

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Virtanen KA, Lidell ME, Orava J, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* 2009;360:1518-25.

SUPPLEMENTARY INFORMATION

METHODS

PET Study. A batch of [¹⁸F]-FDG was only released for use if the standard operating procedure had been followed and the radiochemical purity exceeded 90%. The PET-investigations were performed using a hybrid PET/CT scanner, Discovery VCT scanner (General Electric Medical Systems, Milwaukee, WI, USA). The scanner enables the acquisition of 64 contiguous image planes of 2.42 mm each. The total axial field of view is 15.5 cm. Acquisition can be made in 2D or 3D mode. Final in-plane resolution of the reconstructed PET images was 4.89 mm (Teräs M et al. Performance of the new generation of whole-body PET/CTscanners: Discovery STE and Discovery VCT. *Eur J Nucl Med Mol Imaging* 2007; 34:1683-1692). Photon attenuation was measured with CT that was also used for anatomical reference.

Dynamic scanning (40 min; 4 x 30s, 1 x 60s, 1 x 120s, 3 x 300s, 2 x 600s) of the neck and upper thoracic region was started simultaneously with a bolus injection of 185 MBq [¹⁸F]FDG. Plasma radioactivity samples were collected during each time frame.

After each PET-investigation the resulting dynamic data were reconstructed. During this process the numerical PET data (i.e. number of counts per detection channel) were converted to PET images. All emission scans were reconstructed with iterative ML-OSEM reconstruction, resulting in a transaxial spatial resolution of about 5 mm in the field of view. The matrix included 128 x 128 pixels. The data from the transmission scans were used to correct the corresponding emission scans for photon attenuation. Furthermore, the PET data were corrected for decay, scattered radiation and random coincidences. The summation images of the dynamic PET images were made to obtain a better statistical reference for the co-registration with CT images. The regions of interest were outlined in the CT images to follow the anatomy upon visual inspection of the images. They were subsequently transposed to and checked to be inside the defined structures in the respective summed PET images. Regions of interest (ROIs) were drawn in adjacent image planes for each defined structure of the brown adipose tissue (BAT) and subcutaneous white adipose tissue (WAT) (4-5 image planes). The ROIs were further copied to dynamic PET images and were used to generate time-activity curves (TAC) and glucose uptake rate data for the different regions. TAC data were analyzed against current radioactivity measured in plasma samples at specified time points using the Patlak-Gjedde plot model. In this model the volume of tracer distribution is plotted against normalized plasma integral. As a result, influx rate constant, K_i is produced. The slope of the linear phase of plot is equal to K_i value and is calculated as follows: $K_i = (K_1 \times k_3) / (k_2 + k_3)$, where K_1 is the transfer coefficient from the vascular space into the tissue, k_2 is the initial clearance and efflux coefficient, and k_3 is the phosphorylation rate constant. Metabolic rate of glucose uptake (rGU) is obtained from the equation: $rGU = ([Glc]_p / LC) \times K_i$, where $[Glc]_p$ is plasma glucose concentration and LC is a lumped constant term. The lumped constant accounts for differences in the transport and phosphorylation of [¹⁸F]FDG and glucose. The lumped constant (LC) value of 1.14 for adipose tissue (Virtanen KA et al. Human adipose tissue glucose uptake determined using [(18)F]-fluoro-deoxy-glucose

([(18)F]FDG) and PET in combination with microdialysis. *Diabetologia*. 2001 Dec;44(12):2171-9) was used. The differences between cold and warm conditions were tested in a pair-wise fashion with Student's t-test.

RNA Preparation, cDNA Synthesis and Quantitative Real-time PCR. Total RNA was isolated from 100 mg biopsies using the RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol including the DNase treatment step. The 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche) was used for cDNA synthesis from 200 ng total-RNA according to the manufacturer's recommendations. cDNA synthesis reactions without the addition of reverse transcriptase were used to exclude unspecific amplification in the following analyses. Real time PCR reactions were performed using diluted cDNA samples, the Power SYBR Green PCR Master Mix (Applied Biosystems) and the following primers at a final concentration of 1.15µM:

Beta-actin-forw: 5'-GAGCTACGAGCTGCCTGACG-3'
Beta-actin-rev: 5'-GTAGTTTCGTGGATGCCACAG-3'
UCP1-forw: 5'-CTGGAATAGCGGCGTGCTT-3'
UCP1-rev: 5'-AATAACACTGGACGTCGGGC-3'
DIO2-forw: 5'-CCTCCTCGATGCCTACAAAC-3'
DIO2-rev: 5'-GCTGGCAAAGTCAAGAAGGT-3'
PGC1-alpha-forw: 5'-GCCAAACCAACAACCTTTATCTCTTC-3'
PGC1-alpha-rev: 5'-CACACTTAAGGTGCGTTCAATAGTC-3'
PRDM16-forw: 5'-GAGGAGGACGATGAGGACAG-3'
PRDM16-rev: 5'-CGGCTCCAAAGCTAACAGAC-3'
ADRB3-forw: 5'-TTTGCCAACGGCTCGAC-3'
ADRB3-rev: 5'-CGTCAGGTTCTGGAGGGTAG-3'

The analyses were performed on an ABI PRISM 7900HT (Applied Biosystems). All samples were analyzed twice in triplicates and normalized to the expression level of beta-actin. For each subject, the expression level of each gene in the brown adipose tissue was related to the expression level of the same gene in white adipose tissue. For Statistics Student's t-test was used.

Protein Preparation SDS-PAGE and Western Blotting. Protein extracts were prepared from the organic phases attained during the RNA extraction according to the manufacturer's recommendations for TRI Reagent (Sigma-Aldrich). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce) and 25 µg protein fractions were separated by SDS-PAGE (4-12% or 10% gels, NuPage, Invitrogen), blotted to a PVDF membrane (Immobilon P, Millipore) and detected by anti-human-UCP1 (U6382, Sigma), anti-human Cytochrome C (#4272, Cell Signaling), and anti-human-GAPDH (ab9484, Abcam) antibodies.

Histology. Samples were prepared for morphology by keeping in formalin until moulding in paraffin and staining with haematoxylin-eosin (HE). Tissue sections were imaged using an Eclipse E800 microscope (Nikon) fitted with a 40X/0.75 Plan Fluor objective (Nikon).

Immunofluorescence and Immunohistochemistry. Sections for immunofluorescence and immunohistochemistry were subjected to heat induced antigen retrieval by boiling for 10 min in a pressure cooker in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 9). After washing in PBS sections for immunofluorescence were blocked for 1 h in 1% BSA, 0.1% Tween20 in PBS and incubated with primary antibody in dilution buffer (0.5% BSA, 0.05% Tween20 in PBS) for 2 h. This was followed by 3 x 10 min washes in PBS and incubation with secondary antibody, mitochondrial and nuclear markers in dilution buffer for 1h. After washing 3 x 10 min in PBS sections were mounted in ProLong Gold Antifade (Invitrogen, Molecular probes P-36934) and photographed on a Zeiss LSM 510 Meta system. For immunohistochemistry, the VECTASTAIN Elite ABC Kit (Vector Laboratories, PK-6101) was used according to manufacturer's protocol. Slides were mounted with DPX Mountant for Histology (Sigma, 44581) and photographed using a Nikon Eclipse E800 microscope. For clarity, brightness/contrast and sharpness of photographs were adjusted using LSM Image Browser (Zeiss) and Photoshop CS2 (Adobe). Antibodies used were rabbit anti-UCP1 (1:500, Sigma U6382), Alexa594 conjugated mouse anti-OxPhos Complex IV subunit I (1:200, Invitrogen, Molecular Probes A21297), Cy2 conjugated donkey anti-rabbit IgG (1:100, Jackson Immunoresearch, 711-225-152) and Topro3 (1:1000, Invitrogen, Molecular Probes T3605) was used as a nuclear marker.