In this thesis, we described the effect of Perivascular Adipose Tissue (PVAT) on microvascular insulin sensitivity, an important determinant of metabolic insulin sensitivity and blood pressure regulation. To do this, we used multiple approaches, from human studies to animal studies, and from in-vivo studies to protein signaling. This chapter will put the previous chapters in their context. First, the rationale for the main hypothesis will be shortly discussed, then we will discuss the main findings of the chapters, address methodological issues, and present directions for future research.

Rationale
The relation between obesity, insulin resistance and cardiovascular disease is well established, but the putative mechanisms are not entirely clear. After binding to the insulin receptor, insulin initiates several signaling pathways, of which the IRS-PI3k-Akt pathways is responsible for translocation of GLUT4 to the cell membrane in muscle and adipose tissues, where it facilitates the uptake of glucose in the cell. In obesity, downstream insulin signaling is blunted, including GLUT4 translocation, and therefore leads to a decrease in glucose uptake. Insulin also activates the same IRS-PI3k-Akt pathway in endothelial cells, where it leads not only to glucose uptake, but also to the production of nitric oxide (NO), the most important vasodilator. As insulin-induced NO production is largely mediated by the IRS-PI3k-Akt pathway, this effect is also blunted in obesity. The blunting of insulin’s vascular actions in obesity is one of the characteristics of endothelial dysfunction, and has broad and severe implications. Firstly, in healthy lean people insulin stimulates NO production, which leads to vasodilation, in particular in the microcirculation, a process called microvascular recruitment. Microvascular vasodilation leads to an increase in microvascular blood volume and flow. Both result in increased delivery of glucose and nutrients to target tissues, primarily skeletal muscle. Indeed, microvascular recruitment augments skeletal muscle glucose disposal by approximately 40% in mice and rats. Therefore, when microvascular recruitment by insulin is diminished, such as in obesity, this also has a deleterious effect on glucose uptake. Secondly, parallel to the PI3k-Akt-eNOS pathway leading to NO production, insulin stimulates the mitogen-activated protein kinase
(MAPK) pathway. The MAPK pathway results in endothelin-1 production, but also enhanced cell proliferation, which may lead to increased vessel stiffness, remodeling and loss of blood pressure regulation. This pathway is not inhibited in obesity which, together with blunting of the PI3k-Akt-eNOS pathway, causes diminished insulin-mediated microvascular recruitment. Moreover, altered insulin signaling has been proposed to contribute to atherosclerosis, partly through endothelin-1, and reduced NO production.

In this thesis, we concentrated primarily on the changes in diameter of microvessels initiated by insulin, and the subsequent changes in microvascular blood volume.

The reason for the unbalanced impairment in insulin signaling pathways, and the resulting blunted insulin-induced microvascular recruitment in obesity is not well understood. Individual adipokines have been implicated, but systemic concentrations of these adipokines generally fail to provide sufficient explanation.

The hypothesis of this thesis is that perivascular adipose tissue in the microcirculation regulates insulin-induced microvascular vasomotor responses. Thereby, PVAT could modify the regulation of perfusion in skeletal muscle and other insulin sensitive tissues and therefore glucose and insulin delivery and thus insulin sensitivity.

Several observations have led to the hypothesis underlying this thesis; First, the observation by my co-promotor dr. E.C. Eringa that there are small deposits of adipose tissue surrounding the muscle microcirculation, was essential. Second, others observed that venous concentrations of the most important adipokines are higher than the arterial concentrations, suggesting that there is local production of adipokines in the microcirculation, at least in the heart. Third, the observation that carotid arteries in the mouse, which lack PVAT, are relatively protected from endothelial dysfunction during a high fat diet, compared to arteries that do have PVAT. A more in-depth explanation of the rationale for the main hypothesis is given in chapter 2.
Microvascular recruitment as a determinant of metabolic insulin sensitivity

Obesity is often used as a surrogate inclusion criterion for presumably insulin resistant subjects, but this approach neglects overweight, which comprises a substantial proportion of the population. Insulin-induced microvascular recruitment and the blunting thereof is also often studied in a lean group, comparing them with obese. To study whether insulin-induced microvascular recruitment is already blunted in overweight people, we therefore investigated the relation of insulin-induced microvascular recruitment with metabolic insulin sensitivity in a group with a continuous range of BMIs from lean to obesity in chapter 3a.

Microvascular recruitment was quantified using two different techniques. The first was contrast enhanced ultrasound (CEU), the second capillary videomicroscopy. CEU uses the interaction between gas-filled microbubbles, which are infused intravenously, and ultrasound.\(^{17}\) Circulating microbubbles have rheological properties similar to erythrocytes and enhance ultrasonographic wave reflection.\(^{18-20}\) We studied whether hyperinsulinemia during a euglycemic hyperinsulinemic clamp increases the intensity of reflected signal, and therefore the microvascular blood volume. Simultaneously, we used a second method to directly visualize perfused capillaries per \(\text{mm}^2\) of the skin of the finger. This density is quantified at baseline, and during peak-reactive hyperemia (PRH). The difference between the number of perfused capillaries at baseline and the number during PRH is called capillary recruitment, and has been shown to be related to metabolic insulin sensitivity.\(^{21-23}\) It had however not been studied whether insulin further augments this response. Therefore, we studied the change in capillary recruitment from baseline to hyperinsulinemia, so-called ‘insulin-augmented capillary recruitment’. Another issue addressed in this chapter concerns a long-standing debate in microvascular research: is skin microvascular function an appropriate surrogate for microvascular function in skeletal muscle?\(^{24-43}\) As we describe in this chapter, both methods to quantify insulin-induced microvascular recruitment showed correlations with metabolic insulin sensitivity. Moreover, the amount of insulin-induced microvascular recruitment in muscle and in skin were also related to each other, thereby validating the use of skin as a proxy for skeletal muscle, at least in relation with
insulin-induced vasoreactivity. In chapter 7, where we studied a group of lean healthy women and a group of obese healthy women, we found that microvascular recruitment explains the difference in metabolic insulin sensitivity by 22%. This is smaller in magnitude than the 40% found in an experimental study with rats where microvascular recruitment was inhibited by NO-blockade. Although these studies differ in experimental setup and even species. Together, these results clearly show the contribution of insulin-induced microvascular recruitment to metabolic insulin sensitivity.

One important question is whether improving microvascular insulin sensitivity results in improved glucose disposal. It is known that when insulin signalling in endothelial cells is blocked, or NO production inhibited, this results in reduced metabolic insulin sensitivity, but whether the opposite is true is unknown. One study was able to partly restore metabolic insulin sensitivity by increasing NO production pharmacologically, the anti-anginal compound ranolazine enhances insulin-induced microvascular recruitment, and increases metabolic insulin sensitivity in chow-fed animals, whether it also does this in insulin resistance is unknown. The contribution of blunted insulin-induced microvascular recruitment to cardiometabolic risk must therefore be studied in more detail.

Another unknown is whether microvascular and metabolic insulin resistance develop simultaneously, or whether one follows after the other. As we have shown in chapter 6, microvascular insulin resistance is established after two weeks Western Diet, and mice were also metabolically insulin resistant. We did not restore microvascular recruitment in this experiment to assess the exact contribution of reduced microvascular recruitment to the metabolic insulin resistance. However, since insulin signaling is reduced in macrovascular endothelial cells before skeletal muscle myocytes, we can not exclude that the metabolic insulin resistance was exclusively due to the decreased delivery of insulin and glucose. A recent presentation at the Scientific Sessions of the American Diabetes Association supports this observation with in vivo measurements that microvascular insulin resistance precedes metabolic insulin resistance, in agreement with our data.

We also explored the relation between microvascular blood volume, measured by CEU, and blood pressure, these results are
described in chapter 3b. Here we found that microvascular perfusion in the forearm is negatively related to blood pressure in healthy subjects, even after adjusting for sex and age. This being a post-hoc analysis, we could not further explore the relationship between these two variables. The reduced microvascular perfusion may be due either to structural or functional microvascular rarefaction in skeletal muscle. Further applicability of CEU in blood pressure research needs to be investigated in experimental studies to see whether this defect is amenable for therapeutic gain.

Together, chapters 3a and 3b demonstrate the power of CEU in microvascular research, especially in relation to insulin’s effect on the microvasculature, as also demonstrated in chapters 5, 6 and 7 and by others.4, 6, 48-57 But first and foremost, all these chapters, and most notably chapters 3a and 7 clearly show the contribution of microvascular insulin sensitivity to metabolic insulin sensitivity.

Perivascular adipose tissue as a regulator of microvascular recruitment

Understanding how influences from outside of the vessel wall can affect insulin-induced microvascular responses is important to explain the in-vivo actions of insulin on microvascular blood volume as described in chapter 3a. These external effects can be studied by applying isolated adipokines,58-60 or pharmacological substances,56 to microvessels ex-vivo. However, such experiments will not reveal unknown adipokines, or synergistic or antagonistic effects between adipokines. Therefore, these studies must also be performed with PVAT, or other complete tissues that abut the microvasculature. To explore the effect of PVAT on insulin-induced microvascular responses, we first used an ex-vivo mouse-model in chapter 4. To do this, we performed pressure-myography experiments with isolated resistance arteries (RAs) from the gracilis muscle in the mouse hindleg. In this setup, one can add insulin or other substances of interest abluminally. Isolated RAs without PVAT did not exhibit diameter changes after exposure to increasing doses of insulin, corroborating previous findings,56, 58, 61 and findings in chapter 5 and 6, but also chapter 7, where we studied isolated human resistance arteries from skeletal muscle for the first time. When the RAs in chapter 4 were pre-incubated with PVAT from
lean mice, they exhibited a dose-dependent insulin-induced vasodilation, showing the potential of VAT to regulate microvascular perfusion. This is in line with the anticontractile effects of PVAT in the macrocirculation, and in the microcirculation of subcutaneous adipose tissue as described by other researchers, using vasoactive substances other than insulin.\(^{62-72}\) However, insulin is particularly interesting because of the dual vasoactive effect, stimulating both a vasodilator and a vasoconstrictor pathway in the endothelium, with the balance between these two which determining the vasomotor response. The effect of PVAT from lean mice and humans to enhance insulin-induced vasodilation was also found in chapters 6 and 7. In contrast, PVAT from obese subjects does not uncover insulin-induced vasodilation. In chapter 7 we even found that PVAT from obese subjects elicits insulin-induced vasoconstriction in microvessels from obese women. The resistance arteries from the Western Diet mice in chapter 6 with PVAT from these same mice also showed a trend towards insulin-induced vasoconstriction. We identified adiponectin and downstream AMPK\(\alpha_2\) signaling, as well as inflammatory responses in PVAT, as potential mediators for the change in vasoactive effects from lean to obese PVAT (chapters 4, 5 and 6).

Interestingly, the ex-vivo responses to insulin by by vessels incubated with but not without PVAT, parallel insulin responses seen in-vivo in insulin-sensitive individuals in our own studies.\(^4\) Intriguingly, even the AMPK\(\alpha_2^{+/+}\) and AMPK\(\alpha_2^{-/-}\) ex-vivo results in chapter 4 were later confirmed by the in-vivo responses as described in chapter 5. Therefore, the pressure myography experiment with PVAT is a more physiologic model than without PVAT, which may not be too surprising, as it reflects the in-vivo situation somewhat more, because of the addition of a tissue which is normally left out in the pressure myography experiments.

In this thesis, we did not directly assess the involvement of PVAT in in-vivo microvascular responses, because we first needed to study whether PVAT indeed has some regulatory function at all for insulin-induced microvascular responses, and specific manipulation of PVAT in-vivo is hard. But, apart from ex-vivo modulation of insulin-induced vasoreactivity, we also found that adipocyte size in PVAT mediates the relation between obesity and microvascular recruitment in chapter 7.
Macrophage infiltration in PVAT was not different between the lean and obese group, the latter is at variance with a host of literature.\textsuperscript{16, 62, 73-82} However, this analysis was hampered by the small amounts of PVAT in skeletal muscle, and the associated artefacts. We could therefore not identify the number of macrophages as a critical factor affecting PVAT phenotype in this group. The M1/M2 polarization of macrophages may however be more important than the total number of macrophages.\textsuperscript{83} PVAT adipocyte size per se seems an unlikely culprit of altered microvascular responses, because size alone does not affect the production of adipokines. The pathway from nutrient excess to altered microvascular vasoreactivity by PVAT is therefore likely to be mediated by other factors. When adipocytes increase in size, this might cause hypoxia.\textsuperscript{63} Hypoxia may then lead to ER-stress, which triggers an inflammatory response, in turn this inflammatory response could change the adipokine-profile.

These results, and the results of others identify PVAT as an important regulator of microvascular responses. The true in-vivo effects of PVAT must be studied more deeply, independent of systemic effects of adipose tissue depots. Also, further characteristics of PVAT must be identified to facilitate specific interventions at PVAT alone.

**Factors in perivascular adipose tissue (and the vessel wall) affecting regulation of microvascular insulin responses**

The obese state is associated with metabolic insulin resistance,\textsuperscript{84} microvascular insulin resistance,\textsuperscript{4, 52} and PVAT dysfunction.\textsuperscript{63, 82, 85} Whether the last condition is linked with the former two was unknown. In chapter 4 we have found that when we pre-incubated the RAs of lean mice with PVAT from db/db mice, instead of PVAT from lean mice, they did not relax on increasing doses of insulin. This also happened with the PVAT from mice on a Western Diet (WD) in chapter 6 and the PVAT from obese women in chapter 7, although we could only use those together with microvessels from the obese women themselves. So the state of nutrient excess seems to be associated with an altered PVAT phenotype, which agrees with others.\textsuperscript{63, 82, 85} More importantly, this altered PVAT phenotype changes vascular responses to vasodilators and vasoconstrictors. It has been shown that during a high-fat diet PVAT becomes pro-inflammatory,\textsuperscript{82} and that weight-loss reduces
inflammation. So, when we inhibited inflammation using an inhibitor of total c-Jun N-terminal Kinase (L-JNKi, chapter 4) or JNK2 (through JNK2-deficient bone-marrow transplantation, chapter 6), we completely restored the insulin-responses of lean RAs in presence of obese PVAT. In contrast, adiponectin inhibition concomitantly with JNK inhibition, or adiponectin inhibition with lean PVAT led to a complete loss of vasodilator-effect of PVAT (chapter 4), and adiponectin administration could mimic the vasodilator effect of PVAT. The latter results suggest an important signalling role for adiponectin, AdipoR1 agonists or at least AMPK agonists coming from PVAT. Therefore, we further explored how AMPKα2 activation in the vessel wall affects insulin-induced microvascular reactivity, and indeed found that AMPKα2 activation enhances Ser1177eNOS phosphorylation, and inhibits endothelin-1 activation (Chapter 5). Moreover, AMPKα2−/− mice had a loss of perfused microvascular blood volume during hyperinsulinemia, which may explain the impaired insulin sensitivity of these mice. Together with the anti-inflammatory effect of adiponectin, this might make substitution of adiponectin seem an attractive intervention. However, solely substituting adipokines or AMPK agonists will not improve the endothelial dysfunction associated with obesity (chapter 6), at least not through acute administration. Downregulated AMPK might be an explanation for this, as AMPKα2−/− RAs were also adiponectin resistant (chapter 5). Whether or not PVAT is involved in the genesis of long-term endothelial dysfunction in obesity is not known, further studies are needed to elucidate this. On the other hand, inhibition of inflammation may be an approach suited to preserve or restore microvascular dysfunction in obesity. Others have systemically administered salsalate, thereby completely restoring metabolic and microvascular function. Systemic administration of salsalate inhibits inflammation in both the endothelium and PVAT. Further research is needed to see whether anti-inflammatory treatment in the endothelium alone, or in PVAT alone, is sufficient to restore microvascular responses. One could hypothesise that making the endothelium resistant to inflammation, for example by downregulation of JNK, NFκB or p38, could salvage the microvascular responses, as the endothelium is the integrator of all signals in this interaction.
Intriguingly, it appears that there is not only communication from PVAT directed to the endothelium, but in fact, it is more a PVAT-endothelial-dialogue.\textsuperscript{87} Endothelial deficiency of P-selectin glycoprotein ligand-1, a cell adhesion molecule, protects PVAT against monocyte infiltration and preserves PVATs anticontractile property.\textsuperscript{16} Also, peroxidation products generated in the vessel wall signal to PVAT to upregulate adiponectin gene expression.\textsuperscript{87} That observation suggests that when the endothelium is stressed, it signals to PVAT to generate more adiponectin which has anti-inflammatory properties in order to overcome the inflammatory stressor. This may reconcile the observations that when studied in healthy individuals, higher adiponectin concentrations are associated with lower risk for cardiovascular events and diabetes,\textsuperscript{88-90} whereas in unhealthy individuals higher adiponectin concentrations are related with endothelial dysfunction and thus associated with adverse cardiovascular outcome.\textsuperscript{91-97} However, the results of \textbf{this thesis} only indirectly reflect on this debate. The current findings support the notion that inhibition of JNK2 could positively preserve PVAT function, and that AMPK activation in the endothelium has beneficial effects on insulin-induced vasodilation.

Together, these results strongly suggest a balance in PVAT between adiponectin production and inflammation, which tips the microvascular effects of PVAT towards either insulin-induced vasodilation or no effect.

\textbf{Implications}

In their hypothesis paper, Yudkin et al. described a pad of adipose tissue at the origin of arterioles in the rat cremaster muscle.\textsuperscript{14} Due to this specific location, they suggested a sort of ‘gatekeeper’ function for this pad of adipose tissue, where this pad would secrete more cytokines with increasing adiposity. They continued to hypothesize that these cytokines would act downstream, without entering the systemic circulation, so called “vasocrine signalling”. The implication of this hypothesis was that that specific pad of adipose tissue would control microvascular perfusion in the muscle, and thereby affect insulin and glucose delivery and uptake. In this thesis, we have described that PVAT is present throughout the arteriolar tree in skeletal
muscle of mice and humans, unlike the specific location at the origin of an arteriole in the rat cremaster. Moreover, in this thesis we have demonstrated that even a small amount of healthy PVAT enhances vasodilation (chapters 4, 6 and 7) through secretion of adiponectin. Nevertheless, when PVAT pads, or their adipocyte sizes, expand during nutrient excess (chapters 4, 6 and 7) and PVAT becomes inflamed, this blunts the insulin-induced vasodilator response, through diminished adiponectin secretion (chapter 4). We did not study the hypothesis of the downstream “vasocrine signalling”, which is left for future research, but the hypothesis that PVAT in the muscle microcirculation has at least local effects on microvascular perfusion is supported by our data.

As we have demonstrated in chapter 6, once the endothelium has developed insulin resistance, even PVAT from lean mice, or PVAT which has diminished inflammation, cannot reveal insulin-induced vasodilation. In chapter 7 we have demonstrated that in lean women there is a relation between PVAT adipocyte size and microvascular insulin sensitivity, but that this is lost in obesity. This may further demonstrate that in lean healthy subjects, PVAT is necessary for insulin-induced vasodilation, but that in obesity PVAT phenotype is less relevant. Nevertheless, we have not studied the involvement of PVAT in the origin of this chronic endothelial dysfunction. This involvement may be significant, because as discussed here earlier, Wang et al. found that carotid arteries, which are devoid of PVAT in mice, do not develop endothelial dysfunction during a high fat diet. Nevertheless, it appears that it is more important to prevent endothelial dysfunction, and that PVAT dysfunction can be restored later, at least through an intervention on JNK2.

**Methodological considerations**

The data in this thesis were generated using several techniques, most notably those to study microvascular function. Here we will discuss these techniques briefly, and discuss how these methods helped us answer the research questions.

The major method to image microvascular perfusion and changes in perfusion used in this thesis is the Contrast Enhanced Ultrasound (CEU) technique (Chapters 3a, 3b, 5, 6 and 7). CEU
makes use of gas-filled microspheres with a lipid capsule. These microbubbles have flow-characteristics similar to erythrocytes, and depending on the ultrasound signal they will either burst or oscillate, creating a distinctive reflected signal, which enhances video-intensity in the blood vessels, and therefore contrast. This results in an estimation of the perfused microvascular blood volume, but does not give an absolute measure of flow (ml/g/min). It is however still a powerful tool to measure changes in blood volume. As placing of the ultrasound probe is crucial, it is easier to reliably detect these changes with acute (minutes) or semi-acute (hours) changes in volume. Longer term studies will probably prove harder to perform compared to e.g. capillary videomicroscopy. A more serious drawback however is that destruction of the microbubbles is a prerequisite for CEU measurements. As the destruction of microbubbles may damage the endothelium, and thereby affect microvascular endothelial function, but the extent of this is still uncertain. Nevertheless, CEU is an often-applied technique and one of the major advantages is that it is applicable in multiple organs (skeletal muscle, liver, kidney, heart) and different organisms (humans, mice, rats) with relatively minor adjustments.

Capillary videomicroscopy was used in chapter 3a. With this technique, capillaries in the skin of the finger are directly visualized with a microscope, when they are perfused with erythrocytes. Because the microscope looks at the surface of the skin of the finger, and the penetration of the light into the skin is very shallow, it is used to study an area, rather than a volume. The number of capillaries in one mm² is quantified during three different conditions; 1) baseline: no stimulus is given before this measurement, the amount counted capillaries reflects the number of perfused capillaries at rest, 2) postocclusive reactive hyperemia: this measurement is performed after digital occlusion at 300 mmHg and the number of perfused capillaries counted is a measure of the functional reserve of capillaries that can be recruited in case of higher demand, 3) venous occlusion: this measurement of performed during venous occlusion at 60 mmHg and the number of perfused capillaries is a measurement of the structural amount of capillaries present in the one mm² of interest. The capillary videomicroscope is a
device often used to study the skin microcirculation, and the results obtained with it, are often translated to other vascular beds. We could indeed show in chapter 3a that the results were related to skeletal muscle perfusion. A disadvantage of the capillary videomicroscope is that, in contrast to CEU, a repeated baseline measurement is not sufficient, but extra stimuli (e.g. postocclusive reactive hyperemia) are needed, making interpretation more difficult. Moreover, it assumes that only capillaries perfused with erythrocytes are actually perfused. However, some capillaries will be perfused only by plasma, and will be capable of delivering glucose and insulin to target tissues. The modest strength of the relation may be due to this drawback. Capillary videomicroscopy is nevertheless one of the few dynamic tests to truly measure changes in microvascular perfusion. Recently an accelerated method to (semi-)automatically quantify the number of perfused capillaries was presented, which may further its application for the study of microvascular function in large cohorts.

Ex-vivo microvascular vasoreactivity using pressure myography is an elegant method to study isolated effects of substances, or, as in the case of this thesis, tissues, on microvascular diameter regulation. This method was employed in chapters 4, 5, 6 and 7. To do this, microvessels are isolated, mounted on two glass cannulae, and subsequently pressurized at a level comparable to the in-vivo pressure. Because the vessel is not inside a living organism anymore, and not perfused, there is no influence of circulating adipokines or other tissues that may affect microvascular vasoreactivity. This has the advantage that the effect is not counterregulated by other factors, but one needs to be careful with translation to the in-vivo situation for the same reason. Furthermore, shear stress is also missing in this experimental configuration. Nevertheless, the in-vivo and ex-vivo results in this thesis generally pointed in similar directions. An important consideration with ex-vivo vasoreactivity is the preparation technique, which has to be increasingly careful with smaller vessels. Microvessels with a damaged endothelial layer will yield different results from those with intact endothelium, and therefore the in-vivo situation. One of the quality controls is therefore the minimal 10% vasodilation after the addition of acetylcholine, although this forgoes the fact that also
acetylcholine-induced vasodilation may also be impaired in certain conditions.63

In this thesis, we have combined human studies (chapters 3a, 3b and 7) with murine studies (chapters 4, 5 and 6). While studies in humans give direct insight into human physiology, and are therefore most robust, animal studies have the advantage of higher reproducibility and the possibility for a wider range of methodological options. The most studied animal in this thesis, is the C57Bl/6 mouse, but we also used Wistar rats (chapter 5) and AMPKα2−/− (chapter 4 and 5) and JNK2−/− (chapter 6) mice, but used the latter only to create chimaeric mice by bone-marrow transplantation. The isolated resistance arteries from AMPKα2−/− did not show insulin-induced vasodilation, either with PVAT, or with globular adiponectin. Nor did these mice show microvascular recruitment during a clamp, instead they showed a neutral response where C57Bl/6 would show vasodilation. Furthermore, they would exhibit vasoconstriction in conditions where C57Bl/6 mice would have a neutral response (e.g. without PVAT or adiponectin). Why these mice have altered microvascular responses may be elucidated by further research. But in general the experimental results were comparable, strengthening the observations made in this thesis.

In the animal studies (chapter 4, 5 and 6) we used all male mice and rats, whereas we studied women in chapter 7, and studied both women and men in chapter 3a and 3b. The results of the studies where we examined one gender may apply only to that gender, or be generally applicable. The reason to study only women in chapter 7 is to create a homogenous group. Especially when studying an adipose tissue depot, one should study either only women or only men, because there are known large differences in adipose tissue distribution between the two genders, with women having more subcutaneous adipose tissue and men predominantly visceral adipose tissue. Whether PVAT differs between men and women is currently unknown. The reason to study only women in chapter 7 is because the recruitment of obese women proved to be less troublesome than that of obese men.
Directions for further research

The results in this thesis support the idea that PVAT is involved in the regulation of insulin-induced vasoreactivity, however they do not provide conclusive evidence for the in vivo effects of PVAT, as we have only shown an ex-vivo effect, as well as statistical relations between PVAT-properties and in-vivo vasoreactivity. Future research should further delineate the pathways involved in the change of vasoactive properties of PVAT during nutrient excess, and the effect this in turn has on the vessel wall. Furthermore, the contribution of PVAT in-vivo, where adipokines from subcutaneous and abdominal adipose tissue circulate, needs to be addressed as well. Lipoatrophic mice could be an interesting model to do this, as circulating adipokines are not present.

To further study PVAT and endothelium in obesity, inducible knockouts could be used, where a single gene can be silenced or overexpressed in a specific tissue. This would allow us to disentangle the interactions between PVAT and the endothelium. It would be particularly interesting to test whether early modifications in the PVAT phenotype would prevent the endothelium from being affected by obesity, but also whether the endothelium could be spared from detrimental signals from PVAT, e.g. by using an constitutively active AMPKa2 in the endothelium.

Another interesting direction would be to study what would happen if the PVAT dysfunction and subsequent microvascular insulin resistance would not develop. In other words does (microvascular) insulin resistance prevent the tissues from nutrient excess at a cellular level? Unrestricted uptake of e.g. glucose might lead to excessive glycogen formation, which would have a strong osmotic action, perhaps causing the cell to swell and rupture. Alternatively, caloric restriction seems to increase longevity in humans and animals, insulin resistance may teleologically be a mechanism to promote caloric restriction at the cellular level. Insulin resistance could thereby be a physiological response with pathological consequences (i.e. hyperinsulinemia and ultimately type 2 diabetes and cardiovascular disease).

Examining the in-vivo contribution of PVAT to microvascular function in humans will be considerably more difficult. But when imaging techniques reach higher resolutions, it may be possible to study relations similar to the one performed in chapter 7 with the
histology performed in skeletal muscle biopsies, but without the need for the invasive skeletal muscle biopsy.

**Conclusion**

In conclusion, PVAT enhances insulin-induced microvascular recruitment in healthy lean individuals, and this effect is lost in obesity. Through the loss of this perfusion-stimulating effect in obesity, PVAT contributes to obesity-related insulin resistance. The loss of the vasodilator phenotype of PVAT is mediated by inflammation, and more specifically JNK2 positive cells from the bone marrow. The increased inflammatory state of PVAT reduces the secretion of AMPKa2 agonists (e.g. adiponectin), consequently the vasodilator pathway which is activated by insulin is blunted, and the vasoconstrictor pathway strengthened. The figure depicts the proposed pathways involved in the regulation of insulin-induced microvascular recruitment by PVAT. Perivascular adipose tissue is a new target to improve and protect microvascular endothelial function, but the mechanisms need to be further elucidated. Because obesity is still increasing at an alarming rate in the developed and developing world, a deeper insight into the pathogenesis of obesity’s cardiovascular and metabolic sequelae is paramount, further insight into PVAT may help constrain these problems.
Figure. Proposed regulatory role of PVAT on insulin-induced vasoreactivity and subsequent microvascular recruitment.

A. A schematic representation how insulin regulates insulin-induced microvascular recruitment in skeletal muscle. At the top, a cross-section of an arteriole is depicted in which insulin initiates two signaling cascades in the endothelial cells (orange) after binding to the insulin receptor (blue). Hereby insulin starts insulin-induced relaxation of the vascular smooth muscle cells (pink) of arterioles and resistance arteries in skeletal muscle. This results thereby in insulin-induced microvascular recruitment in that skeletal muscle (at the bottom). The vascular wall is covered in perivascular adipocytes (yellow). Some bone-marrow derived cells have infiltrated the PVAT (purple). IRS1/2=Insulin Receptor Substrate 1 and 2, PI3-k=Phosphoinositide 3-kinase, eNOS=endothelial Nitric Oxide Synthase, NO=Nitric Oxide, MAPK=Mitogen-activated protein kinase, ET-1=endothelin 1.

B. In lean, healthy women, there is less PVAT, and the individual adipocytes are smaller. More importantly, the adipocytes secrete adiponectin, an AMPKα2 agonist. Activation of AMPKα2 subsequently leads to inhibition of the insulin-induced endothelin-1 production, and enhancement of NO production. This shift in the balance between vasoconstrictor and vasodilator effectors results in vasodilation, and thereby insulin-induced microvascular recruitment. AMPKα2=5’-AMP-activated protein kinase subunit α2.

C. In obesity, the adipocytes from PVAT become larger and bone-marrow derived cells infiltrate the PVAT. Moreover, JNK2 is activated in these bone-marrow derived cells, leading to an inhibition of adiponectin production and increased endothelin-1 production. The resulting effect is either no insulin-induced vasomotor response, or even insulin-induced vasoconstriction. ‘X’=unknown mediating protein, JNK2=c-Jun NH₂-terminal Kinase 2.
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