Annex 3.2.4.1 – Found documentation for A84
Investigation into the research of Milena Penkowa

Overview of paper

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of exps and ID of health ethics permit</td>
<td>01-069/02. exercise type 2 diabetes patients. 8 weeks of rowing exercise. Muscle biopsies</td>
</tr>
<tr>
<td>Type and no. of persons</td>
<td>8 men with type 2 diabetes, 5 controls</td>
</tr>
<tr>
<td>MP’s exps and time of these exps</td>
<td>2004-2005. preparation of slides, stainings, eval. of NITT, 8-oxoguanine and MDA. Technician Hanne Hadberg contributed acc.to.ackn.</td>
</tr>
</tbody>
</table>

Documentation and information requested by Panel, and further keywords and comments for identifying the wanted documentation:
-Examine the slides showing MT-I+II and NITT
-Assess the raw data / lab notes on the assessment of the slides stained for MT-I+II and NITT.
-Interview of C. Scheede-Bergdahl about how the analyses and assessments were done, and who were responsible for the compilation of the assessment.
CS-B is not at Panum any longer. Flemming Dela is there, though, and Hanne Hadberg
-F Dela has made an extended co-author declaration on www.xlab.dk See Dela’s declaration, as well as CS-B’s declaration on bmi.ku.dk/English/research/systemsbiology/xlab/ (Both declarations are inserted further below)

The documentation and information found, including the “animal key”, if found:
We did not succeed with finding keys, samples or other documents or material for the exps described in this paper in the MP archive. However, we received slides and information from F. Dela and C. Scheede-Bergdahl:
In telephone conversation 16.03.12, F.Dela informed the Secretariat that:
1. Celena Scheede-Bergdahl lives in Canada. F.Dela is in Salzburg 11-13 April. None of the two can thus meet the Panel 12 April.
2. The NITT lab work was all done by FD/CS-B.
3. FD has recently, i.e. after the publication of his declaration on the collaboration with MP, tried to repeat the exps of MP on content of MT-I+II. FD did his exp on some of the remaining unstained samples that he and CS-B got from MP. He was not able to re-find the sample expression that MP has found earlier on. However he emphasises that this does not prove the MP’s results to be wrong, because FD did not use the same method as MP had used originally – he used Western Blot instead.
4. FD repeated what is written in their questionnaire answers, namely that they found it strange that MP had broken the corners of the sample-glasses off, so that there was no ID on the stained samples that she returned to FD/CS-B.
5. FD encouraged to call CS-B in Canada, and to interview her and ask for photos of the original slides stained for MT-I+II.
6. The declaration of FD on www.xlab.dk is copied into this document, further below.
PJ’s telephone conversation with Celena Scheede-Bergdahl 19.03:
Concerning supplementary material for paper A84 from Celena Scheede-Bergdahl and Flemming Dela:

The Secretariat did not succeed with finding keys or slides, in the MP archive, that may be associated with paper A84. However, some slides, supplemented with written material and photos, were obtained from two of the co-authors, Flemming Dela (FD) and Celena Scheede-Bergdahl (CSB).

Already in the questionnaire response of August 2011, CSB had informed that when Milena Penkowa (MP) returned the slides to her, some of the ID numbers of the slides were broken off. CSB informs further: “When I asked her [MP] about it (Clara Prats was also present), she claimed that her technician had done that in order to “blind” herself to the results. We thought it was strange - when we (Clara and myself) looked at the slides "blinded" I had just covered the information with paper so that the subject IDs were not visible. However, as I said before, at the time I did not think anything else of it, except that it was a strange thing to do.”

CSB was a PhD student at the time she conducted the research in, and co-authored, the paper A84.

In the following is inserted the following reporting and information:

1. The slides received from CSB and accompanying descriptions (page 1)
2. Report on experiment conducted by FD aiming at verifying experiment in A84 (page 4)
3. Copy of declaration of F.Dela and C.Scheede-Bergdahl (page 6)
4. Photos of the slides (page 10)

1. The slides received from CSB (report)
The slides were received ahead of the Panel’s meeting at Panum 11-13 April. Further below in this document (under point 4) are some photos (received from FD 10 April) of the slides. In an email of 04.04.2012, CSB explains about the slides, as follows:

“There are 3 trays of slides:
-1 contains slides that MP gave me to show the specificity of her antibody (I don't know where those slides come from as she did not use my tissue).
-2 contains slides that are identifiable: On the top row (and it has been indicated in pen by me) are control subjects (3 subjects out of the 5 that were in the data set) and on the bottom row are the diabetics (I believe that there are 3 subjects with full data and 3 others where there are only either the pre training or post training). All slides that contain a 1 in the ID are pre training and 2 are post training. These slides are consistent with the data that is in the Diabetes paper.
-3 contains the slides that were broken. I have no idea any more who these are. These were the slides that were "blinded" by someone from MP’s lab (at the time, she had claimed that it was a technician of hers). I put them in a possible division of who could have been diabetic (top row, I think- but they are lighter in colour than the controls) and the darker controls. As indicated in my initial report to you, I have no idea why this was done. At the time, I thought that it was because MP was either frustrated or angry at us for asking her all our questions. Clara Prats also thought it to be strange.

The full set of slides had initially been blinded (I hid the data, Clara Prats did the actual categorization and I generated the table in the paper) and the data that we included in the paper was put together before the slides were broken. I can't remember why we had her look at the slides again- sorry- there has been 3 children, a PHD thesis, 2 countries and about 3 different jobs since I worked on this paper so my memory is not so accurate anymore ;) If there are more questions about this, I will be happy to help you in any way that I can.”

In addition, CSB elaborated the following description in connection with the slides, received by mail from FD 10 April 2012:

**Metallothionein study with Milena Penkowa**

(C. Scheede-Bergdahl et al, Diabetes, 2005)

- The following subjects were included in the study:
  - Control: HT, JG, LC, PK, SB

- On Tray 2 of the original slides that were sent: On the top row (and it has been indicated in pen by me) are control subjects (3 subjects out of the 5 that were in the data set) and on the bottom row are the diabetics (There are 3 subjects with full data and 3 others where there are only either the pre training or post training). All slides that contain a 1 in the ID are pre training and 2 are post training. These slides are consistent with the data that is in the Diabetes paper.
- On Tray 3, there are the slides that were broken by someone in MP’s lab. I have no idea which subject these belong to anymore because the name IDs were removed. These were the
slides that were "blinded" by someone from MP's lab (at the time, she had claimed that it was a technician of hers). I put them in a possible division of who could have been diabetic (top row, I think- but they are lighter in colour than the controls) and the darker controls. As indicated in my initial report to Pia and subsequent discussions, I have no idea why this was done. At the time, I thought that it was because MP was either frustrated or angry at us for asking her all our questions. Clara Prats also thought it to be strange.

- Tray 1 includes some of the “control” slides that MP gave to me to prove the specificity of the antibody that she used.

- On the slides: the name Hidalgo was given to the Metallothionein antibody (MP received it from Juan Hidalgo)

- Please note that the slide quality is poorer now than when the initial analysis had been conducted as they had been stored at room temperature conditions.

- Timeline of events:
  - Late 2003/early 2004: the slides were brought to MP's lab. At the time, we did not plan to do work on MT1+2 but on another protease that we were interested in (matrix metalloproteinase) and associated matrix proteins.
  - Shortly after delivering the slides to MP, she had asked if she could go ahead and have a technician start to prepare some slides. All biopsies were left with their initial IDs (example: XXST, where XX were the subject’s initials, S was the status- either D for diabetic or H for healthy and T was the time point- in the case of these slides either 1 for pre training or 4 for post training), thus making this information available to MP.
  - Early 2004, MP reported to me that she had some interesting data regarding MT1+2, which she had included because she was familiar with it.
  - I took the slides back to our lab to have someone from my lab (Clara Prats) to blindly record the data (which was put into a table by me and included in the Diabetes paper). *The data included in the paper did not change from this time point.*
  - There were some questions from Clara Prats’ supervisor (Thorkil Ploug) as to the specificity of MP's antibody so we brought the slides back to MP to have further testing conducted.
  - As reported to Pia, I sensed that MP became more frustrated, and perhaps insulted, by our questions. She had her technician run some tests (she did not specify exactly what tests and, since I am not an expert in these techniques, I did not think to question this). As it was told to me, this technician had broken the slides and removed the IDs in order to blind some of the samples. At the time Clara Prats and I had thought that removing the IDs from the slides was a very strange way to blind samples. This was now early summer 2004 and, from what I recall, after the submission of the manuscript to Diabetes. The paper was returned to me for
comments in October 2004 and resubmitted the manuscript in the summer of 2005 after my maternity leave. The paper was accepted shortly afterwards.

The Secretariat returned the slides to FD after the Panel’s meeting 11-13 April.
2. Report on experiment conducted by FD aiming at verifying experiment in A84:

**Supplementary experiment**

Due to the fact that the scientific integrity of one of the co-authors (Milena Penkowa) is presently being examined by a scientific committee on behalf of the University of Copenhagen and by The Danish Committees on Scientific Dishonesty, we attempted to catch our immunohistochemical (IHC) findings regarding MET-kinase (MET) in skeletal muscle by immunostaining (II).

A researcher outside of our author group (Dr. Andreas Frangia, Concordia University, Canada [please see disclaimer below]) kindly agreed to perform the protein analysis of MT by II, using a commercially available antibody. Although we no longer have the muscle samples that were used for the IHC staining (embedded in OCT), we did have muscle samples saved from the original biopsies that were flash frozen in liquid nitrogen for protein analysis. These original muscle samples had been stored at −80°C in a buffer containing protease inhibitors.

**Materials and methods**

**Protein determination and immunostaining.**

Cell lysates were mixed with lysis buffer containing 250 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 0.1 mM Na₃VO₄, and centrifuged at 13,000g for 10 min. Supernatant was collected, and protein was measured using Pierce BCA Protein Assay Kit (Thermo Scientific). 10 μg of lysate was separated on a 15% SDS-PAGE and transferred to a nitrocellulose membrane (0.2 μm, Bio-Rad) using 10 mM sodium borate buffer. The membranes were blocked in 1% BSA in Tris buffer (10 mM Tris-HCl, 126 mM NaCl, pH 7.5) for 1 h at room temperature followed by overnight incubation at 4°C with primary antibody. Metastathisium (UR11/II) (ab12320) from Abcam (Cambridge, MA, USA). The blots were washed 3×10 min in TBS-T (0.1% Tween), incubated with horseradish peroxidase-conjugated secondary antibody (anti-mouse, ab6778; Abcam), again washed 3×10 min in TBS-T, and visualized with a chemiluminescence system (ECL Healthcare, AnorexinaE9, Western Blotting Detection Reagent, RN2105). The bands were analyzed using the ImageJ software.

**Chemicals and materials.**

All chemicals and materials, if not stated, were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada).

**Statistics.**

Values are means ± SEM, unless otherwise stated. n refers to the number of subjects. Statistical significance was determined using two-way repeated measures ANOVA.
Results
The IHC analysis revealed that there was an overall effect of diabetes on skeletal muscle type I and II metattractin (MT) levels but that MT was higher in the diabetic subjects versus the controls (please see below for graph). There was no effect of exercise. This is contrast to the data reported.

![MT I+II Skeletal Muscle Protein graph]

Discussion
Had the IHC data been in full agreement with the IHC data, it would have strengthened the findings. However, the discrepancy between the IHC and the IIB data is clear. Despite this, the IIB data cannot fully exclude that the IHC may still be valid. There are a number of reasons for this, for example, there are many isoforms of MT and different antibodies may have different specificities for the different isoforms. Dependent on the specific epitope of the antibody different metattractin-2a may be revealed. Also, the IHC may reveal non-muscle staining, while the IBB done on freeze-dried and dissected muscle.

Conclusion
We are not able to confirm or reject the IHC data by this approach. Thus we will await the conclusion by "The Danish Committees on Scientific Dishonesty". All recommendations will be promptly reported to the Journal.

Renee King, Andreas Bergdahl, Celine Schweede Bergdahl.

* Disclaimer: Andreas Bergdahl is not independent as he is married to the first author of the paper.
3. Copy of declaration of F. Dela and C. Scheede-Bergdahl:

Declaration of work assignments and tasks in relation to the article:

Metabolism-Mediated Antioxidant Defense System and Its Response to Exercise Training Are Impaired in Human Type 2 Diabetes

Authors: Celena Scheede-Bergdahl (CSB), Milena Pankova (MP), Juan Hidalgo (JH), David B. Moore (DBM), Jette Schjerling (JS), Lars Prats (CP), Robert Boukal (RB) and Pernille Dela (PD).


Written by Celena Scheede-Bergdahl and Flemming Dela.

The text was uploaded on Friday 29th of May, 2012.

03-05-2011 addition: The text is approved by JH, PK, CP and RB. It has not been possible to get in contact with DBO or MP.

09-07-2011 addition: The text is approved by DBO.

RESEARCH DESIGN AND METHODS (the text that appears in blue italics has been taken directly from the 2005 Diabetes paper):

Thirteen matched male subjects (type 2 diabetes n=6; control subjects n=5) were accepted for participation in this investigation after informed written consent as approved by the ethical committee of Copenhagen and Frederiksborg (01-106/02) and in accordance with the Declaration of Helsinki. Physical characteristics of the subjects are presented in Table 1. It was established that none of the subjects regularly performed exercise before the commencement of the study. Normal glucose tolerance was confirmed in all healthy control subjects through a standard 75-g oral glucose tolerance test. None of the control subjects were taking any medications, while type 2 diabetes was treated with antidiabetic drugs (metformin [n=7], gliclazide [n=2], alogliptin [n=1], orlistat [n=1], orlistat [n=1], enalapril [n=2], glimepiride [n=2], or angiotensin II receptor antagonists [n=1]), or cholesterol-lowering drugs (simvastatin [n=3]).

This study was a part of a larger one that investigated the effects of exercise training on endothelial function, insulin action and cardiovascular disease markers in type 2 diabetes. FO, DBO, RB and CSB were involved in generating the original study hypotheses and design of the main project. Subject recruitment was undertaken primarily by BBO. Anthropometric measurements, patient medical histories, patient physical activity status and oral glucose tolerance tests were performed primarily by BBO. CSB and a medical student who participated in the main study (Danny Reving)
helped DBO to obtain these parameters. Table 1 in the text was prepared by CSB, including related statistics.

All participants undertook 8 weeks of exercise training (65% of maximal heart rate, 3–4 sessions per week, 50 min per session) on a running ergometer. In order to minimize effects of acute exercise, subjects were instructed to not exercise on the day immediately before coming into the laboratory for testing.

The participants were instructed in their training protocol by DBO, with the help of CSB and the medical student mentioned earlier (Danny Revig). Training log books were followed up by the same researchers.

Muscle biopsies were obtained from the vastus lateralis (Bergström technique) under local anaesthetic at baseline and after completion of the training period (same leg for both biopsies). Tissue was immediately set in Tissue Tek and flash frozen in liquid nitrogen-cooled isopentane. Samples were stored at -80°C.

All muscle biopsies were taken by FD and DBO. All biopsies were handled immediately following extraction by Regina Cramer (a post doctoral fellow at the Copenhagen Muscle Research Centre, teaching CSB) and, eventually, CSB alone. Samples were preserved for mRNA, immunochemistry, protein (related to this study).

Blood was sampled from a brachial artery catheter.

All blood samples were taken by FD, KS and CSB.

Blood sample processing: Blood samples were immediately mixed with 10% EDTA and centrifuged at 4,000 rpm for 10 mins at 4°C. Plasma was then maintained at -80°C until analysis.

All blood samples were processed by the technicians in FDLab.

Tissue processing: Muscle tissue was cut in 6-μm consecutive sections on a cryostat, and the sections were immediately collected on glass slides to be used for immunohistochemistry. For some immunohistochemical stainings, sections were preincubated in 20 μg/ml Protease K (Sigma-Aldrich, St. Louis, MO) for 5 min or sections were incubated overnight in tris-EGTA buffer (1.211 g Tris, 0.953 g EGTA, one l dist. H2O, pH 8.0) for epitope retrieval. Afterwards, sections were incubated in 1% H2O2 to block endogenous peroxidase, followed by incubation in 10% goat serum to block unspecific background staining.

For epitope retrieval, some sections were pretreated in 20 μg/ml Protease K (Sigma-Aldrich) for 5 min or sections were incubated overnight in tris-EGTA buffer (1.211 g Tris, 0.953 g EGTA, one l dist. H2O, pH 8.0) followed
by blocking of endogenous peroxidase by incubation in H2O2, and, afterward, sections were incubated in 1% goat serum to block nonspecific background staining.

**immunohistochemistry:** Sections were incubated overnight at 4°C with primary antibody: rabbit anti-MT-I-I diluted 1:500 (opo), rabbit anti-nitrotyrosine (NTT) diluted 1:100 (rabbit peroxynitrite-induced oxidation of tyrosine residues/oxidative stress), rabbit anti-malondialdehyde (MDA) diluted 1:100 (marks MDA, a byproduct of fatty acid peroxidation/oxidative stress), and mouse anti-apolipoprotein A-I 1:200 (Chemicon, Hounslow, U.K.) (marking of free radical-induced base modification in the genome/oxidative stress).

The primary antibodies were detected using biotinylated mouse anti-rabbit IgG diluted 1:400 (Sigma-Aldrich), goat anti-mouse IgG diluted 1:2000 (Sigma-Aldrich), or goat anti-mouse IgM 1:20 (Jackson Immunoresearch, West Grove, PA) for 30 min at room temperature followed by streptavidin-peroxidase complex (StrepULARcomplex/RRP: DakoCytomation, Glostrup, Denmark) prepared at manufacturer's recommended dilutions for 30 min at room temperature. Afterward, sections were incubated with biotinylated streptavidine and streptavidin-peroxidase complex (New England Nuclear Life Science Products) prepared following manufacturer's recommendations. The immunoreactions were visualized using 0.3% H2O2 in 3,3'-diaminobenzidinehydrochloride/tri-buffered solution for 10 min at room temperature.

In order to evaluate the extent of nonspecific binding in the immunohistochemical analysis, control sections were incubated in the absence of primary antibody or in the blocking serum. Results were considered only if controls were negative. For the simultaneous examination and recording of the staining, a Zeiss Axioplan two-light microscope was used.

All tissue processing and immunohistochemistry was performed by MP lab. Photography of slides (for publication purposes) was done by CSB and MP. Slides that included muscle biopsies stained for MT-I-I were analyzed ‘blinded’ by CP and CSB. CSB prepared the slides so that the identifications (IDs) were not visible to CP. CP then graded the slides with a series of + and +, representing either no reaction (+) to strong reaction (+++). CSB (when the slide IDs were not visible) also checked the slides. CSB then recorded the results and prepared Table 2 (semi-quantitative data). Slides with staining for NTT, 8-oxoguanine and MDA were visually evaluated by CSB and MP. This was not done blinded as this was not the focus of the manuscript. A slide representing the trend for each group (control, diabetic, pre and post training) was photographed for publication purposes.

**Evaluation of nonspecific binding was performed by MP.**

MT-I-I mRNA: Total RNA was isolated from muscle biopsies by phenol extraction (Trizol reagent; Molecular Research Center, Cincinnati, OH) as previously described (48). Intact RNA was confirmed by denaturing agarose gel.
Electrophoresis: Five hundred nanograms total RNA was converted into cDNA in 20 μl using the Omniscript reverse transcriptase (Qiagen, Valencia, CA) according to the manufacture’s protocol. For each target mRNA, 0.25 μl cDNA was amplified in a 25 μl reaction using PCR containing: 1 × Quantitect SYBR Green Master Mix (Qiagen) and 100 nmol/l of each primer. The amplification was monitored real time using the MX3000P real-time PCR machine (Stratagene, LaJolla, CA). The primers for metallothionein were designed to target mRNA for all but one target gene: TCC TCC TCG CTT GGG GGC TGG CTC TCT G (used CATCAAGG CAG CAG CTC CTC TCT). The Ct values were related to a standard curve made with pooled cDNA.

The quantities were normalized to mRNA for the large ribosomal protein P13 (RPL13) as internal control (4) using the primers GGA AAG CTA TGC TGG TGG CTT TCT CTC TCT CCA CTA ATC CGT TCT GCT G.

All mRNA work was performed by PS lab. CSB was involved in the total mRNA isolation as well as cDNA conversion, under the supervision of PS. Analysis of data and statistics were performed by PS. Figure was prepared by CSE.

Plasma MT1-III: Circulating MT1-III levels were measured in arterial plasma as an acute phase marker of the adsorptive phase response to training and/or diabetes. MT1-III levels were measured by the radioimmunoassay (39). The antibody was raised against rat MT-III and showed full cross-reactivity with human MT-I (the latter provided by Dr. Milan Vacek). Undetermined factors present in human plasma eventually bind labeled metallothionein, and thus in the radioimmunoassay, the non-specific binding (i.e., binding of 125-I-Metallothionein in the absence of primary antibody) was determined for each sample, which was then subtracted from total binding (i.e., binding in the presence of primary antibody) to get the specific binding.

Blood samples were prepared by CSB, according to JH specifications. Samples were shipped to JH (Barcelona), where all work was done. Raw data was sent back to CSE. Statistics were performed by CSE and FD. Figure was prepared by CSE.

Statistics: All data are presented as means ± SE. Two-way repeated measures ANOVA testing was utilized to determine statistical significance in results. Differences between type 2 diabetic and control groups (Tables 1) were established with Student’s t-tests.

All statistics, except for mRNA (PS), was performed by CSE, with input from FD.

CSB prepared manuscript, with input from MP, JH and FD.

All authors approved final manuscript.
4. Photos of the slides, received from F. Dela