al* reported that the Bellomo mice were on a partly mixed background[222], inconsistent with our results. To further analyse the effects of reducing VEGF-B signalling during HFD conditions, would therefore be of importance.

Taken together, in the diabetic experimental animal models studied here, peripheral lipid accumulation mediated by VEGF-B is a major part of the diabetic pathology (Fig. 10 a-b). Importantly, we show using both genetic and pharmaceutical tools that VEGF-B signalling can be targeted, to reduce IMCL accumulation and to increase glucose uptake into peripheral tissues (Fig. 10c). However, whether VEGF-B signalling pathway can be used to treat T2D also in patients remains to be established.

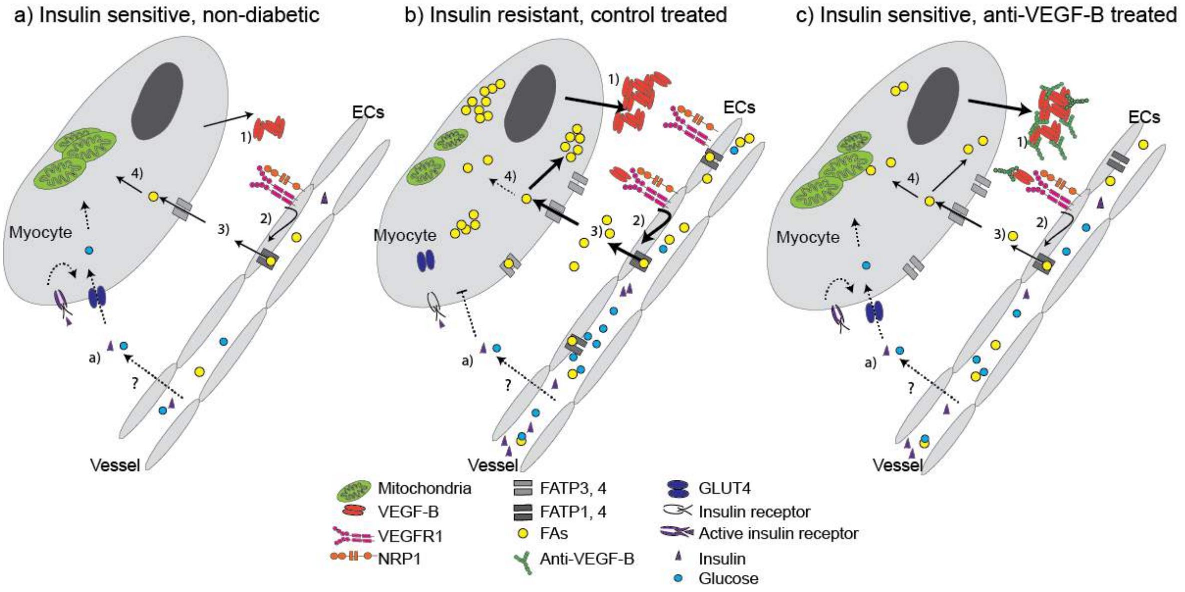
a) Insulin sensitive, non-diabetic
b) Insulin resistant, control treated
c) Insulin sensitive, anti-VEGF-B treated
Myocyte
ECs
Vessel
1)
2)
3)
4)
a)
?
Mitochondria
VEGF-B
VEGFR1
NRP1
FATP3, 4
FATP1, 4
FAs
Anti-VEGF-B
GLUT4
Insulin receptor
Active insulin receptor
Insulin
Glucose

Fig. 10 ***Schematic illustration of the role of Vegfb in physiological and pathophysiological states***.
a) *In the insulin sensitive state, Vegfb is secreted and binds to 1) endothelial VEGFR1 and NRP1 2). FATP3 and FATP4 are upregulated and subsequently FAs are transported across the endothelium and through the 3) tissue cell and transported to the 4) mitochondria for β-oxidation. a) Insulin and glucose are transported through the endothelium, insulin signalling is activated and glucose is taken up by the cell.* ***b)*** *In the insulin resistant, diabetic state, the expression of Vegfb is upregulated 1) and consequently endothelial FATP expression is increased 2), more FA are fed to the tissue cell 3) which leads to an 4). increased ectopic lipid accumulation. a). Ectopic lipid accumulation inhibits insulin signalling and thus glucose uptake.* ***c)*** *By inhibiting Vegfb via anti-VEGF-B treatment 1) Vegfb signalling is reduced 2) and less FAs are transported across the endothelium 3). Thus, ectopic lipid accumulation is reduced 4) and the tissue cell is a). re-sensitized to insulin and glucose is taken up*

3.3 PAPER III; REDUCING VEGF-B SIGNALLING AMELIORATES RENAL LIPOTOXICITY AND PROTECTS AGAINST DIABETIC NEPHROPATHY

In *db/db* mice with genetic ablation of *Vegfb,* reduced levels of glucosuria, were detected in comparison to control mice (paper II). Therefore, we aimed to investigate whether lipotoxicity is a cause of not only diabetes, but also of DN, and if inhibition of VEGF-B signalling can improve the outcome of DN.

Firstly, the kidney phenotype of a standard mouse model of DN, *db/db* was examined with regards to VEGF-B signalling. In *db/db* mice, the progression of diabetes was associated with increased expression levels of *Vegfb* in whole kidney lysates, together with an increase in glomerular lipid accumulation and podocyte loss. Therefore, we hypothesized that inhibition of VEGF-B signalling, and thus less glomerular lipid accumulation might be beneficial in DN. To explore this, we first analysed how genetic deficiency of *Vegfb* affected the development of DN in two different mouse models, *db/db* and HFD-feeding. Reduced glomerular lipotoxicity, improved renal function as well as diminished GBM thickening, GME and podocyte loss were detected upon *Vegfb* deletion in both animal models.

Next, the therapeutic potential of reducing VEGF-B signalling was studied using antibody administration to *db/db* BKS and HFD-fed mice. Importantly, improved effect on insulin resistance and dyslipidaemia was confirmed in anti-VEGF-B antibody treated HFD- fed mice, thus questioning the findings of Dijkstra *et al*. However, in contrast to Paper II only modest effects on blood glucose levels were detected upon VEGF-B inhibition in *db/db* BKS mice. Two different mouse backgrounds were used in these studies. Paper II used a *db/db* BKS/ C57/Bl6 mix whereas here we used a pure *db/db* BKS mouse line. It has been shown that the *db/db* mice on a BKS background develop a more severe hyperglycaemia that progress much faster than on a C57/BL6 background[223], which we also could confirm (Paper III). Therefore, anti-VEGF-B antibody treatment seems to be efficient at targeting hyperglycaemia only if the diabetic pressure is intermediate (Paper II), and cannot halt hyperglycaemia when the progression is very aggressive (Paper III). Nevertheless, several hallmarks of DN could be improved with anti-VEGF-B antibody treatment, even despite that hyperglycaemia was not greatly altered. Reduced glomerular lipotoxicity, improved renal function as well as diminished GBM thickening, GME and podocyte loss were detected upon anti-VEGF-B antibody treatment. Hence, hyperglycaemia does not contribute significantly to the pathogenesis of DN, in this setting,

Renal dysfunction is not only a comorbidity of T2D, but also frequently found in T1D subjects. Lipotoxicity is being contributed to an increasingly larger role also in the pathology of T1D. Therefore, we next sought to investigate whether anti-VEGF-B antibody treatment could be beneficial in STZ-injected mice, a mouse model for T1D. Animals injected with STZ in combination with control antibody treatment, displayed severe renal dysfunction and glomerular lipotoxicity causing podocyte loss, traits that were near to normalized with anti-VEGF-B antibody administration.

To fully understand the therapeutic potential of reducing VEGF-B signalling, we next investigated whether VEGF-B expression was altered in human DN. Microarray analysis of renal biopsies from patients with DN revealed that glomerular VEGF-B production is increased compared to healthy subjects. This suggests that VEGF-B signalling is present and activated in human DN and that lipotoxicity could presumably be targeted using anti-VEGF-B antibody treatments.

Next, as glomerular VEGF-B expression levels were increased in human DN subjects, we aimed to explore the consequences of increased VEGF-B signalling specifically in the podocytes. Interestingly, in mice with podocyte-specific overexpression of VEGF-B, microalbuminuria could be induced in unchallenged mice, however with age the level of leakage was not aggravated. Upon HFD-feeding, a steady rise in albuminuria that developed into macroalbuminuria was detected in mice with podocyte-specific overexpression of VEGF-B. Hence, both elevated VEGF-B levels, as well as dyslipidaemia achieved by HFD-feeding, are needed for the progression of albuminuria to occur in these mice.

Our data in paper III suggest that glomerular-derived VEGF-B will promote ectopic lipid accumulation. During persisted elevated circulating FA levels, as in diabetes, podocyte VEGF-B expression is increased, causing lipotoxicity and podocyte insulin resistance with subsequent podocyte loss and impaired glomerular structure and function (Fig 11). Therefore, renal lipotoxicity is an important element of DN, and reducing VEGF-B signalling can ameliorate renal lipotoxcicity. This represents a novel approach to treat DN (Fig 11).

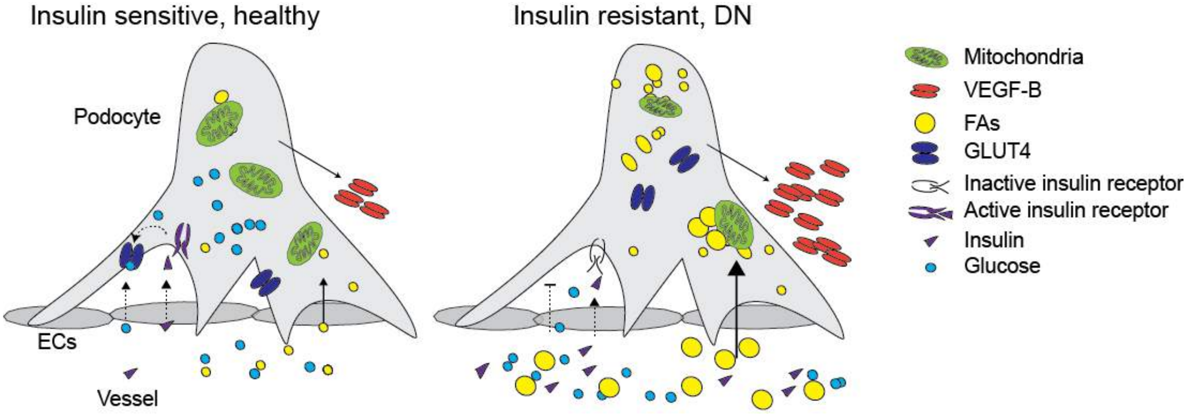

Fig 11, Schematic illustration of VEGF-B signaling in podocytes in healthy subjects and DN
In the healthy, insulin sensitive state, the circulating levels of FAs are low, as well as podocyte expression of Vegfb. Consequently FA uptake is low and insulin sensitivity maintained. In DN, plasma levels of FAs are increased, podocyte expression of Vegfb is upregulated and thus the uptake of lipids from the circulation into the podocyte is enhanced, causing lipotoxicity, insulin resistance and proteinuria (Modified from Falkevall et al. in submission).

3.4 PAPER IV; IMAGING OF NEUTRAL LIPIDS BY OIL RED O FOR ANALYZING THE METABOLIC STATUS IN HEALTH AND DISEASE

All of the papers presented previously in this thesis required a method that enabled the detection of lipids within tissue cells. Not many easy and reliable techniques existed that could enable both quantification and visualization of intra tissue lipids.

Therefore, we aimed to develop a protocol that enabled the detection of neutral lipids as well as LD morphology, using Oil red-O (ORO). By using ORO, the lipid content could readily be quantified. To validate the robustness of the protocol, three different mouse models were analysed for lipid accumulation in three different tissues. Both in liver, heart and muscle, the highest lipid accumulation was detected in *db/db* animals, followed by HFD-fed and lastly normal diet. Hence, a protocol enabling easy quantification of neutral lipids and distribution is presented, that can be performed using only basic laboratory and computer equipment.

4 FUTURE PERSPECTIVES

The results included in this thesis are exciting for a broad scientific audience. We show that lipotoxcicity is an important contributor to the progression of T2D (paper II) and DN (paper III), and that it can be targeted. This opens up for several interesting future implications. Could the development of other comorbidities associated with diabetes be halted using anti-VEGF-B agents as well, such as diabetic foot ulcers, stroke, hepatic steatosis and cardiovascular disease? Dyslipidaemia is a known risk factor for all of these comorbidities, however little research on the subject particularly in regards to intra-tissue lipid accumulation are available.

Furthermore, the identification of PGC-1α as a regulator of VEGF-B in paper I prompts several interesting questions. Mainly, may inappropriately high PGC-1α upregulate VEGF-B expression levels also in a human diabetic patients and if so, does increased VEGF-B signalling drive IMCL accumulation? Also, what are the underlying factors behind atypically high PGC-1α levels in obesity and T2D?

Several questions of a more specific character also rise with the studies presented in this thesis. Among others, is reduced podocyte loss by inhibiting VEGF-B signalling due to a relief of lipotoxicicity-mediated insulin resistance? Interestingly, pioglitazone, an agonist for PPARs, was effective in reducing albumin excretion and podocyte injury in early-stage diabetic nephropathy[224]. Pioglitazone functions as an insulin sensitizer and decreases lipid content in peripheral tissues[225,226]. Even though pioglitazone has been implicated with several side effects[227,228], this study shows that podocyte survival can be targeted using a drug that decreases lipid accumulation, in line with our findings in paper III.

Finally, an important question that rises from our studies is whether hyperglycaemia is the pathology to target for diabetes and diabetic complications. VEGF-B acts by lowering intra-tissue lipid levels and in paper III we can almost halt the progression of DN in several mouse models, without reducing glucose levels. This is in line with large patient cohort studies where modest or no effect was found on the outcome of diabetic complications after intensively controlling and lowering blood glucose levels. High glucose levels are obviously toxic for the cell and can interfere with, among others, the insulin signalling pathway. However, if hyperglycaemia were the main pathological driver behind diabetic complications, a larger effect after intensively controlling it, would have been expected. Thus, the large cohort data as well as our studies suggest, that diabetic complications are not primarily caused by increased glucose levels, instead focus of research and drug development could be shifted towards lipotoxcicty.

Your theory is crazy, but it's not crazy enough to be true. Niels Bohr

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