Research Article

Presence of Vasculature Results in Faster Insulin Response in Adipocytes in Novel in vitro Vascularized Adipose Tissue Model

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Abstract

Besides being an energy storage, adipose tissue is an endocrine organ closely associated with vascular system. Human relevant in vitro models are needed to study adipose tissue and related diseases. Vasculature plays a central role in the development and inhibition of adipose tissue related diseases. Here, adipocyte culture was established from hASC (human adipose stromal cells), and a vascularized adipose tissue model was established from hASC and HUVEC (human umbilical cord vein endothelial cell) co-culture, utilizing the same differentiation procedure. Using these models together allowed analysis of the effect of vascularization on adipocytes. Adipocyte culture and Vascularized adipose tissue model were characterized on gene (adipocyte and vasculature-related), protein (von Willebrand factor, CollagenIV, CD140b and CD144, secretion of leptin, adiponectin and FABP4) and functional (triglyceride accumulation, glucose uptake and lipolysis) levels. Additionally, vascularized adipose tissue model was exposed to chemicals with known effects on adipogenesis and angiogenesis (rosiglitazone, chlorpyrifos, prochloraz, thiazolidinediones, have been problematic in developing vascularized adipose tissue as they have multiple negative effects on...
endothelial cells, including delayed growth (Kang et al., 2009). Volz et al. (2018) developed an elegant, fully serum-free defined medium for in vitro vascularized adipose tissue, but the need to pre-differentiate the adipocytes prior to combining the endothelial cells into the culture hampers its usability and lengthens the culture time.

In present study, a novel vascularized human adipocyte tissue model was established. This vascularized adipose tissue adipocyte model is based on protocols of our previously established insulin-sensitive adipocytes (Huttala et al., 2016) and vascularized adipose tissue model (Huttala et al., 2018). All these models utilize the adipose tissue extract (ATE) which is natural adipogenesis inducer that also enables angiogenesis (Sarkanen et al., 2012a). The novel vascularized adipose tissue model was compared to adipocyte culture that was established from human adipose stromal cells (hASC), and the vascularized adipose tissue model was established from hASC and human umbilical cord vein endothelial cells (HUVEC) co-culture, using the same differentiation procedure. The use of the same differentiation procedure for both provides a reliable model pair for studying the effect of vasculature on adipocytes. With the vascularized adipose tissue model, effects of chemical on both angiogenesis and adipogenesis can be investigated simultaneously.

In order to ensure that the vascularized adipose tissue model is suitable for human adipose tissue research, it was characterized comprehensively. The gene expression of markers of adipocyte tissue and vasculature, secretion of adipokines produced in in vivo white adipose tissue (leptin, FABP4 and adiponectin), accumulation of triglycerides, insulin responses (inhibition of lipolysis and induction of glucose uptake) and proper vascular formation were investigated. In addition, the proper function of the vascularized adipose tissue model was confirmed by using reference chemicals with known effects on angiogenesis and adipose tissue angiogenesis. Nine chemicals (rosiglitazone, chlorpyrifos, prochloraz, mancozeb, butylparaben, 15-deoxy-Δ12,14-prostaglandin J2, bisphenol A, bis-(2-ethylhexyl) phthalate, tributyltin chloride) were used to ensure the correct adipogenic and angiogenic responses of the vascularized adipose tissue. Three of the chemicals (rosiglitazone, chlorpyrifos and prochloraz) on both the adipocyte culture and vascularized adipose tissue model to determine the impact of vasculature on the chemical effects.

2 Materials and methods

2.1 Ethical considerations

This in vitro study conforms to the ethical principles outlined in the Declaration of Helsinki. The human adipose tissue samples were obtained from the excess material of surgical operations and human umbilical cords were received from caesarean sections with written informed consents at Tampere University Hospital, Tampere, Finland. The use of human adipose stromal cells (hASC) and human umbilical cord endothelial cells (HUVEC) were approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland with permit numbers of R15161 and R15033, respectively.

2.2 Cells

hASC used in the study were heterogeneous cell populations obtained by isolating the stromal vascular fraction cells of human adipose tissue. HUVEC used in the study were isolated from umbilical cords. hASC and HUVEC were isolated as described by Sarkanen et al. (2012b). Before cryopreservation, all cell batches were tested for mycoplasma using Mycoplasma kit (Mycoblastik Detection Kit, Lonza Group LTD, Basel Switzerland). Only Mycoplasma-free cell batches were used. hASC were characterized for markers CD73, CD90 and CD105 (BD biosciences, Franklin Lakes, NJ, USA) using flow cytometer FACSCanto II (BD Biosciences) as published previously (Huttala et al., 2015). hASC were expanded in hASC medium (Tab. 1) and HUVEC in Endothelial Cell Growth Medium-2 BulletKit (EGM-2, Lonza). In the co-cultures, hASC were at passage 2 and HUVEC at passage 4.

2.3 Adipose tissue extract

Adipose tissue extract (ATE) was produced as described previously (Huttala et al., 2018). Briefly, the human adipose tissue sample was manually cut into small pieces and incubated in DMEM/F12 (Gibco, Carlsbad, CA, USA) for 24 hours in 37 °C. Resulting extract was filtered through 0.2 μm filter. The protein content of the extract was determined with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer’s instructions using bovine serum albumin (BSA) as a standard. Results were measured after 30 min incubation at 37 °C at 562 nm with Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific).

2.4 Vascularized adipose tissue model

The culturing scheme of the vascularized adipose tissue model can be seen in Tab. 2 and the different media composition in Tab. 1. Undifferentiated control contained hASC and HUVEC cells cultured in basic media corresponding to the media used in the differentiation (hASC medium corresponding to ATE medium, SFM corresponding to SM and MM). The adipocyte culture and the vascularized adipose tissue model were produced using a protocol published previously as “protocol 7” (Huttala et al., 2016) with some modifications: one additional medium change and one additional macromolecule component (Ficol-paque plus) were included. Day 0, hASC were plated in hASC medium on the density of 20 000 cells/cm². Tryple™ express (Gibco) was used for the detachment of cells. On days 1, 4, 8 and 11 the media were changed according to Tab. 2. Total medium volume used per 48 well

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1 Abbreviations: hASC, human adipose stromal cells; HUVEC, human umbilical cord vein endothelial cells; ECM, extracellular matrix; ins, insulin; SFM, serum free medium; ATEm, ATE medium; SM, stimulation medium; MM, maintenance medium (SM without insulin and troglitazone)
plate well was 500µl and after each medium change macromolecule Ficol-paque plus (GE Health care, Buckinghamshire, UK) was added into each well 25 µl per 500 µl of culture media. For the vascularized adipose tissue model; on day 7, the HUVEC (4400 cells/cm²) were plated by first removing 100 µl of culture media from the wells and then adding the HUVEC in 100 µl of EGM-2 (Lonza). Analyses were performed on day 14.

### 2.5 Effect of chemicals on the properties and functions of vascularized adipose tissue model

Tab. 3 shows the used chemicals and their final concentrations in the test. Vascularized adipose tissue model was exposed to test chemicals on day 8 to investigate the effect of study chemicals on both adipogenesis and angiogenesis. After one week of exposure the analyses (viability/mitochondrial activity, lipid accumulation, amount of vasculature and with three chemicals also the adipocyte related gene expression) were performed. The actual purity of the chemicals was used in the dilution calculations. Vehicle controls for all chemicals were exposed to the same concentration of solvent that was present in the chemical dilutions.

Both adipocyte culture and vascularized adipose tissue model were exposed to three chemicals (on day 8): Rosiglitazone, Chlorpyrifos and prochloraz, to investigate the impact of vascularization on the effects of the chemicals on adipocyte lipid accumulation and adipocyte related gene expression. The impact of chemicals on angiogenesis was also investigated. Three independent experiments with two parallels were performed.

To further evaluate the performance of the vascularized adipose tissue model, Mancozeb, Butylparaben, 15-Deoxy-Δ12,14-

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### Tab. 1: List of cell culture media and their components and manufacturers

<table>
<thead>
<tr>
<th>Medium</th>
<th>Abbreviation</th>
<th>Content</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hASC medium</td>
<td>-</td>
<td>DMEM/F12 10 % Human serum 2 mM L-glutamine</td>
<td>Gibco PAA laboratories, Gibco</td>
</tr>
<tr>
<td>Serum free medium</td>
<td>SFM</td>
<td>DMEM/F12 2.56 mM L-glutamine 0.1 mM 3,3’,5-Triiodo-L-thyronine sodium salt ITS™ Premix: - 1.15 µM: 6.65µg/ml insulin - 6.65 µg/ml Transferin - 6.65 ng/ml selenious acid 1 % Bovine serum albumin (BSA) 2.8 mM Sodium pyruvate</td>
<td>Gibco Gibco Sigma BD biosciences PAA laboratories Gibco</td>
</tr>
<tr>
<td>ATE medium</td>
<td>ATEm</td>
<td>1800 µg/ml Adipose tissue extract (ATE) DMEM/F12 10 % Human serum 2 mM L-glutamine 50 IU/ml Penicillin/ 50 µg/ml streptomycin</td>
<td>Gibco PAA laboratories Gibco Gibco</td>
</tr>
<tr>
<td>Stimulation medium</td>
<td>SM</td>
<td>Serum free medium supplemented with 200 µg/ml Ascorbic acid 0.5 µg/ml Heparin 5.5 µM: 2 µg/ml Hydrocortisone/ cortisol 10 ng/ml Vascular endothelial growth factor 1 ng/ml Fibroblast growth factor β 9 µM Troglitazone</td>
<td>Sigma Sigma Sigma Sigma R&amp;D systems R&amp;D Systems</td>
</tr>
<tr>
<td>Maintenance medium (stimulation medium without insulin and troglitazone)</td>
<td>MM</td>
<td>DMEM/F12 2.56 mM L-glutamine 0.1 mM 3,3’,5-Triiodo-L-thyronine sodium salt 6.65 µg/ml Transferin 6.65 ng/ml Selenious acid 1 % BSA 2.8 mM Sodium pyruvate 200 µg/ml Ascorbic acid 0.5 µg/ml Heparin 2 µg/ml Hydrocortisone 10 ng/ml VEGF 1 ng/ml FGF2</td>
<td>Gibco Gibco Sigma Sigma Sigma Sigma Sigma Sigma Sigma Sigma Sigma Sigma Sigma R&amp;D Systems R&amp;D Systems</td>
</tr>
</tbody>
</table>

### Tab. 2: Culture schedule for the undifferentiated control, adipocyte culture and vascularized adipose tissue model.

<table>
<thead>
<tr>
<th>Name</th>
<th>Day0</th>
<th>Day1</th>
<th>Day4</th>
<th>Day7</th>
<th>Day8</th>
<th>Day11</th>
<th>Day14</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undifferentiated cells</td>
<td>hASC seeded in hASC medium on 48 well plate 20,000 cells/cm²</td>
<td>hASC medium+ 25 µl Ficol</td>
<td>SFM+25 μl Ficol</td>
<td>HUVEC in EGM-2: 4400 cells/cm²</td>
<td>SFM+25 μl Ficol</td>
<td>SFM+25 μl Ficol</td>
<td>SFM+ 25 μl Ficol</td>
<td>Analysis</td>
</tr>
<tr>
<td>Adipocyte culture</td>
<td>hASC medium+ 25 µl Ficol</td>
<td>SFM+25 μl Ficol</td>
<td>HUVEC in EGM-2: 4400 cells/cm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascularized adipose tissue model</td>
<td>hASC medium+ 25 µl Ficol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATEm+25 µl Ficol</td>
<td>SM+25 µl Ficol</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
prostaglandin J2, Bisphenol A, bis-(2-ethylhexyl) phthalate and tributyltin chloride were tested for their effect on lipid accumulation, viability/mitochondrial activity and amount of vasculature. Two independent experiments with two parallels were performed. Half maximal effective concentration (EC50) values were calculated using GraphPad Prism 6.05 software.

2.6 Mitochondrial activity, triglyceride accumulation and protein secretion

Mitochondrial activity, as an index of living cells, was analyzed by WST-1 (Roche Diagnostics, Basel, Switzerland) with one-hour incubation. Absorbance was measured at 450 nm with Varioskan flash multimode reader (Thermo Fisher Scientific). The WST-1 results are used as they are, without normalization to depict the cell numbers as well as mitochondrial activity.

Triglyceride accumulation to the cell cultures was measured with Adipored assay reagent (Lonza) with 10 min incubation at room temperature. The radioactivity of the samples was measured by liquid scintillation counter Wallac1410 (Perkin Elmer) using OptiPhase HiSafe 2 oscillation liquid.

2.7 Glucose uptake and lipolysis

The glucose uptake test was performed as follows. On day 14, DMEM/F12 (Gibco) was changed on the cells and incubated for 2 h in 37 °C 5 % CO2. Then cells were exposed to 100 nM or 500 nM insulin and incubated in 37 °C 5 % CO2 for 30 min and [3H]-2-deoxy-D-glucose (0.2 μCi/well, Perkin Elmer, Waltham, MA, United States) was added for another 20 min. The cells were lysed using 0.1% sodium dodecyl sulfate. The radioactivity of the samples was measured by liquid scintillation counter Wallac1410 (Perkin Elmer) using OptiPhase HiSafe 2 oscillation liquid (Perkin Elmer).

Lipolysis i.e. glycerol release was analysed using EnzyChrom Adipolysis Assay Kit (BioAssay Systems, Hayward, CA, USA) according to manufacturer’s instructions. On day 14 fresh DMEM/F12 (Gibco) was changed. After one hour, 2μM Isoproterenol was added and incubated for two hours after which 100 nM or 500 nM insulin (Sigma, Saint Louis, MO, USA) was added. After 15 min or 30 min incubation medium was collected and the fluorescence was measured with Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific) at I0 and I∞.

2.8 Visualization of vasculature by immunocytochemical stainings

The immunocytochemical staining was performed as described earlier (Hurtlala et al., 2015) except the fixative used here was 4 % formaldehyde at room temperature for 20 minutes. Antibodies used were anti-von Willebrand factor IgG (produced in rabbit, Sigma), anti-Collagen IV IgG (produced in mouse, Sigma), FITC-labeled goat polyclonal antibody anti-mouse IgG (Sigma), TRITC-labeled goat polyclonal antibody anti-rabbit IgG (Sigma). In addition, for confocal imaging anti-collagen IV (produced in Rabbit, ab6586, Abcam, Cambridge, UK), CD140b-PE (BD Biosciences, 558821) and CD144-FITC (BD Biosciences, 560411) were used. Nuclei were stained by DAPI in Fluoroshield™ with DAPI (Sigma).

2.9 Microscopy and quantification of the vascular structures

Microscopic imaging was done with Nikon Eclipse Ti-s inverted fluorescence microscope (Nikon, Tokyo, Japan) and Nikon digital sight DS-2/-2–camera (Nikon) and images were further processed with NIS Elements (Nikon), ZEN 2012 software (Carl Zeiss, Oberkochen, Germany). Confocal imaging was done with LSM710 and with Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss) and images were further processed with ZEN 2012 software (Carl Zeiss). All image types were further processed using Adobe Photoshop CS3 software (Adobe Systems Incorporated, San Jose, CA, United States).
In order to investigate the area of tubular networks, cell cultures were imaged with Cell-IQ (CM Technologies Oy, Tampere, Finland) as described earlier (Huttala et al., 2018). Briefly, culture plates were imaged with 10x objective and 5x5 grid per well. Grids were stitched together with Cell-IQ Analyzer (CM Technologies Oy) and further analyzed with ImageJ software (The National Institutes of Health (NIH), Bethesda, MD, USA). Images were converted to an 8-bit gray scale, background was subtracted and binary threshold was adjusted to determine the total tubule area in pixels. By size and circularity restrictions, the fluorescence from adipocytes was removed from the count. This analysis method does not take length, number or branch points of structures into consideration. Hence, the analysis focuses only on changes in the area covered by the vascular network.

2.10 Gene expression studies
Expression of adipose tissue related genes Glut1, PPARγ, leptin, Glut4, FABP4, PPARα, adiponectin and PPARγ2 was studied as follows. Gene expression studies of insulin exposed cultures were performed by adding 500 μM insulin for 24 hours into the culture prior to RNA isolation. RNA isolation was performed using the Purelink RNA mini kit (Invitrogen, Carlsbad, CA, USA) and PCR was performed with iTaq universal SYBR green one-step kit (Biorad, Hercules, CA, USA), both according to the manufacturer’s instructions. Each reaction contained 30 ng of the template, and primer concentration was 300 nM. Primer sequences and annealing temperatures can be seen in Tab. S1. Melt curve analysis was performed in each run. PCR amplification was performed with CFX96 Real-Time System (Biorad). Results were calculated with ΔΔCT method 36B4 as housekeeping gene. Unexposed and insulin exposed samples were analyzed using undifferentiated cells as control. The chemical exposed cultures were compared to corresponding vehicle control.

2.11 Fluidigm analysis of vessel related genes
The developmental stage of vasculature in the model was further analyzed with a panel of 30 genes by Fluidigm system. Genes were VEGFA, VEGFR-2/FLK-1, VEGFR-1/FLT-1, FG2, FGFR2, EGFL7, EGFR, DLL4, Notch 1, Notch 4, AGGF1, PDGF, PDGFRβ, ANGPT1, ANGPT2, TIE2, Dkk1, SMO, TGFβ, TGFBR1, CD34, VE-cadherin 5, GJA1, Occludin, Fibronectin, ITAG5, CSPG4, NG2, S1PR1, enOS and Hprt1. Results were concentrations based on the standard curve and they were normalized to housekeeping gene GAPDH.

Integrity of the RNA samples was ensured by using 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the concentration of the samples was measured with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). cDNA synthesis was performed using Reverse Transcriptase Master mix (Fluidigm, South San Francisco, CA, USA) according to the manufacturer’s instructions and using T100 thermal cycler (Biorad). The no template control contained water instead of Total RNA. In the No amplification controls, the Reverse Transcriptase Master Mix was omitted. The cDNA synthesis reaction was performed with 50 ng of total RNA. Pre-amplification was performed using preAmp Master Mix (Fluidigm) and the instructions provided from Fluidigm for “Fast Gene Expression Analysis Using EvaGreen” using annealing temperature of 60°C and T100 thermal cycler (Biorad).

Primers were designed with Primer3 software. Amplicons were designed to overlap intron sequences when possible. Amplicons were designed to be between 100-250 bp in length. Exonuclease I treatment was performed with Exonuclease I (E.coli) (Lot no 0201507, New England Biolabs, Ipswich, MA, USA) and T100 thermal cycler (Biorad). After the Exonuclease treatment 10 mM Tris-0.1 mM EDTA was added to give a 10-fold dilution of the cDNA. No template control and No amplification control samples.

For the gene expression analysis, 96.96 Dynamic Array Chip for Gene Expression (Fluidigm) was utilized according to manufacturer’s instruction using Fluidigm IFC ControllerHX (Fluidigm) and BioMark HD (Fluidigm). Sample pre-mixes were prepared by mixing 2x SsoFast EvaGreen Supermix with low ROX (Biorad), Supermix with low ROX (Biorad 172-5211), 20X DNA Binding Dye (Fluidigm 100-7609), Preamplified and Exo 1-treated samples. Sample pre-mixes were then added to sample inlets of the primed 96.96 IFC. Assay mixes contained 2X Assay Loading Reagent (Fluidigm, 100-7611), 10 mM Tris-0.1 mM EDTA buffer, 100 μM forward and reverse primer mix (primer sequences Tab. S2). Assay mixes were added to assay inlets of the primed 96.96 IFC. The 96.96 IFC was then loaded to the Fluidigm Biomark HD selecting; Gene Expression, ROX, single probe, EvaGreen and GE 96x96 PCR+Melt v1 Thermal protocol. Results were analyzed using Fluidigm’s BioMark Data Collection Analysis program (version 4.1.5).

2.12 Statistical analysis
All calculations were made with Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and statistical analyses performed in GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Results are depicted as mean ± Standard deviation. Differences were considered significant when *p<0.05 (significant), **p<0.01 (very significant) and ***p<0.001 (extremely significant). Results from triglyceride accumulation (n=5), ELISA (n=6), Glucose uptake (n=5), lipolysis (n=3) and one-step RT-qPCR (n=6, in insulin exposure n=3) were subjected to one-way analysis of variance (ANOVA), followed by Fisher’s LSD test.

3 Results
3.1 Adipocyte culture and vascularized adipose tissue model show similar lipid accumulation and secretion of adiponectin, leptin and FABP4
The characteristics of vascularized adipose tissue model were compared to adipocyte culture. These both were differentiated with modified protocol of the previously published in vitro adipocyte model (Huttala et al., 2016). Both adipocyte culture and vascularized adipose tissue model had lipid accumulating cells which the undifferentiated cells did not contain (Fig. 1). In morphological inspection, the lipid accumulation of vascularized adipose tissue model was more evenly spread out than in adipocyte culture, where there were lesser number of adipocytes but those contained larger lipid storages. This morphology was also seen in previous

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2 doi:10.14573/altex.1811271s
study (Huttala et al., 2018) but could not be confirmed by the quantitative results on lipid per cell measurements (Fig. 1). Out of the studied secreted proteins adiponectin, leptin and FABP4, adiponectin showed significant increase in the vascularized adipose tissue model (Fig. 1). The secretion of the other two proteins did not change whether vessels were present or not.

![Morphology, lipid accumulation and secretion of adiponectin, leptin and FABP4 in vascularized adipose tissue model at day 14](image)

Fig. 1: Morphology, lipid accumulation and secretion of adiponectin, leptin and FABP4 in vascularized adipose tissue model at day 14

3.2 Faster lipolysis response to insulin in vascularized adipose tissue model
The responses to insulin were studied in both adipocyte culture and vascularized adipose tissue model to see whether there was a difference in the presence of vasculature. Inhibition of lipolysis was found to be faster in vascularized adipose tissue model; inhibition could be seen after 15 min at 500 nM insulin concentration (Fig. 2). In adipocyte culture, the response was seen after 30 min. Both cultures increased glucose uptake in response to insulin equally (Fig. 2).

When comparing vascularized adipose tissue model and adipocyte culture at gene expression level, significant differences were found in the upregulation of Glut1 and leptin in vascularized adipose tissue model (Fig. 3). However, the secreted leptin did not show difference between the cultures (Fig. 1). In gene expression analysis of the effect of insulin and vascularization, Glut1 was shown to be significantly down regulated in response to insulin (Fig. 3). In addition, trend of upregulation by insulin can be seen in Glut4 and FABP4 for both cultures. Leptin and adiponectin increase in vascularized adipose tissue model in response to insulin. PPARα, PPARγ2 and general PPARγ did not seem to respond to insulin.

![Response to insulin in adipocyte culture and vascularized adipose tissue model at day 14](image)

Fig. 2: Response to insulin in adipocyte culture and vascularized adipose tissue model at day 14
A) Change in glucose uptake in response to 100 nM and 500 nM insulin. B) Inhibition of lipolysis after 15 min and 30 min of 1000 and 500 nM insulin exposure. ins=insulin

3.3 Markers of different stages of vascular formation present in vascularized adipose tissue model
The vascularization was analyzed with immunostaining and expression analysis of panel of angiogenesis related genes. The gene panel was also analyzed from insulin-exposed samples. The vascular network was well formed in the vascularized adipose tissue model as seen in the Collagen IV staining in Fig. 4. Both CD144 i.e. v-cadherin and CD140b i.e. PDGFRb were expressed as they should in well-developed vasculature (Fig. 4). CD144 junctions are located in the tubules and CD140b on the surface of the tubules. CD140b indicates presence of pericytes that have been shown to be present in the vascular structures in previous publication (Huttala
et al., 2015). Gene expression results show presence of ongoing angiogenesis as well as mature tubules (Tab. S3). The individual genes which were upregulated (2 fold or more compared to the non-insulin treated cultures) in response to insulin were PDGF, FGFR2 and ve-cadherin 5 (Tab. S3). In vascularized adipose tissue model prior to insulin exposure, PDGF and ve-cadherin were expressed in lower level than the housekeeping gene. FGFR2 was upregulated also before insulin exposure.

Fig. 3: Expression of adipose tissue related genes (Glut1, PPARγ, leptin, Glut4, FABP4, PPARα, adiponectin and PPARγ2) with or without 500 nM insulin in both adipocyte culture and vascularized adipose tissue model at day 14 ins=insulin

3.4 The effect of adipogenesis stimulator seen in gene expression changes
Rosiglitazone was used to test the response of the vascularized adipose tissue model to adipogenesis stimulators (Fig. 5). Exposure time was 7 days. The mitochondrial activity/viability determined by WST-1 showed increase for adipocyte culture (EC50 7.97 µM) but no effect was seen in vascularized adipose tissue model in the tested concentration range of 0.0100-140 µM. Only slight increase was seen in lipid accumulation, vascularized adipose tissue model responding at higher concentration than adipocyte culture. There was increase in tubules in the lower concentrations up to 1.4 µM but no statistically significant angiogenic effect was seen at the studied concentration range of 0.0100-140 µM but. Vascular network was intact and well branched in all exposed cultures. Gene expression of FABP4 and adiponectin greatly increased with 4.4 µM Rosiglitazone compared to the corresponding unexposed cultures in both adipocyte culture and vascularized adipose tissue model. In the vascularized adipose tissue model, also the expression of leptin was increased. Glut4 was also slightly upregulated in both cultures.
Fig. 4: Confocal images of the vascularization in vascularized adipose tissue model at day 14
Top row: CD144 (green) i.e. ve-cadherin junctions can be found in the tubules surrounded by collagen IV stained basement membrane (red). Nuclei stained with DAPI (blue). Bottom row: CD140b (green) i.e. PDGFRb staining aligns along the tubules together with Collagen IV staining (in red). Scale bars 100µm.

Fig. 5: Adipocyte culture and vascularized adipose tissue model exposed to rosiglitazone
A) Mitochondrial activity/viability measured with WST-1. B) Lipid accumulation analysed by AdipoRed. C) The area of vasculature shown on few example concentrations of rosiglitazone. D) Gene expression of Adipocytes and Vascularized adipose tissue model after 4.4 µM Rosiglitazone exposure. Red line indicates the level of unexposed corresponding cultures.
3.5 Effects of prochloraz and chlorpyrifos on vascularized adipose tissue model

Effects of prochloraz and chlorpyrifos were studied in the similar manner to rosiglitazone with 7 days exposure time. Prochloraz was toxic at EC50 of 182.2 µM with adipocyte culture and 181.6 µM on the vascularized adipose tissue model (Fig. 6). Inhibition of lipid accumulation for adipocyte culture reached EC50 at 164 µM and for vascularized adipose tissue model at 187.2 µM. No effect on vasculature was observed at tested concentrations of 0.01-5000 µM. The exposure to 50µM Prochloraz increased the expression of PPARα significantly in the vascularized adipose tissue model. Other studied markers were expressed on a lower level than in the unexposed cultures.

Chlorpyrifos caused viability/mitochondrial activity to increase at EC50 of 48.54 µM for adipocyte culture and 73.51 µM for vascularized adipose tissue model (Fig. 6). Chlorpyrifos seemed to inhibit lipid accumulation in lower concentrations and increase it in the higher concentrations before inhibiting it again. No effect on the amount of vasculature was detected. Expression of leptin was significantly higher in vascularized adipose tissue model exposed with 10 µM Chlorpyrifos than in adipocyte culture.

![Fig. 6](image): Adipocyte culture and vascularized adipose tissue model exposed to prochloraz and chlorpyrifos
A) Expression of selected genes after the exposure to 50 µM prochloraz. Red line indicates the level of unexposed cells. B) Mitochondrial activity/viability measured with WST-1 (EC50 of 182.2 µM in adipocyte culture and 181.6 µM for vascularized adipose tissue model) C) Lipid accumulation measured with AdipoRed (EC50 of 48.54 µM for adipocyte culture and 73.51 µM for vascularized adipose tissue model) D) Expression of selected genes after the exposure to 10 µM Chlorpyrifos. Red line indicates the level of unexposed cells. E) Mitochondrial activity/viability. F) Lipid accumulation.

3.6 Results of further chemical tests

To test further the responses of the vascularized adipose tissue model, the model was exposed to six more chemicals for 7 days. These were analyzed for the lipid accumulation, mitochondrial activity and amount of vasculature only in the vascularized adipose tissue model. Results of these chemicals can be found in Tab. 4. The effect on angiogenesis is determined from the changes in the total area of vasculature.
Tab. 4: EC50 values for chemicals given by vascularized adipose tissue model. EC50, Half maximal effective concentration

<table>
<thead>
<tr>
<th>Chemical</th>
<th>EC50 Mitochondrial activity</th>
<th>Mitochondrial activity (Increase/decrease)</th>
<th>EC50 lipid accumulation</th>
<th>Adipogenesis (Inhibitor/stimulator)</th>
<th>Angiogenesis (Increase/decrease)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mancozeb</td>
<td>96.24 µg/ml</td>
<td>decrease</td>
<td>84.91 µg/ml</td>
<td>inhibitor</td>
<td>increase at non-toxic concentrations</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>433.8 µg/ml</td>
<td>decrease</td>
<td>340.8 µg/ml</td>
<td>inhibitor</td>
<td>no effect</td>
</tr>
<tr>
<td>15-Deoxy-A12,14-prostaglandin J2</td>
<td>2.675 µg/ml</td>
<td>increase</td>
<td>0.1038 µg/ml</td>
<td>inhibitor</td>
<td>increase in higher concentrations</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>65.3 µg/ml</td>
<td>decrease</td>
<td>0.5442 µg/ml</td>
<td>inhibitor</td>
<td>increase at higher non-toxic concentrations</td>
</tr>
<tr>
<td>Bis-(2-ethylhexyl) phthalate</td>
<td>0.1634 µg/ml</td>
<td>decrease</td>
<td>0.03 µg/ml and 10 µg/ml</td>
<td>inhibitor (biphasic effect)</td>
<td>significant increase</td>
</tr>
<tr>
<td>Tributyltin chloride</td>
<td>106.2 ng/ml</td>
<td>increase</td>
<td>312.8 ng/ml</td>
<td>Stimulator</td>
<td>inhibition in lower concentrations</td>
</tr>
</tbody>
</table>

4 Discussion

Our previous study found two suitable methods for combining adipocytes and vascularization to produce vascularized adipose tissue model (Huttala et al., 2018). From these previously published methods, one was further modified here to reach mature adipocyte marker expression profile at the end of the 14-day culture period. In the vascularized adipose tissue model presented here utilized the same differentiation protocol that produced insulin responsive adipocytes (Huttala et al., 2016) with few modifications. The differences to the previously published protocol (Huttala et al., 2016) were the additional medium change at day 8 and the addition of macromolecule ficol to the media. Now this differentiation protocol can be used for differentiating adipocyte culture from hASC and inducing both angiogenesis and adipogenesis in co-culture of hASC and HUVEC. This allows the proper comparison of the effects of vascularization in adipose tissue in for example mechanistic studies, as the only variable between adipocyte culture and vascularized adipose tissue model is the presence of vascularization.

This in vitro human vascularized adipose tissue model was studied at gene, protein and functional levels and results were compared to the adipocyte culture. Functional tests included insulin and chemical responses. The vascularized adipose tissue model was exposed to nine chemicals and their effect on lipid accumulation, mitochondrial activity/viability and vascularization was quantified. Both adipocyte culture and vascularized adipose tissue model were exposed to three of these chemicals, i.e., rosiglitazone, prochloraz and chlorpyrifos; and these cultures were also analyzed for gene expression.

The presence of the key biological characteristics confirmed that the vascularized adipose tissue model mimics the in vivo adipose tissue characteristics and functional properties well. The adipocytes in the model accumulated triglycerides and showed upregulation of adipocyte related genes PPARγ and PPARγ2, adiponectin, FABP4, leptin and Glut4. Those selected genes, which are less relevant in adipocytes (Glut1 and PPARα) were not upregulated and hence correlate with human white adipose tissue expression profile. The adipocytes also secreted leptin, FABP4 and adiponectin; proteins produced by native adipose tissue. The results showed that lipolysis was inhibited and glucose uptake increased as a response to insulin as they do in vivo. Vascularization was seen to induce a faster response to insulin in lipolysis and also had effect on responses to chemicals. Based on these parameters the vascularized adipose tissue model depicts well the characteristics of adipose tissue.

The importance of vascularization in adipose tissue has been shown in multiple studies. Especially the changes in vasculature in disease conditions are important step in disease progression. Vasculature has been shown to counteract obesity and even insulin resistance when induced with VEGF (Robciuc et al., 2016; Elias et al., 2012). In our study the results showed that adding vascularization into the model, it induced upregulation of Glut1 and leptin. This would also indicate the anti-obesity effect of vasculature, as leptin is hormone regulating energy balance suppressing food intake and thereby inducing weight loss (Halaas et al., 1995). Glut1 on the other hand is a non-insulin dependent glucose transporter, which is found in variety of tissues including fetal and adult tissues; red blood cells, endothelia and muscle (Huang et al., 2015; Ciaraldi et al., 2005; Olson and Pessin, 1996). Hence the seen upregulation is likely due to the presence of Glut1 in the formed vascular endothelial cells. The angiogenic vessels are known to produce various cytokines and growth factors that promote adipogenesis (Varzaneh et al., 1994), which further enable the success of the adipogenesis process.

When developing relevant adipose tissue cell models, the source of cells is crucial. In 2016 Ruiz-Odeja et al. noted that most of in vitro adipose models are still performed with mouse 3T3-L1 cells (Ruiz-Ojeda et al., 2016). These are stable cell lines but not suitable when aiming for the human relevant models. For human models, one commonly utilized cell type is the human adipose stromal cell population isolated from human fat (Ruiz-Ojeda et al., 2016). These are often primary cells as the ones used here in our study.

The use of primary cells has its concerns, as there is the possibility of lot-to-lot variations. When using primary cells, each isolated cell lot should be tested against quality criteria known for the cells in question. With increasing knowledge of cell type specific marker expression, more relevant quality control criteria can be set to the cells. One recent example is the marker CD142 that has been found be expressed in hASC which can suppress adipogenesis (Schwalie et al., 2018). Through quality control the cells positive for CD142 could be sorted out of the population when studying adipogenesis. On the other hand, the diversity of the population could well be advantage making the population more relevant for human studies.
The role of extracellular matrix (ECM) composition has been brought up in the context of in vitro models and differentiation of stem cells. Our vascular network has been shown to produce own ECM in the culture (Huttala et al., 2015) and the staining for collagen IV was positive in the current vascularized adipose tissue model. Gene expression results (Tab. S3) showed that the vasculature contains tubules in all different developmental phases of angiogenesis including mature vascular structures expressing PDGFRb and ANPGT1, which would indicate that the tubules also contain basal lamina layer. Due to the vast vascularization of adipose tissue, the basal membrane collagen IV is one component of natural adipose tissue ECM (Nakajima et al., 2002). As the vascularized adipose tissue model is expressing variety of angiogenesis related genes it can be utilized for studying the angiogenic potential of chemicals in addition to adipogenic properties.

The utilization of macromolecular crowding in the cultures has been shown to enhance the accumulation of ECM in cultures (Zeiger et al., 2012; Chen et al., 2011). We used ficol as macromolecule, aided with presence of BSA in the medium. Combination of these large molecules had similar effect to the culture that is aimed for in the concept of macromolecular crowding. Protein concentrations of in vivo interstitial fluids could be 30-70 g/l (Bates et al., 1993) and although the concentrations used here were not high enough to lead to corresponding in vivo protein concentrations, the effect was seen in the better handling properties of the resulting model. The premature detachment issues encountered earlier (Huttala et al., 2018) were not found now with the use of ficol. This would indicate that ECM proteins were accumulated to the cultures which provided more attachment sites of the cells. Detachment of cells in tissue models is a serious problem when considering the applicability and reliability of the model. Detection of the possible effect of the chemical on the adipogenesis and/or angiogenesis would be impossible if the cells detached. Previous studies have reported that serum free medium combined with macromolecular ficol crowding is not beneficial for the cells (Patrikoski et al., 2017), the effects seen in our study were positive probably due to the lower concentration of the crowders. Serum free medium would be beneficial as it enables defined culture conditions. Serum free defined medium has already previously been shown to produce vascularized adipose tissue in vitro (Volz et al., 2018).

In addition to the natural composition of the in vivo environment, the stiffness of the surface on which the cells grow on influences the cells. It has been shown that cells grown on low stiffness matrix are more likely to become adipocytes than cells seeded on stiffer matrices (Winer et al., 2009; Chum et al., 2006). The ECM produced by the vasculature would lead to the low stiffness platform for the adipocytes to grow on. The vascularized adipose tissue model presented here shows improvement in the expression of key adipose tissue genes compared to the previously published (Huttala et al., 2018). The model presented here also shows secretion of adipocyte proteins leptin, adiponectin and FABP4, that are secreted from young (few lipid vacuoles) and mature (one big lipid vacuole) adipocytes, indicating the relevance of this model on depicting mature white adipose tissue.

The interactions between tissues are important in creating in vivo like in vitro tissue models. The influence of these interactions is seen for example in altered insulin responses in muscle cells caused by adipokines secreted from adipocytes (Kudoh et al., 2018). In native adipose tissue, insulin represses lipolysis in adipocytes (Choi et al., 2010) and leads to translocation of Glut4-containing vesicles from intracellular storage sites to the plasma membrane where they dock and fuse with the membrane (Bryant et al., 2002; Watson and Pessin, 2007) for glucose uptake. To ensure the central insulin responses were present in the culture, both adipocyte culture and vascularized adipose tissue model were exposed to insulin. Responses to insulin were confirmed by the increased uptake of glucose and inhibition of lipolysis. The lack of further increase in glucose uptake with higher insulin concentration (Fig. 2) is most likely due to a negative feedback response to excessive insulin hyperstimulation (Ma et al., 2013).

Response time to lipolysis inhibition was found to be shorter in vascularized adipose tissue model. As discussed above, the increase in vasculature has previously been shown to protect from insulin resistance (Robciuc et al., 2016; Elias et al., 2012) and our results in faster responses seem to support these findings. In our results (Fig. 2) the level of secreted triglycerides was slightly higher in Vascularized adipose tissue model than in the adipocytes alone with little or no insulin addition. Robciuc et al. found that the induction of excess vasculature improved lipid metabolism and they linked the activated VEGF/VEGFR2 pathway to better insulin responses in adipose tissue Robciuc et al., 2016). This also led to the higher basal metabolic rate (Robciuc et al., 2016). In our results the VEGF/VEGFR2 is upregulated and increases in response to insulin (Tab. S3). This further supports the hypothesis that similar mechanism of improved lipid metabolism is seen in our in vitro model and would explain the increased lipolysis when little or no insulin is present in the Vascularized adipose tissue model.

At gene expression level, the exposure to insulin led to upregulation of Glut4 and FABP4 in both adipocyte culture and vascularized adipose tissue model. Increase of FABP4 would indicate that some adipocytes were still further maturing as FABP4 is the indicator of late stage adipogenesis (Gregoire et al., 1998). Glut4 is the insulin dependent glucose transporter (Birnbaum, 1989; James et al., 1989) and hence the upregulation would be expected. In contrast, Glut1 was down regulated in the vascularized adipose tissue model. In mouse 3T3-L1 cells, Glut1 mRNA has been upregulated in response to insulin (Sargeant and Paquet, 1993). It has also been shown that the expression of Glut1 in 3T3-L1 cells is downregulated as ECM rigidity decreased whereas the Glut4 mRNA levels do not respond to the rigidity (Li et al., 2009) which would probably be closer to the situation in our vascularized adipose tissue model than the plastic rigidity. It has also been reported that there is negative feedback loop between the expression of Glut1 and Glut4 (Ebeling et al., 1998). Also, there are studies which show no change in glut1 expression in response to insulin (Machado et al., 1994). The response of the Glut1 should be studied further to confirm the result.

In vasculature, insulin upregulated PDGF, FGFGR2 and v-cadherin (Tab. S3). Since PDGF recruits pericytes and v-cadherin is tight junction protein, it would seem that insulin would increase maturation of vessels. It has been shown that in retinal newovascularization reduction of IGF-1 expression leads to the increase of angiogenic markers angiotensin II and VEGF (Li et al., 2018). It could be that the opposite happens with insulin exposure, as proper mature vessels are needed for the functioning glucose homeostasis.

In response to rosiglitazone, gene expression of FABP4, adiponectin and Glut4 were elevated in both cultures. In the vascularized adipose tissue model, also the expression of leptin was increased. Our vascularized adipose tissue model shows good concordance with the findings of others on the effects of rosiglitazone on adipocytes. Taxvig et al. showed that Rosiglitazone increases the expression of leptin and adiponectin with no effect on PPARα (Taxvig et al., 2012). Rosiglitazone is also used for the induction of adipogenesis (Cheng et al., 2016; Foley et al., 2015) hence it was expected for the markers of mature adipose tissue to
increase. Thiazolidinediones like rosiglitazone are also known to enhanced Glut4 action (Martinez et al., 2010). Although we did not see increase in PPARγ expression or lipid accumulation seen by others (Taxvig et al., 2012), the gene expression changes in our model support the increased adipogenesis. It is possible that with longer exposure time or longer culture time after exposure (here 7 days exposure), the increased lipid accumulation could be seen. To further improve the vascularized adipose tissue model presented here, it might be possible to lower the concentration of Troglitazone. Compared to our previously published adipocyte protocol (Huttala et al., 2016), we added one additional change of fresh Troglitazone medium and this could be masking some of the lipid accumulating effects of the chemicals even though effects were seen on gene expression level. Regarding vascularization, there are studies showing increase in adipose tissue vascularization in in vivo exposure of humans (Gealekman et al., 2012) and inhibition of endothelial proliferation, migration and angiogenesis in vitro (Sheu et al., 2006) in response to rosiglitazone. The quantification of vascular network formed in our vascularized adipose tissue model showed increase in angiogenesis in response to rosiglitazone being in concordance with the in vivo exposures.

We found that prochloraz was cytotoxic (EC50 181.6 -182.2 µM) in both adipocyte culture and vascularized adipose tissue model. In adipocyte culture inhibition of lipid accumulation was seen in the non-cytotoxic concentrations, EC50 being 164µM, however inhibition of lipid accumulation was only seen in toxic concentrations in vascularized adipose tissue model. In vascularized adipose tissue model, the expression of PPARα increased whereas expression of other studied markers decreased compared to the unexposed cultures. Previous results have shown that Prochloraz decreases adiponectin and leptin expression and decreases lipid accumulation in 3T3-L1 adipocytes already at 50µM (Taxvig et al., 2012). Taxvig et al did not see any effect on PPARα or PPARγ in their study (Taxvig et al., 2012). The increased level of PPARα in vascularized adipose tissue model is intriguing finding. The result of Taxvig et al is obtained with monoculture of mouse adipocytes and hence the difference in result observed here seems to be due to the presence of vasculature. PPARα is found also in endothelial cells (Bishop-Bailey and Swales, 2008) and the activation of endothelial PPARα could be one reason to the upregulation seen here. However, this would likely impair the angiogenesis which was not seen here nor seen by others (Kleinstreuer et al., 2011). Further mechanistic studies would be needed to confirm the reason for PPARα upregulation.

Chlorpyrifos caused increase in viability/mitochondrial activity with EC50 of 48.54 µM for adipocyte culture and 73.51 µM for vascularized adipose tissue model. Chlorpyrifos seemed to inhibit lipid accumulation in lower concentrations and increase it in the higher concentrations before inhibiting it again. It has been reported that chlorpyrifos decreases lipid accumulation in 3T3 (Taxvig et al., 2012). In our results leptin expression was significantly increased in in vascularized adipose tissue model exposed with 10µM Chlorpyrifos and this effect at 10µM has been shown by others on released leptin protein levels (Taxvig et al., 2012). Angiogenic effect was not detected for chlorpyrifos neither in our study nor in other reports (Jeon et al., 2016).

The six additional studied chemicals induced various responses in vascularized adipose tissue model. Tributyltin chloride increased lipid accumulation as reported in literature (Biemann et al., 2014; Pereira-Fernandes et al., 2013). Mancozeb has been reported to decrease body weight (Kwon et al., 2018) and inhibited lipid accumulation here. Bisphenol A has been shown to decrease mitochondrial activity on triglyceride accumulation (Biemann et al., 2012) in concordance with our results although contrary results have also been reported (Ariemma et al., 2016). Based on literature butyl paraben (Pereira-Fernandes et al., 2014), 15-Deoxy-Δ12,14-prostaglandin J2 and bis-(2-ethylhexyl) phthalate (Biemann et al., 2012) should increase lipid accumulation but the opposite was seen in our vascularized adipose tissue model.

The effect of the studied chemicals was also assessed in terms of their influence on the amount of vasculature in the vascularized adipose tissue model after the exposure to chemicals. The results obtained in our model correlated well with the results reported in the literature. In addition to correct angiogenic responses with Rosiglitazone, prochloraz and chlorpyrifos; butylparaben (no effect neither found here nor reported based on literature search), Bisphenol A, Mancozeb and Tributyltin showed correlation to literature. Bisphenol A should induce tubule formation based on the literature (Tait et al., 2015; Helmestam et al., 2014) but can also inhibit tubule formation depending on the concentration (Tait et al., 2015). In our results, induction was seen in higher concentrations. Mancozeb has been found to influence the TIE2 pathway (Kleinstreuer et al., 2011) and in our model the effect of mancozeb was seen as slight induction of angiogenesis. Tributyltin chloride has been shown to influence for example ICAM and hence angiogenesis process (Hu et al., 2015) and slight angiogenesis inhibition was seen in the tubule formation here. Based on these results our vascularized adipose tissue model can also predict the angiogenic effects of chemicals in adipose tissue.

On the contrary, the results with Bis-(2-ethylhexyl) phthalate and 15-Deoxy-Δ12,14-prostaglandin J2 showed opposite results to those reported in literature. Bis-(2-ethylhexyl) phthalate has been shown to decrease PIGF and increase soluble VEGFR-1:PIGF ratio in fetus which would indicate inhibition of angiogenesis (Ferguson et al., 2015), but we found induction of angiogenesis. 15-Deoxy-Δ12,14-prostaglandin J2 has been reported to be an anti-angiogenic factor and induces endothelial cell apoptosis, although the mechanism remains unclear (Ho et al., 2008) but the opposite was seen here as higher concentrations induced angiogenesis. The effects of these two chemicals should also be studied on gene expression level to confirm the result and correlation with previous results.

5 Conclusion

Due to the growing obesity problem, interest in safety testing of chemicals, in drug development and biomedical research, has grown. In order to conduct reliable research, relevant models are needed. The need for relevant human cell-based models is evident based on the species-to-species differences between animals and human. Especially needed are those human cell in vitro models that are confirmed to be relevant and which are thoroughly characterized. The vascularized adipose tissue model developed here is a valuable research model as it is a relevant replacement option for animal models used for adipose tissue research.

The vascularized adipose tissue model presented here contains well-formed extensive vascular network and mature adipocytes, analyzed on gene and protein level, and shows correct responses to insulin. The study also found that the response of adipocytes to insulin was faster in the presence of vasculature. This finding should be studied further as it might lead to development.
of new therapeutic strategies in insulin resistance. Based on the results this novel vascularized adipose tissue model is relevant for studying adipose tissue, especially the interaction of vasculature and adipocytes, and for studying effects of chemicals on adipogenesis and adipose tissue angiogenesis.

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Conflict of interest

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