CIM
Centre of Inflammation and Metabolism

Udvalgene vedr. Videnskabelig
Uredelighed (UVVU) and
The University of Copenhagen

12 August 2011

Professor Henrik Galbo has submitted a report to the University of Copenhagen, which is to be made available to the independent investigation panel that the University has established to evaluate Milena Penkowa's research. I received Dr. Galbo's report on 19th July 2011 from Prorektor, Professor Thomas Bjørnholm.

In the conclusion to his report Dr. Galbo states: *"Even with its limitations… the present investigation, primarily based on review of published papers, leaves little room for doubt that BK Pedersen is at least guilty of gross negligence of her scientific duties both as a group leader and supervisor, as a senior author and, indeed, as a professor of the University of Copenhagen. Considering not only the glaring obviousness of Penkowa´s fraudulent manipulations, but also the numerous inconsistencies in other parts of the work and its interpretation, one has to conclude that it is likely that BK Pedersen chose to close her eyes, i.e. that her negligence was willfull. However, bearing in mind also the obvious motive, deliberate scientific fraud must be suspected."*

This is an unprecedented and serious accusation and I must clear myself. Therefore, in agreement with the co-authors of the 12 publications in question, I have prepared the present report.

In short,

1. We think it would be unethical not to take advantage of all information that can be extracted from human samples. However, Dr. Galbo raises serious accusations against us with regard to reuse of biological material, although he, himself, on several occasions has followed the same tradition regarding the optimal use of human material (tables 2-4). Moreover, Dr. Galbo has extensively reused his data.

2. Throughout his report, Dr. Galbo does not only criticize the fact that there is not always parallelism between mRNA and protein levels, but he also insinuates that by neglecting such findings, we are guilty of fraud. We show that his criticism is based on an outdated and wrong perception of the "Central Dogma" of molecular biology: that DNA is transcribed into RNA, which is translated into protein. Dr.Galbo is apparently not up-dated on new era phenomenon such as microRNA, etc.

3. Dr. Galbo criticizes the fact that we express our data as arbitrary units or fold-changes, although this is the common praxis when evaluating the effects of an intervention, and although he, himself, has co-authored at least 10 publications in which mRNA levels are expressed in the same way; http://www.ncbi.nlm.nih.gov/pubmed?term=galbo%20h%20mRNA.

4. Dr. Galbo is confused with regard to basic endocrinological terms and the limitations of various techniques, which has made it sometimes difficult to provide a response to his criticism.

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5. We demonstrate that our procedures are or were at the time of publication of the articles in question commonly used in the research field of integrative human physiology. When applying the same main criteria that Dr. Galbo uses when reviewing our papers to another muscle physiology article coauthored by Penkowa (with Flemming Dela as senior author), it appears that the Dela group has used exactly the same procedures as we have.

6. Dr. Galbo has voluntarily decided to evaluate our papers. Although all papers have undergone previous peer review and several have been included in PhD theses, we acknowledge that Dr. Galbo identifies mistakes in two of the papers and points to some issues that could have been dealt with differently. However, not once in his lengthy report is he able to give a specific example of potential scientific dishonesty other than those related to the work performed by Milena Penkowa, which we ourselves have previously reported to UVVU and the scientific journals in questions. In consequence, we do not find that Dr. Galbo is able to provide any valid support for his serious and potentially very damaging accusations against us.

Below I shall respond in a point-by-point fashion to the charges put forward against me and my research group. To back my response, I include all original data. However, I shall start by providing the readers of this document with some background information and general comments.

Finally, I cannot help noticing the coincidence that this particular critisism should come from Dr. Galbo when bearing in mind the decade-long and wellknown antagonism between Dr. Galbo and the undersigned and indeed also the positive relationship that exists between Dr. Galbo and Professor Jamie Timmons. Thus, one might suspect that Dr. Galbo's motives for bringing forward his accusations could be personal rather than scientific. However, as this is irrelevant in relation to the scientific evaluation to be performed by UVVU, I shall refrain from elaborating further on the subject. Instead I hereby ask to be cleared of the flagrant, unprofessional, and erroneous accusations that Dr. Galbo has raised against my research group and myself.

The present report will be submitted also for information to the authorities at the University of Copenhagen and Rigshospitalet as well as to the Danish National Research Foundation.

Sincerely

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Background information

Collaboration with Milena Penkowa

Dr. Penkowa was an independent senior scientist at the Panum Institute with a research group of her own, whereas my research group has always been located at Rigshospitalet. When she and I initiated collaboration (2002), we were both associate professors. Thus, I (BKP) have never had a role as mentor to Penkowa.

When the Danish Nation Research Foundation's Centre of Inflammation and Metabolism (CIM) was founded in 2005, Penkowa was included as a senior partner. The last immunohistochemistry (IHC) data used in CIM publications, on which Penkowa had "hands-on", are from 2005. From 2005-2006 CIM personnel used the Penkowa laboratory for IHC. The last IHC experiments were carried out in September 2006 (Diabetologia paper, published 2009) by CIM personnel. In August 2007, I (BKP) informed Milena Penkowa that I had decided to exclude her from CIM. The arguments for this decision are given in the attached letter (C_2 and C_3). My decision was based on the fact that she did not demonstrate any interest in the research plan of CIM. I/we did not suspect fraud and I even invited her to collaborate on e.g. IHC in the future, should she be interested.

We do not try to hide the fact that Milena Penkowa has misled us as she has probably done it to her numerous other scientific collaborators in Denmark and abroad. However, we had no doubts about her data before the Penkowa case appeared in the press in 2010. In March 2011, we became aware of manipulations with the immuno-blot pictures in 4 of our 12 Penkowa co-authored papers. I reported these four articles to the Danish Committees on Scientific Dishonesty (in Danish: UVVU) on 02.04 2011 and 03.04 2011, respectively, and informed the involved scientific journals on 04.04.2011.

I have co-authored 12 papers with Penkowa. On three of these papers, the immunoblots were performed by PhD students or Master students from my laboratory in the Penkowa laboratory. Penkowa did not have "hands on" and we have no reason to believe that these data are fake. On two of the articles CIM personnel performed the IHC in collaboration with Penkowa and we know for sure that the proper muscle samples were cut and stained correctly. On another four papers, we now have strong reasons to believe that the immunoblot data made by Penkowa are fraudulent (and as mentioned above this was communicated to UVVU in separate reports already in April 2011) and we have retracted the papers in question. We are of course deeply concerned about the apparent misconduct performed by Penkowa on immunoblot data in four CIM papers. Therefore, we have decided to initiate a new exercise study, with multiple muscle biopsies, for the measurement of protein levels of specific cytokines in order to determine whether the protein levels of these cytokines increase in skeletal muscle cells following exercise.

Please see our comments with regard to the 12 publications in question (C_4). Among these publications, only two studies can be considered key-myokine papers. Although the IHC data appear to be flawed, later work from our group or another group did find support for the idea that IL-6 (C_1) and IL-8 (C_5) are expressed in/released from contracting muscle cells. The hypothesis that skeletal muscle is an endocrine organ is supported by numerous other studies including proteomics studies showing that C2C12 muscle cells have the capacity to secrete more than 600 myokines (Henningsen et al., Mol Biosyst 2011; 7(2):311-21, Henningsen et al., Mol Cell Proteomics 2010; 9(11):2482-96). Furthermore, other researchers, e.g. Kenneth Walsh at Boston University School of Medicine, have identified several other myokines.

Collaboration with Henrik Galbo

I have known Dr. Galbo since 1987, where we met at the Department of Rheumatology at Rigshospitalet. Later Dr. Galbo and I were both associated with the Copenhagen Muscle Research Centre (CMRC) as in principle equal senior partners. From 1994 to 2003 the CMRC was funded by the Danish National Research Foundation and led by Professor Bengt Saltin. From 2004 to 2008 the CMRC was continued on a trial basis with financial support from the University of Copenhagen and the Copenhagen Hospital Corporation (later the Capital Region of Denmark) during which period Dr. Galbo was the chairman of the board of CMRC and member of its steering committee. The University did not wish to continue their support to CMRC after 2008. However, the support from the Capital Region of Denmark was made permanent and the administration of the grant was placed at Finsencenteret, Rigshospitalet (RH) at which occasion I was appointed administrative head of CMRC at RH.

Dr. Galbo has co-authored 17 publications with me (1988 to 2003)

http://www.ncbi.nlm.nih.gov/pubmed?term=pedersen%20bk%20galbo%20h

Collaboration with Jamie Timmons

My research group and I collaborated with Jamie Timmons from 2005-2010. At present Prof. Timmons is affiliated with Royal Veterinary College, University of London, UK.

When the Penkowa case came up in the media late in 2010, Jamie Timmons took the opportunity to start accusing members of my research group, me, and professor Mark Febbraio, Melbourne, Australia of scientific fraud. At first the accusations were in the form of a stream of slanderous e-mails with "cc" to numerous people and offensive and threatening phone text messages. The harassment culminated in April 2011, when Prof. Timmons filed a report to UVVU (D_1), in which he accuses me, professor Mark Febbraio, and six people in our groups of scientific fraud. In addition to filing his report to the UVVU, Timmons has chosen to circulate it to the Danish and international press and to a vast number of people in the scientific community around the world.

Professor Timmons' conduct in this matter has been inappropriate and damaging to the basis of CIM and its researchers. Therefore, in order to clear ourselves, I submitted in May 2011 a response to UVVU regarding the charges put forward against us by Timmons (D_2). My report includes documentation, e.g. original data and e-mails, which refutes the accusations (A1_1-20, A2_1-16, A3_1-5, A4_1, A5_1-2, B_1-9, C_1-5).

I find it of interest to mention the above collaborations, as the report written by Dr. Galbo seems to be inspired by the report submitted to UVVU by Jamie Timmons. In support of the latter, please see the attached e-mail correspondence (D_3).

General comments to Dr. Galbo's criticsm

Dr. Galbo describes himself as a researcher who has worked "in endocrinology as it relates to muscle". It appears, however, that cell biology and cell signalling are not areas of his expertise, considering his assertions with regard to our publications.

Below I shall respond in a point-by-point fashion to his specific criticism regarding the 12 publications that are co-authored by Penkowa. However, I shall start by commenting on a number of issues that are general in Dr. Galbo's report and to which I shall further refer below while responding to the specific comments.

1. Basic knowledge with regard to signalling in endocrinology

An example of Dr. Galbo's general criticism is the following paragraph:

"In the discussion they concluded that it is likely that muscle-derived BDNF "works in an autocrine and/or paracrine manner. They did not mention that this view was in conflict with the fact that they did not find any difference in release of BDNF to the medium in contraction-treated versus control C2C12 cells."

It appears that Dr. Galbo is confused with regard to basic endocrinological terms. Autocrine signalling is a form of signalling in which a cell secretes a hormone or chemical messenger (called the autocrine agent) that binds to autocrine receptors on the same cell, leading to changes in the cell. Paracrine signalling is a form of cell signalling in which the target cell is near the signal-releasing cell. The fact that we found no increase in BDNF either in the medium of the cells or in the circulation of the animals, is precisely why we concluded that the effects was either paracrine or autocrine and not endocrine (which is what occurs when the tissue releases the protein to act upon another tissue). Throughout his report, Dr. Galbo overlooks the fact that proteins may be up-regulated within a muscle cell without being secreted or they may be secreted without being involved in organ-to-organ signalling.

2. mRNA and protein expression and regulation

Throughout his report, Dr. Galbo does not only criticize the fact that there is not always parallelism between mRNA and protein levels, but he also insinuates that by neglecting such findings, we are guilty in fraud.

His criticism is based on an outdated and wrong perception of the "Central Dogma" of molecular biology: DNA is transcribed into RNA, which is translated into protein.

However, differences in fold changes between mRNA levels and protein levels are common since protein expression involves a balance between translational efficiency and protein turnover and stability. There are several ways that proteins may change without a (large) change in the mRNA levels, e.g.:

- Alterations in translational efficiency, without altering transcript abundance.

- Alteration of protein degradation rate, while maintaining the same translational rate.

- miRNA regulation: Some papers have shown that miRNAs have a larger role on regulating protein abundance without an effect on mRNA levels. For example, our own paper with professor Timmons shows no change in mRNA using the microarray, but the differences in miRNAs are predicted to result in a difference in protein. In fact, BDNF was shown to be changed in the proliferating cells. (Gallagher IL et al. Genome Med. 2010 Feb 1;2(2):9.

There are large-scale studies that have measured all mRNAs and all proteins in the same study, which show that there is a substantial difference between mRNA and protein expressions. Also, the difference is greater in eukaryotic cells than in more simple cell types suggesting that with evolution there are more post-translational modifications (De Sousa Abreu R et al., Molecular Biosystems, 2009 Dec;5(12):1512-26. Epub 2009 Oct 1).

3. Expressing RT-PCR data

Throughout his report, Dr. Galbo criticizes the fact that we express our data as arbitrary units or fold-changes. In our intervention studies, we normalize pre-exercise CT values to 1 and express RT-PCR data obtained in subsequent samples as fold changes. This is the relevant way of expressing data, when looking for an effect of an intervention. Moreover, this is common practice; this is "how it is done". Thus, it is incomprehensible why Dr. Galbo would criticize this procedure as

1) He appears to be no expert in molecular biology.

2) He has co-authored at least 10 publications in which mRNA levels are also expressed as arbitrary units: http://www.ncbi.nlm.nih.gov/pubmed?term=galbo%20h%20mRNA

4. Immunohistochemistry

Throughout the report, Dr. Galbo appears to be confused with regard to the IHC technique as he expresses himself as if he believes that IHC is a quantitative technique. That is not the case.

Immunohistochemistry or IHC refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies specifically to antigens in biological tissues. IHC is widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Visualising an antibody-antigen interaction may be accomplished in a number of ways. In the most common instances, an antibody is conjugated to an enzyme, such as peroxidase that can catalyse a colour-producing reaction. Optimal staining depends on a number of factors including the antibody dilution, the staining chemicals, the preparation and/or fixation of the cells/tissue, and length of incubation with antibody/staining reagents. These are often determined by trial and error rather than by any sort of systematic approach. Immunohistochemistry is not a quantitative method per se and the staining intensity may vary, depending on the procedure used.

5. Reuse of biological material

Integrative human physiology is often based on highly advanced physiological studies in humans. The voluntary human subjects may be asked to participate in strenuous experiments that last for hours or days with several appointments to the laboratory. Catheters are often inserted in the veins and arteries, biopsies are often obtained from muscle and/or adipose tissues, large volumes of blood are obtained, etc, etc.

When designing a physiological study, several different scientific questions are often addressed. They may be so diverse that they are unsuited for presentation in one article. In addition, new scientific questions may arise after the first publications from a study have appeared. Such questions may be addressed by the use of biological material, which has been stored in the freezer.

In some cases, there may be lack of material in order to answer the new hypotheses and more experiments will have to be added using the same protocol. Considering the strain on the human subjects, we think it would be highly unethical not to try to get the most out of the human samples that have already been obtained. Alternatively, new test persons or patients would have to be recruited and undergo experimental procedures, just for the sake of avoiding using biological material that was obtained in former studies.

The common practice has been either to give a reference in the methods section to a previously published study or to give a thorough description of the design and the procedures in each paper. It is also common practice that supporting data, which describe the study population, but are not related to the research focus of the new article, may be "re-used".

Dr. Galbo claims that "the material indicates extensive reuse of subject populations and tissue material as well as reuse of data, both without notifying the reader."

In table 1, we provide an overview of the main experimental studies that have been included in the 12 publications reviewed by Dr. Galbo.

It is correct that we have used biological material (muscle biopsies) from the same test persons in more than one article. In regard to data we have included the same description of muscle fibre types in 3 papers as we regarded such information as a description of the study population (table 1).

In some cases, we have neglected to include a proper reference in the method section, but we have typically referred to other papers using the same material in the results section and discussion section.

However, it is highly peculiar that Dr. Galbo finds it proper to raise serious accusations against us on this basis, given that on several occasions he himself has followed the same tradition with regard to optimal use of human material. Moreover, Dr. Galbo has extensively reused his data.

In order to show that this procedure is common practice in physiological research environments, we would like to provide some examples, showing that many researchers within integrative physiology, and indeed also Dr. Galbo, adhere to this tradition.

One such example is given in table 2. With regard to the lack of cross-references between papers, where material from the same subjects is used, Galbo is found "guilty" in exactly the same neglect. A few examples are provided below - including a 2011 publication in which a 2010 publication is referenced – but the reader gets the impression that the papers represent two independent experiments. There is no description of reuse of subjects:

1. Elevated muscle interstitial levels of pain-inducing substances in symptomatic muscles in patients with polymyalgia rheumatica. Kreiner F, **Galbo H**. Pain. 2011 May;152(5):1127-32. Epub 2011 Mar 8.

2. Increased muscle interstitial levels of inflammatory cytokines in polymyalgia rheumatica. Kreiner F, Langberg H, **Galbo H**. Arthritis Rheum. 2010 Dec;62(12):3768-75. doi: 10.1002/art.27728

Subject characteristics in the PAIN 2011 and Arthritis Rheum 2010 articles are identical. The Ethical Committee of Copenhagen approval number is KF[01]261665 for both trials.

Moreover, table 1 of both articles are not just containing the same information, but represent **double publication**, not just of data, but also in format. There are reuse and double publication of data for **ESR and CRP as well as for blood flow (outflow/inflow ratio).**

In addition, it is possible that the subjects included in the paper below are included in the two papers above:

3. Insulin sensitivity and related cytokines, chemokines, and adipokines in polymyalgia rheumatica. Kreiner F, **Galbo H.** Scand J Rheumatol. 2010;39(5):402-8. Jan.

Another example is given below and outlined in table 3. The below-mentioned three papers seem to be based on the same experiment with the same subjects, although there are minor differences in the method description (see table 3). Only the last paper makes a reference to the second paper and only in the discussion section (i.e. not in the method section). Paper 3 (the IL-6 paper) is not referenced in any of the papers. Dr. Galbo is last author on paper 2 (2004), which does not provide any cross-reference to paper 3 (2003):

1. Muscle metabolism during graded quadriceps exercise in man. Helge JW, Stallknecht B, Richter EA, **Galbo H**, Kiens B. J Physiol. 2007 Jun 15;581(Pt 3):1247-58. Epub 2007 Mar 22.

2. Interstitial glycerol concentrations in human skeletal muscle and adipose tissue during graded exercise. Stallknecht B, Kiens B, Helge JW, Richter EA, **Galbo H**. Acta Physiol Scand. 2004 Apr;180(4):367-77.

3. The effect of graded exercise on IL-6 release and glucose uptake in human skeletal muscle. Helge JW, Stallknecht B, Pedersen BK, **Galbo H**, Kiens B, Richter EA. J Physiol. 2003 Jan 1;546(Pt 1):299-305.

Yet another example is given in table 4:

1. Myosin heavy chain isoform transformation in single fibres from m. vastus lateralis in spinal cord injured individuals: effects of long-term functional electrical stimulation (FES). Andersen JL, Mohr T, Biering-Sørensen F, **Galbo H**, Kjaer M. Pflugers Arch. 1996 Feb;431(4):513-8.

2. Long-term adaptation to electrically induced cycle training in severe spinal cord injured individuals. Mohr T, Andersen JL, Biering-Sørensen F, **Galbo H**, Bangsbo J, Wagner A, Kjaer M. Spinal Cord. 1997 Jan;35(1):1-16. Erratum in: Spinal Cord 1997 Apr;35(4):262.

3. Increased bone mineral density after prolonged electrically induced cycle training of paralyzed limbs in spinal cord injured man. Mohr T, Podenphant J, Biering-Sorensen F, **Galbo H**, Thamsborg G, Kjaer M. Calcif Tissue Int. 1997 Jul;61(1):22-5.

4. Insulin action and long-term electrically induced training in individuals with spinal cord injuries. Mohr T, Dela F, Handberg A, Biering-Sørensen F, **Galbo H**, Kjaer M. Med Sci Sports Exerc. 2001 Aug;33(8):1247-52.

6. <u>Further evidence that our procedures are "common praxis"</u>

Dr. Galbo has chosen to review only 12 out of the 13 muscle physiology papers with Penkowa as co-author. He has left out one paper on skeletal muscle Methallothionein expression in diabetic patients following training. The senior author of the paper that is not included in Dr. Galbo's list of Penkowa coauthored scientific muscle physiology papers is professor Flemming Dela, a former student of Dr. Galbo and a close collaborator with professor Jamie Timmons.

Below please find a short review of the Dela-Penkowa paper, in which we use the same main criteria as employed by Dr. Galbo, when reviewing our papers:

- Metallothionein-mediated antioxidant defense system and its response to exercise training are impaired in human type 2 diabetes.
  Scheede-Bergdahl C, Penkowa M, Hidalgo J, Olsen DB, Schjerling P, Prats C, Boushel R, Dela F. Diabetes. 2005 Nov;54(11):3089-94.

    - *Lack of mRNA and protein parallelism*
      There are no changes in MT-I+II plasma levels or muscle mRNA levels with training. Only changes in IHC! If anything there was a trend for a higher MT-I+II mRNA expression in the Diabetic subjects both pre and post training, whereas there is a marked expression of MT-I+II protein expression in controls, but not in diabetic individuals following training.
    - *mRNA: Only arbitrary units are shown!*
      There are no CT-values and thus the article is in line with the way we presented mRNA data at that time. It is noteworthy that molecular biologist Peter Schjerling who is indeed also very knowledgeable in mRNA analysis is a co-author on the paper. As said, at the time of publication it was common practice to present mRNA data as arbitrary units without CT values.
    - *Quality of Immunohitochemistry*
      It is stated that the MT-I+II IHC was performed as "Blinded, nonbiased evaluations". Does that mean that Penkowa did not know the blinding codes etc.? Further, does this also apply to the other IHC stainings (including those where data are not shown)?
      When looking at Fig 1 and 2 the quality with regard to colour intensity, staining patterns etc. are indeed in line with the data presented in the MT-I+II exercise paper that we have published with Penkowa.
    - *Other papers which seems to be based on same experiment/ subjects:*
      There are later papers by the same authors where 8 weeks rowing ergometer training of patients with T2D has been used – but more subjects were included in these papers – were the subjects in the MT paper a subgroup of these?

Specific responses to claims in the introduction of Dr. Galbo's report

In regard to Dr. Galbo's highly slanderous comments about the lenient scientific ethics of work performed in my laboratory and his outrageous accusations about scientific misconduct by my group and myself, I refer to the background information above and my request to be cleared by UVVU. Moreover, to disprove the criticism regarding lack of supervision, I hereby include:

a) Organizational structure of CIM showing that each PhD student belongs to a thematic research group with daily or almost daily supervision by a group leader.
b) Protocols for various laboratory analyses.
c) Our internal rules for conducting clinical studies.

(A1_1 to A1_20)

HG

Immunohistochemistry.

Key analyses were immunohistochemical determination of various peptides and biochemical dete*rmination of corresponding mRNAs in muscle. The histochemical data* clearly indicate fraud, e.g. the same stained section being used to illustrate presence of different peptides or of the same peptide in different situations.

Response

As said, when we became aware of the fact that the IHC pictures had been manipulated, we reported the relevant articles to UVVU and the scientific journals in questions and subsequently retracted the papers.

HG

Importantly, she also had the highest interest in positive results and has, by far, taken most of the credit for the studies. See e.g. the recent paper "BK Pedersen: Muscles and their myokines. J Exp Biol 214: 337-346, 2011" ,in which you may also note that, surprisingly, even some of the above papers that have now been retracted continue to be acknowledged.

Response:

The revised and final version of "J Exp Biol 214: 337-346, 2011" was submitted on December 8, 2010 and published in January 2011 (J Exp Biol. 2011 Jan 15;214(Pt 2):337-46). The fact that Milena Penkowa had performed manipulation with figures included in four of our scientific publications became clear to us in March and April 2011. We reported this fact to UVVU and the relevant scientific journals. The papers have been retracted and subsequently no reference has been made to them in any of our work.

Specific responses to Dr. Galbo's criticism of articles 1-12:

The original data from all 12 articles are included (E_1-12). Folders with description of volunteers and patients are kept in the CIM office and shall be put at your disposal upon request.

Paper 1. Penkowa M, Keller C, Keller P, Jauffred S, Pedersen BK. Immunohistochemical detection of interleukin-6 in human skeletal muscle fibres following exercise. FASEB J 17(14): 2166-2168, 2003.

Status of article: We have retracted the paper.

HG

As judged from the two figures, the IL-6 staining seen after exercise was surprisingly high considering the complete lack of staining at rest.

Response

Please see general comment above regarding IHC.

IHC is used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. However, it is not a quantitative method.

Given that muscle tissue expresses a low amount of IL-6mRNA at rest, which increases with exercise, we were not surprised that the IL-6 protein level was low/absent at rest and increased by exercise.

HG

Also, it was not clearly stated how sections were selected for illustration of findings after exercise. The legend to the key Fig. 1 mentioned "exercising subjects", but because only one section was shown for each time point of tissue sampling, it would not be correct to include samples from more than one subject. Although no attempt was made to quantify the IL-6 staining, and although it was not explicitely mentioned, how many sections were evaluated, it was stated in both Results section and legend that IL-6 immunoreactivity/ expression increased "significantly" with exercise. It was not stated, whether more than one scientist examined the sections, nor if this was done in a blindfolded manner.

Response

Penkowa performed the IHC analysis, evaluation and data presentation. It is stated that we show a representative subject. We do not think that any journal would allow the presentation of multiple subjects.

As IHC is not a quantitative method, it is apparent that the term "significant" is used in its non-statistical meaning, i.e. important, major, considerable, large.

HG

An 11-fold increase was found in IL-6 mRNA from pre-exercise to end of exercise. Unfortunately, however, the pre-exercise values were not given. Furthermore, the absence of IL-6 protein in muscle fibers in the presence of the corresponding mRNA at rest requires an explanation.

Response

Again it is well-known that IHC is not a quantitative method, as opposed to real-time PCR. The real time-PCR technique was developed into a hitherto unseen sensitive method, which allowed the identification of small amounts of mRNA. The finding that we could identify IL-6 mRNA by PCR, while protein was low/absent when measured by IHC, does not represent a contradictory finding. Please, also see the general comment above.

As for the pre-exercise levels, IL-6 mRNA CT levels were on average 34,2, whereas the levels were 30,7 in the post-exercise samples. When looking at individual levels, the average was 11-fold at peak increase following exercise.

HG

The need for an explanation becomes more urgent, when a previous paper from the group is taken into account. In the latter they reported that IL-6 mRNA could not be detected in muscle before a marathon race, and only in 5 of 8 subjects after the race (Ostrowski et al, J Physiol 508, 949, 1998). These findings were not mentioned.

Response

The Ostrowski paper is an early “PCR” – article. This pioneering work, which has become a citation classic, was highlighted in an editorial by Lancaster in J. Physiol 2009. As stated in the methods section, we used a ” Semiquantitative polymerase chain reaction” method, which is less sensitive than real-time qPCR.

Real-time qPCR was introduced as a technique to measure mRNA abundance following the Ostrowski et al. paper. This provided a faster and much more sensitive system for determination of mRNA levels allowing detection of low-abundance transcripts. Therefore it was now possible to determine pre-exercise levels of cytokines. The first determination that IL-6 mRNA increased in human skeletal muscle measured by real time PCR was performed by the Mark Febbraio (MF) group and was the catalyst for the collaboration between BKP and MF (Starkie RL, et al J. Physiol 533: 585-591, 2001).

HG

It was stated that the finding of a peak in mRNA at the end of exercise fitted nicely with a peak in intramyocellular IL-6 protein 3h later. Again, this statement could not be evaluated from the sparse immunohistochemistry data presented. The possibility exists that the evaluation of IL-6 stainings was influenced by knowledge about mRNA behavior in the present or previous studies.

Response

We do not accept the critique. Given that so far only IL-6 mRNA levels had been measured in muscle, we wanted to evaluate whether the IL-6 mRNA was translated into protein and whether this was present within the myofibers. Given that the IHC technique is not quantitative, we can only say

that the protein data fitted nicely with the IL-6 mRNA levels identified in this paper and in former papers.

HG

The above deficiencies raise serious doubts above the article as a whole, and as described below these doubts are confirmed by the fact that one of the sections shown in this article (Fig.1, panel H) was also used to illustrate an oxidative stress marker (NITT) in paper 4 (Fig. 3, Panels D and F).

Response

As said, when we became aware of the fact that the IHC pictures had been manipulated, we reported this to UVVU and the scientific journal in questions, and subsequently retracted the papers.

HG

Furthermore, the group has subsequently published conflicting findings as regards IL-6 protein expression in resting myocytes and distribution among various fiber types (see comments on Paper 6 below).

Response

We refer to our general comments with regard to IHC. The finding that the sensitivity in protein expression differs between two independent studies is not surprising. The quality of the staining depends on a number of factors including the antibody dilution, the staining chemicals, the preparation and/or fixation of the cells/tissue, and length of incubation with antibody/staining reagents. Immunohistochemistry is not a quantitative method per se and the staining intensity may vary depending on the procedure used. Even with quantitative analyses, one should be careful with regard to making direct comparison between actual levels measured in independent studies carried out with an interval of years.

HG

However, even if the data had been correct they would not justify the conclusion. An accumulation of IL-6 in myocytes was found, whereas a depletion would be necessary to account for the release of IL-6 from an exercising leg and the accompanying increase in plasma IL-6 concentrations. Furthermore, from endocrinology It should be common knowledge that generally an endocrine gland has hormone stores which are depleted early, when hormone secretion is stimulated. This knowledge should have made the authors suspicious as regards the data.

Response

It is clear that HG demonstrates a major lack of knowledge when it comes to the biological role of muscle-derived IL-6. The myokine IL-6 exerts major autocrine and/or paracrine effects within the muscle itself. This has been demonstrated in numerous publications (for reviews see e.g. Rudermann Diabetes. 2006 Dec;55 Suppl 2:S48-54. Review; Steinberg GR, Jørgensen SB. Mini Rev Med Chem. 2007 May;7(5):519-26. Review; Pedersen BK, Febbraio M Physiol. Rev. 2008 Oct;88(4):1379-406.), not the least in regard to the ability of IL-6 to enhance fat oxidation within skeletal muscle. The kinetics of exercise-induced IL-6 protein expression in the present study is totally in agreement with the fact that IL-6 is accumulated within myocytes following exercise and following the peak in IL-6 release from muscle.

HG

And if not earlier they knew these arguments from a CMRC (Copenhagen Muscle Research Centre) Scientific Meeting the 19th of June, 2003, before acceptance of the paper for publication. At that meeting the group presented its IL-6 findings, and during the discussion I pointed out the lack of consistency between their results and what would be expected from endocrine physiology.

Response

Pernille Keller tells me (BKP) that she, as a young PhD student, was the person, who presented the IL-6 data on the CMRC meeting in question. I cannot remember if I was present at the meeting, nor do I recall the particular discussion referred to by HG. However, throughout the years Dr. Galbo has often expressed his scientific views in an aggressive, rude and destructive fashion, not the least against me and people from my research group.

While I do not remember the discussion to which Dr. Galbo refers, I remember that I once invited him to a meeting in my office in order to try to sort things out. This was after a CMRC meeting, where again he had been rude against me and/or people from my group. At that occasion, he told me that he was angry that I had presented preliminary data from my group on hormone sensitive lipase (Am J Physiol Endocrinol Metab. 2004 Jan;286(1):E144-50. Epub 2003 Sep 23 and Diabetologia. 2005 Jan;48(1):105-12. Epub 2004 Dec 18). His criticism focused on the fact that I should not enter his field of research interest. I explained to Dr. Galbo that my collaborators were actually more experienced in the HSL-field than he and that I had presented the data at the CMRC meeting in the hope that the CMRC group would find the findings of interest to discuss. Dr. Galbo did not raise any criticism with regard to our science. As I remember that particular meeting between Dr.Galbo and I, we came to an agreement.

It is true that Dr. Galbo and I have disagreed on and disputed a number of issues over the years; however, I am absolutely sure that Dr. Galbo has never told me that he believed our data was flawed or dishonest.

HG

After the meeting I received a letter from a foreign student working in BK Pedersen´s (BKP) laboratory blaming me for criticizing such an extraordinary scientist (BKP). Because I had a special responsibility within the CMRC, I wrote him a lengthy reply stating, among other things, that the progress of science depends on competent criticism, that the criticism basically is not personal, and that, in the discussion, apart from the critique of the data, I had also made constructive proposals.

Response

Dr. Galbo's statement seems to speak for itself.

HG

Other criticism:

A one-way ANOVA was used. Considering the fact that two groups were included in the study, either this was not a correct approach (should be a two-way ANOVA) or the data analyzed statistically (IL-6 mRNA and plasma concentrations) were, in contrast to the impression given to

the reader, only obtained in the exercise group. In the present, as in the other papers, it was not apparent, whether p-values represented one- or two-tailed statistical testing.

Response

In the initial paper, only IL-6 IHC was presented as several publications had already demonstrated that exercise induces a fold-increase in IL-6mRNA. However, the reviewers requested presentation of the IL-6 mRNA data in order to confirm the IL-6 protein IHC data. We analysed IL-6 mRNA on a subset of subjects to confirm several previous studies. We did not provide any data for the resting control group. Therefore, a one-way ANOVA was used.

Overall comments

Reflection on validity of data: We believe that the IHC data are flawed.

Although we suspect that the IHC data are flawed, it appears that the conclusion from the above paper may actually be sound. At the same time that we published the first immuno-blots on IL-6 protein in skeletal muscle cells following exercise, supportive IHC as well as in situ hybridization data were published by Hiscock et al., demonstrating that IL-6 mRNA expressions increased in the muscle cells following exercise (C_4).

In the latter study, the authors aimed to determine whether skeletal muscle cells per se were a source of IL-6 during contraction and whether IL-6 production is fibre type-specific. Muscle biopsy samples were collected from seven males before (PRE) and after (POST) completing 120 min of continuous bicycle ergometry. Biopsies were sectioned and analyzed for the following: IL-6 protein detected by IHC, IL-6 mRNA content detected by in situ hybridization, fibre type measured by either IHC or myofibrillar ATPase activity stain, and glycogen content measured by periodic acid schiff (PAS) assay. They found that at PRE, IL-6 protein was evenly distributed across all fibres at low levels, consistent with glycogen distribution. At POST, IL-6 protein was greater ($P<0.05$) in HIGH compared with LOW glycogen fibres, which coincided with type 2 fibers. IL-6 mRNA was distributed peripherally in all fibers at PRE. At POST, however, IL-6 mRNA appeared predominantly in type 2 fibres, which also had higher glycogen content ($P<0.05$). These data clearly demonstrated that myocytes per se are a source of IL-6 produced during contraction. Their data also suggested that type 2 fibres predominantly produce IL-6 during muscle contractile activity. Thus, the study by Hiscock et al was in agreement with our study. The only difference was with regard to which muscle fibres that would express IL-6.

The main findings were that muscle fibres could express the IL-6 protein in response to contraction. The difference with regard to muscle fibres was believed to be due to a difference in exercise protocols, e.g. that the fibres that were recruited during the exercise would also express IL-6.

The idea that IL-6 is expressed and produced by muscle cells has been supported by several subsequent studies, e.g. by Serrano AL, Cell Metab. 2008 Jan;7(1):33-44; Glund et al Endocrinology. 2009 Feb;150(2):600-6. Epub 2008 Sep 25; Green et al, Diabetes 2011 in press.

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us, other than those related to the work performed by Milena Penkowa.

Paper 2. Fischer CP, Hiscock NJ, Penkowa M, Basu S, Vessby B, Kallner A, Sjoberg LB and Pedersen BK: Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle. J Physiol. 558: 633-645, 2004.

HG

In this investigation Penkowa was the third and BKP the senior among eight authors indicating a non-dominant responsibility for the final paper. Fourteen young healthy males were randomized to receive either a supplement of vitamins C and E or placebo for 28 days. They then had femoral arterial and venous catheters inserted and carried out moderate knee-extensor exercise for 3 h. Biopsies from the vastus muscle were sampled before exercise as well as 3 and 6 h later. It was concluded that supplementation with vitamins C and E attenuated the systemic IL-6 response to exercise primarily via inhibition of IL-6 protein release from the contracting skeletal muscle fibers.

Major concerns:

The presented findings disagreed with “common sense” and were mutually disagreeing to an extent that should have made the authors cautious as regards the data and the conclusions; the more so because they had been warned as described above.

Thus:

1.During exercise, in control subjects the histochemical data indicated accumulation of IL-6 in muscle cells, while IL-6 was released from the leg into the venous blood. This is not in obvious accordance with the belief that released IL-6 originated in muscle cells.

Response

The PCR data (Figure 3 in the paper) showed that IL-6 mRNA accumulated within contracting skeletal muscle tissue, while repeated measurements of IL-6 protein concentration in arterial and femoral venous samples showed that IL-6 was released from the contracting leg, but only during exercise in the placebo-treated control group (Figure 5 in the paper). These findings are in accordance with those reported in numerous other studies (for review and metaanalysis, please see Fischer CP, Exerc Immunol Rev. 2006; 12: 6-33).

The IHC data (Figure 4 in the paper) suggested that the increased IL-6 mRNA corresponded with an increased protein content within skeletal muscle during exercise and recovery. The observation that IL-6 protein accumulates within tissue, while also released into the circulation, does not seem to be a contradicting finding: First, the circulating amounts of IL-6 could represent a “spill-over effect” where most of the IL-6 is retained in the contracting muscle tissue. The suggested autocrine and paracrine effects of IL-6 are accordance with this explanation. Second, it is possible that the stainings represent immature precursors of IL-6. Of note, mature IL-6 is the produced from a propeptide (EMBO J. 1997;16(5):989-97), which subsequently is cleaved, phosphorylated and glycosylated (J Immunol. 1989;142(3):948-53; J Immunol. 198; 143(7):2153-9; Cell Immunol. 1990;130(2):437-45). Third, a simple time lag in the release would produce a similar result.

It seems quite unlikely that all IL-6 translated into protein should be released immediately without any – at least transient – intracellular accumulation of IL-6 protein including its precursors.

HG

2.During exercise, the accumulation of IL-6 in myocytes was similar in the two groups, even though IL-6 was released from the leg in control subjects, but not in vitamin treated subjects.

Response

Again, the observation that translation (of IL-6 mRNA into protein) and translocation (of IL-6 into the circulation) are two separate steps regulated separately does not seem to be contradicting. Both posttranslational modifications (cleavage, phosphoration and glycosylation) and translocation are most likely to be steps regulated by other mechanisms than those regulating translation. In vitro findings suggest that antioxidants like vitamin C and E regulate IL-6 synthesis in myotubes at the level of transcription, but IL-6 mRNA and IHC data from our paper suggested that posttranslational mechanisms are involved as well. Of note, the represented IHC data support the PCR data, but the interpretation that posttranslational mechanisms are involved is not solely based on the IHC data. Moreover, the PCR and IHC data both represent just snapshots, thus providing no information about actual turnover of either IL-6 mRNA or protein within the muscle tissue. Accordingly, it makes little sense to try to make direct comparisons with the IL-6 net release data based on repeated samples from the femoral artery and vein.

HG

3.During the 3 h postexercise recovery period a similar marked decrease in intramyocellular IL-6 took place in both groups. This was, in fact, accompanied by release of IL-6 from the legs of both groups. However, the release was much higher in controls than in vitamin treated subjects. The difference between the groups in the relationship between IL-6 depletion and leg release, respectively, was not explained.

Response

Again, several explanations for the observed findings are possible: First, a release of IL-6 was indeed observed in both groups, but mainly during recovery in the vitamin treated group. Second, intramyocellular IL-6 could be degraded locally without being released. Third, neither the PCR nor the IHC data provide information about turnover of IL-6 mRNA or protein within the tissue. Accordingly, it is possible that both the synthesis and degradation were attenuated in the vitamin treated group. Changes in rate of synthesis/degradation within the tissue could probably produce similar PCR and IHC results, but different IL-6 release data when comparing the two groups.

Indeed, the above points could have been discussed in more detail in the paper, but several aspects besides translation/translocation had to be included in the discussion

HG

To reconcile these various findings within the frames of the overall conclusion is probably not possible, and an attempt was not made.

The objections concerning the histochemical data are the same as described for Paper 1. “Significant increases” in IL-6 expression in muscle fibers in response to exercise were reported, but this was not based on quantification and statistical testing. It was not described how sections were evaluated; it should have been done blindfolded, preferably by more than one investigator. Single sections from three time points obtained from a subject from each group were presented in a

figure. However, it was not stated, whether all samples representing controls or vitamin treated subjects were from the same subject.

Response

As was the case for paper 1, Penkowa performed the IHC analysis, evaluation and data presentation of the present article. And again, as IHC is not a quantitative method, it is apparent that the term "significant" in the context of IHC was used in its non-statistical meaning, i.e. clear, marked, major, considerable, large. Of note, no attempt was made in order to hide the fact that the IHC data were qualitative rather than quantitative: For example, the IHC data (Figure 4) is entitled "Representative stainings". Also, it was stated in the discussion that "However, we could not determine whether the skeletal muscle IL-6 protein content was higher in Treatment than in Control, since the intensity of the stainings for IL-6 protein was not quantified".

HG

Based on comparisons between neighbouring sections stained for ATPase and IL-6, respectively, the authors concluded (in contrast to findings in Paper 1) that in response to exercise IL-6 was accumulated predominantly, but not exclusively, in type 1 muscle fibers. It was not reported how many fibers were evaluated, and no attempt was made to give a quantitative estimate of the distribution of IL-6 positive fibers among fiber types.

Response

During the writing and review process of this manuscript, only two other studies evaluating IL-6 protein in contracting skeletal muscle were available (Paper 1 and Hiscock N et al, FASEB J 2004, 18: 992-4). Although both studies also found that IL-6 protein could be detected in skeletal muscle following exercise, the distribution of the staining differed between studies. Both we and the reviewers assigned by the Journal of Physiology wanted a paragraph in the discussion about these apparent differences in IL-6 protein distribution. Possible explanations were provided, but no final conclusion regarding the apparent fibre-type specific distribution was made. In fact, it was concluded that "muscle biopsies obtained during exercise of different modes and intensities would add useful information to the observed discrepancies in skeletal muscle IL-6 protein expression found in the different studies at this point".

HG

IL-6 mRNA increased similarly with exercise in the two groups, and responses were comparable to those seen in Paper 1. Again, unfortunately only fold changes were presented. This makes it impossible to evaluate the actual amounts in the tissue and, accordingly, whether the mRNA most likely originated in myocytes or in less dominant cells. In the present, in contrast to the previous study (Paper 1), IL-6 mRNA and protein levels in muscle declined in parallel after exercise. This discrepancy was not mentioned, even though the time relationship between the two variables was emphasized in paper 1.

Response

Again, it should be noted that regardless of the mode of PCR data analysis, IL-6 mRNA data only represent snapshots. No information about rates of synthesis or degradation was provided with the employed method. The "true" amount of newly synthesized IL-6 in response to exercise was therefore unknown.

In addition, the delta-delta-CT method was considered standard method for evaluating quantitative PCR data at the time when the experimental part of study was carried out. Several other studies at the time used exactly the same method for evaluating quantitative PCR data. Of note, the delta-delta-CT method is simple, but requires that the level of mRNA before the intervention is set to 1, while following data are presented as fold changes from this "pre" value. In later studies, a standard curve was included in the PCR amplification process. The standard curve made it possible to evaluate linearity and efficiency as well as making it easier to compare different groups.

Finally, the discrepancies between paper 1 and 2 especially regarding IL-6 protein in skeletal muscle was indeed discussed in paper 2. And it was concluded that differences in mode and intensity possibly played a role.

HG

Other concerns:

Subject characteristics of the two groups ought not to differ. Correspondingly, no indication of statistical difference was shown in Table 1 regarding BMI (23.5+/- 0.3 (SE), vitamin group, vs 25.9 +/- 0.3, control group, kg/m2 (unit incorrectly stated)) and maximal power (150 +/- 3 vs 140 +/- 3 W). Nevertheless, the values appear to differ. In line with this supposition, during exercise at 50% of maximal power, leg blood flow also appeared lower (20%) in controls compared with vitamin treated subjects (Fig. 1).

Response

Neither age, height, BMI, nor maximal power (Pmax) differed statistically when comparing the two groups. Indeed, this could have been stated more clearly, although comparisons between groups were evident in the lower part of table 1, containing plasma vitamin concentrations. We agree that the BMI unit is incorrectly stated (a minus is lacking in the final print version of the manuscript).

Regarding femoral blood flow, the employed 2-way ANOVA showed no statistical difference between groups, but effect of time alone. Later (in the PhD thesis by Fischer CP, 2005), additional comparisons using an "area-under-curve" approach were performed, but this still did not provide a statistically significant difference between groups (P=0.134). When adjusted for workload, the apparent difference in femoral blood flow when comparing groups was even lower (P=0.360).

Overall comments

Reflection on validity of data: We think this a strong paper and we do not agree that the presented findings in paper 2 disagreed with "common sense" and were mutually disagreeing. There is an agreement between PCR and IHC data in the present study. However, given the knowledge we have today, it is uncertain whether we can trust the IHC, which was performed solely by Penkowa. Still, the major conclusions drawn in the article do not rely on IHC.

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us. We are uncertain as to whether this is also true in regard to the work performed by Milena Penkowa.

Paper 3. Akerstrom T, Steensberg A, Keller P, Keller C, Penkowa M and Pedersen BK: Exercise induces interleukin-8 expression in human skeletal muscle. J Physiol 563: 507-516, 2005.

Status of article: We have retracted the paper.

HG

In this investigation Penkowa was fifth among six authors indicating a non-dominant responsibility for the final paper. BKP was senior and corresponding author. Two protocols were used. In one (study 1), young healthy men were randomized to carry out moderate ergometer cycling for 3 h or to rest, while in the other (study 2), young men performed moderate knee extensor exercise for 3 h. Muscle biopsies were taken in both experiments (but apparently only used for histochemistry in study 1). In study 2 femoral catheterization was used.

Concerns:

Subject population: It is not clear, whether, in study 1, eleven, or twelve, or maybe eighteen subjects participated. Thus, in the Methods section it was stated that the number was 11. However, according to the legends of Figs. 1 and 3, six subjects were included in each of the two groups (exercising or resting). The suspicion arises that one subject may have been excluded without this being mentioned, because it would be natural to have equal numbers of subjects in the two groups. Furthermore, in papers 1 (see above) and 4 (see below) 18 subjects (12 exercising and 6 resting) carried out a protocol identical to the one used in the present study (exercise and repeated biopsies for 24 h). The characteristics of the subjects in the three papers were remarkably similar (age, height, weight and Vo2max), a fact leading to the question, whether the subjects (and, accordingly, tissue samples) used in the present study were a subgroup of the subjects participating in paper 1 and 4, the material studied in the three papers being basically identical.

The suspicion that the three studies represented the same material is further augmented by the fact that in the present paper (on p. 511), it was stated that "IL-8 protein was not expressed in muscle tissue before exercise (n=12)", indicating that the exercise group overall included 12 subjects. Moreover, although in Paper 4, according to information given in the Methods section, twelve subjects participated in exercise, while 6 subjects rested, the legend of Fig. 1 reports 6 exercising and 5 resting subjects – corresponding to the numbers stated in the Methods section of Paper 3.

Response

We refer to our general comment (and table 1) with regard to study populations.

HG

Histochemistry: Data were illustrated by one section stained for IL-8 protein from each of the six time points at which muscle was sampled (Fig. 2). In addition, two sections at 2-4 times higher magnification, and a section colored for various fiber types were shown. It was not stated, how sections were selected, e.g. if they were from one or more subjects, or evaluated (e.g. one investigator?, blindfolded?). They were all of poor quality and did not confirm the text, which stated that IL-8 was expressed in cytoplasm, membranes (which?), nuclei, and intermittently (meaning?) in endothelium.

Response

The IHC part of the study was carried out by Penkowa.

HG

Also, in the text (p. 511), it was stated that 21 h after exercise there was a strong immunoreaction for IL-8, whereas in the legend of Fig. 2 it was stated that at this time point, the staining was mildly increased. More devastating, however, the section representing the 21 h time point appears to be the same as the one representing the 0 h (pre-exercise) time point. And this section was also used in Paper 5, where in Fig. 3D, it was said to illustrate the presence of IL-6 receptor protein in muscle fiber membranes.

Response

As said above, when we became aware of the fact that the IHC pictures had been manipulated, we reported this to UVVU and the scientific journal in questions and subsequently retracted the paper.

HG

IL-8 mRNA: It was stated in the Discussion section (p. 513) and in the conclusion (p. 514) that exercise induced a marked increase in IL-8 mRNA within muscle fibers. However, the real time PCR analysis used does not allow localization of the measured mRNA.

Response

We made the assumption in the discussion that the increase in muscular IL-8 mRNA corresponded to the IHC image demonstrating an increase in muscular IL-8 protein.

HG

Furthermore, only fold-changes in mRNA from pre-exercise values were reported. Critical threshold/ number of cycles needed to reach detection (CT values) for the analysis should have been reported in order for the reader to be able to evaluate how big a cell population might contribute to the mRNA measurements.

Response

We refer to our general comment with regard to expression of mRNA levels.

IL-8 Ct levels went from 36 (pre) to 31 (post) in the exercise study and from 34 (pre) to 27 (post) in the 2-legged knee extensor exercise model.

HG

Furthermore, the absence of IL-8 protein (according to histochemistry) in the presence of the corresponding mRNA at rest requires an explanation.

Response

The Ct levels before exercise would only represent <10 mRNA copies per cell, thus, the protein level would be expected to be low.

HG

Calculation of IL-8 release from the leg: Femoral veno-arterial plasma concentration differences (in Fig. 5 with the wrong unit: ng/min) were incorrectly multiplied with leg blood flow instead of with plasma flow. It is surprising that in the previous paper with the same senior author (Paper 2) the corresponding calculation for IL-6 release was correctly carried out. In the middle of the exercise period a significant increase in IL-8 release above basal levels was found. However, before as well as after that time point, at rest and during exercise and recovery, IL-8 tended to be taken up in the leg rather than being released into the bloodstream.

This pattern is quite unusual, yet the authors did not comment on that. No change in arterial (systemic) IL-8 concentration occured. The conclusion drawn by the authors that muscle-derived IL-8 may "exert its effect in an endocrine or paracrine fashion" (p. 514), even though tentative (and ambiguous), was therefore not justified by their results.

Response

Dr. Galbo is confused when he he mixes up the measurement of net IL-6 release and net IL-8 release (and the respective use of plasma and blood flow). For IL-6, a study has suggested that this cytokine does not interact with the red blood cells (Castell 1988, Eur J Biochem 177: 357-361). This is why we use plasma flow to calculate IL-6 net release across a working limb.

IL-8 on the other hand is known to interact intimately with the red blood cells (Van der Laken 1998, Rennen 2003). Therefore, it is most appropriate to use blood flow when calculating net IL-8 release. In fact, the net release would have been underestimated by a factor of approximately 2 if plasma flow had been used. Even considering Dr. Galbo's confusion we are surprised that he brings this up as a major concern since the conclusions of the study in question would not have changed even if we had wrongly used plasma flow. The data demonstrating a transient IL-8 net release across a working limb only support local production, since no systemic increases in plasma IL-8 were seen – as also clearly presented in the paper.

It is correct that a small net release of IL-8 across the working limb was only observed at the 1.5 hour time-point. It is also correct that we used blood flow and not plasma flow when calculating the net release of IL-8 across the working limb, which is indeed the correct way to perform the measurement.

There was no significant release or uptake of IL-8 across the limb at any time-points other than at 1.5 hours of exercise. Dr. Galbo's observation that figure 5D may suggest that IL-8 was taken up by the muscle at other time points was not supported by statistics (as also stated in the article at p. 513), and we did not find any reason to discuss this further (an observation that we believe represents analytical variance). It is clear from reading the paper that we spend a great effort discussing the finding that a release was only seen at the 1.5 hour time-point and that it occurred without any changes in systemic plasma IL-8 levels. We clearly concluded that IL-8 seems to exert its effect locally in the working limb (p.514). It is correct that we write "endocrine or paracrine fashion" just 17 lines above this conclusion - this should of cause have been "autocrine or paracrine fashion" (something that would be obvious to most unbiased readers). The fact that we did not see any systemic increases in plasma IL-8 (as also observed by other groups) and only a transient and small net release of IL-8 across the working limb was thoroughly discussed in the paper and in the overall conclusion of the study (last 4 lines p. 514).

Overall comments:

Reflection on validity of data: Although there is an agreement between PCR and IHC data in the present study, we suspect that the IHC data may be flawed. Therefore, we have retracted the paper.

However, the IHC data were not crucial for the major conclusion of this paper, which we believe contains important information. We demonstrate that IL-8 is released from an exercising limb, a finding that is not based on IHC data. Also, later studies confirm that exercise induces mRNA and protein expression of CXCL1 (IL-8 like gene) in murine muscle (Pedersen L et al. Exercise-induced liver CXCL-1 expression is linked to muscle derived IL-6 expression. J Physiol. 2011 Jan.).

We maintain that the IL-8 data were presented and discussed in a balanced way taking all current literature into account and that the conclusions drawn in the paper were fully justifiable.

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us, other than those related to the work performed by Milena Penkowa.

Paper 4. Penkowa M, Keller P, Keller C, Hidalgo J, Giralt M and Pedersen BK: Exercise-induced metallo-thionein expression in human skeletal muscle fibres. Exp Physiol 90, 477 – 486, 2005.

Status of article: We have retracted the paper.

HG

In this investigation Penkowa was first and corresponding author, while BKP was senior author.

Concerns:

Histochemistry: Histochemical findings were presented in Fig. 2 (metallothionein expression) and Fig. 3 (expression of the oxidative stress marker nitrotyrosine, NITT). The figures illustrated time courses by showing one section from each time point of muscle biopsy. However, in Fig. 3 the section representing muscle at the end of 3 h of exercise (3D) was the same as the one representing muscle 3 h after exercise (3F), and the very same section was used in Paper 1 to illustrate IL-6 expression in muscle 21 h after exercise (1H).

Response

When we became aware of the fact that the IHC pictures had been manipulated, we reported this to UVVU and the scientific journal in questions and subsequently retracted the paper.

HG

As was the case with histochemical stainings in the other papers, it is not clear how the presented sections were selected, nor how sections were evaluated. No attempt was made to quantify staining; nevertheless the term “insignificant immunoreactivity” was used. Even though the finding of exercise-induced metallothionein expression was ascribed to oxidative stress, it was not studied on neighbouring sections, whether observed variation in metallothionein content between fibers was accompanied by variation in NITT.

Response

No comments. This work was entirely performed by Milena Penkowa. We, ourselves, do not have expertise in IHC. That is the reason why we collaborated with Penkowa on this issue.

HG

Subject population: As mentioned above (see Paper 3 “Subject population”), discrepancy exists between the number of participants stated in the Methods section (12 exercising and 6 resting=18) and legend to Fig. 1 (on metallothionein mRNA, 6 exercising and 5 resting=11), respectively. Furthermore, albeit not stated, there is reason to suspect that subjects and muscle samples were identical to those of Papers 1 and 3 and also overlapped with those of Paper 5 (see below). The anthropometric data presented in the three papers were compatible with this suspicion.

Response

We refer to our general comments and table 1. We inform the reader that the mRNA measurements were only performed on a subgroup. The reason was “lack of material”.

HG

Metallothionein mRNA: It was stated on p. 480 that mRNA levels were hardly detectable, i.e. essentially not quantifiable, at rest. Nevertheless, fold changes with exercise were calculated. CT-values should have been included.

Response

MTII mRNA data in figure 1 have been calculated and presented as MTII/GAPDH ratios, as the pre-exercise levels were hardly detectable. However, for the ease of the reader, an approximate fold change was presented in the results section. The reader was fully able to independently judge the fold change given from the ratios presented in figure 1. There was no misleading of the reader.

HG

The conclusion in Abstract and beginning and end of Discussion that metallothionein mRNA is expressed in both type 1 and 2 muscle fibers after exercise was not justified by the findings, because in situ hybridization was not performed.

Response

The muscle biopsies were obtained from musculus quadriceps, which represents a mixture of type 1 and type 2 fibers. As the MT protein appeared to be uniformly expressed by muscle fibres, we concluded that there was no difference between the ability of the fibre types to express MT.

HG

Minor:

In Fig. 1 data were stated to present geometric means +/- SEM. This is not correct as dispersion from means was not symmetrical.

Response

Dr. Galbo is confused. Dispersion from mean is not symmetrical, when using geometric means.

Overall comments:

Reflection on validity of data: Today, we believe that the IHC data are flawed.

However, initially we did not find any reason to suspect Penkowa's data. There was an agreement between PCR and IHC data in the article. Pernille Keller states "as I recall the events, Penkowa showed me the IHC data before I showed her the mRNA data, and the data seemed to match fine. In an email we discuss the fact that MTII protein increases almost at the same time as the mRNA which Penkowa is unsure of, while I (Pernille Keller) think it matches fine."

This paper is not a key paper for CIM. We did not conclude that metallotheionein was released from muscle cells and CIM associates did not follow this line of research. MP later published a paper on muscle and MT together with Flemming Dela (Scheede-Bergdahl C. Diabetes. 2005 Nov;54(11):3089-94). Apparently, the latter group was inspired by Penkowa's findings and did not

at the time find reasons to suspect her data. Of note, in the Dela paper, there is no agreement between mRNA and protein expression in muscle biopsies.

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us, other than those related to the work performed by Milena Penkowa.

Paper 5. Keller P, Penkowa M, Keller C, Steensberg A, Fischer CP, Giralt M, Hidalgo J and Pedersen BK: Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6. FASEB J 19: 1181-1193, 2005.

Status of article: We have retracted the paper.

HG

P. Keller was first and corresponding author, while Penkowa was second and BKP senior author on this paper. Two human and one mouse experiment were carried out.

Major concerns:

Subject population: In one of the human studies, the same ergometer exercise protocol and the same time schedule for blood and vastus muscle biopsy sampling as used in Papers 1, 3 and 4 were applied. Furthermore, compatible with some of the numbers given in papers 3 and 4, in the present protocol six subjects exercised, while only five subjects rested throughout. The anthropometric data presented suggest that study populations and tissue material were identical in the four studies.

Response

Please, see our general comments as outlined in detail in table 1 of this document.

HG

Correspondingly, in the Discussion it was written that the IL-6 plasma levels obtained were higher in infusion experiments (see below) than in exercise experiments. This is so, although plasma concentration measurements had not been mentioned earlier in the paper. Probably, the measurements referred to are those presented in Paper 1.

Response

In order to avoid double-publication of data, we report (but do not show) the plasma-IL-6 levels from the original study and we provide the proper reference.

HG

Finally, the muscle section illustrating IL-6 receptor presence 21 h after exercise in the present study (Fig. 3 D) was in Paper 3 used to illustrate IL-8 presence before exercise.

Response

When we became aware of the fact that the IHC pictures had been manipulated, we reported this to UVVU and the scientific journal in question and subsequently retracted the paper.

HG

If the material of the four papers was derived from the same experimental study, this was not mentioned in Paper 5, and papers 3 and 4 were not quoted, while the citation of Paper 1 did not mention an intimate relationship with the present study.

Response

As said, we reported the plasma-IL-6 levels from the original study and gave the proper reference. Please, see general comment above on this issue.

HG

In the other human study recombinant human IL-6 or saline were infused into a femoral artery. This appears to be an unethical approach, if not justified by another purpose than that of the present study. It was not stated from where blood samples were drawn. However, in a preceding study of FFA kinetics from BKP´s laboratory one femoral artery was used for infusions identical to those of the present study, while blood was sampled from the other femoral artery (van Hall et al, J Clin Endocrinol Metab 88: 3005-3010, 2003). The subject characteristics and the plasma IL-6 concentrations appear to be identical in the two studies. So, one may suspect that although it was not stated the two resulting papers were based on the same study.

Response

It is correct that we included a former human study to investigate a new scientific question. We included the reference from that study in the discussion (ref 38), but did not mention the original study in the methods section. However, there is no double publication of data. Again, we refer to the general comments above on this issue. It appears that Dr. Galbo has overlooked the fact that the study had been approved by the ethical committee.

HG

Histochemistry: Time courses of IL-6 receptor immunohistochemistry in exercise and infusion experiments, respectively, were shown in Figs. 2 and 3. One section was shown for each time point of muscle biopsy. Most devastating, the section used to illustrate IL-6 receptor presence 21 h after IL-6 infusion (Fig. 3D) was the same as the section used in Paper 3 to illustrate the presence of IL-8 in muscle before (Fig. 2A) and 21 h after (Fig. 2G) exercise. It was not reported how sections were selected for illustration (e.g. representing the same person at all time points?), neither how they were evaluated (e.g. blindfolded?, by more than one investigator?).

Response

As said above, when we became aware of the fact that the IHC pictures had been manipulated, we reported this to UVVU and the scientific journal in questions and subsequently retracted the paper.

HG

In the Discussion it was stated that IL-6 receptor was uniformly expressed in both type 1 and type 2 muscle fibers. However, no evidence for this was presented, and in the Methods section staining for fiber types was not mentioned.

Response

The muscle biopsies were obtained from musculus quadriceps, which represents a mixture of type 1 and type 2 fibres. As the IL-6 receptor protein appeared to be uniformly expressed by muscle fibres, we concluded that there was no difference in fibre type expression.

HG

While after exercise a very prolonged increase in IL-6 receptor mRNA was paralleled by an increase in staining of the receptor in plasma membrane, in response to IL-6 infusion an increase in receptor staining was, surprisingly, seen in the absence of an increase in mRNA.

Response

In general, we do not find it scientifically correct to exclude “surprising data”, “not expected data” or “data that did not fit with the hypothesis”. In the present study, we provided the reader with our data and discussed possible explanations to the unexpected finding.

HG

Here as in the other papers, Western blotting of IL-6 protein would have been appropriate to support histochemical findings.

Response

Yes, but in those days, such analysis was not carried out in our laboratory.

HG

IL-6 receptor mRNA: In the human experiments the evaluation of data is rendered difficult by the fact that values were reported as fold changes from unreported basal levels. CT- values should have been presented.

Response

Please, see general comments above about the CT values.

The CT value for IL-6R mRNA is on average 33,5 pre-exercise, while it is 31,3 at 5 h post-exercise, indicating a clear increase in the amount of IL-6R mRNA.

HG

Other concerns:

Mouse study: Mice had muscle sampled either before or after swimming. In order for this experiment to be reproducible by other investigators swimming conditions should have been better described (i.e. water depth, number of mice swimming simultaneously per unit water surface). Because skeletal muscles differ, the exact muscle from which samples were obtained should have been stated.

Response

Whenever writing a manuscript the authors must consider what is an appropriate level of detail regarding the information provided. It should be sufficient to provide the reader with the knowledge that is necessary to fully understand and evaluate the performed study, but should on the other hand also be limited to what is relevant. It is well known that you can always contact the corresponding author, should you be interested in additional information such as in this case “water depth”.

HG

Conclusion in human study: It was stated in the final paragraph that "contractions induce post-exercise expression of IL-6 receptor mRNA and protein levels in human skeletal muscle, possibly to sensitize muscle to the decreasing IL-6 levels elicited by exercise". However, exercise does not elicit a decrease in IL-6 levels.

Response

In the beginning of the discussion, we write the following "Increased expression of the IL-6 receptor in muscle fibers after an exercise bout suggests that the muscle is sensitized by IL-6. The peak in IL-6 receptor production occurs several hours after the end of the exercise bout, at the time when IL-6 plasma levels are decreasing (4). Expression of the IL-6 receptor may therefore be a mechanism whereby muscle is sensitized to the effects of IL-6 when IL-6 levels are sparse".

We believe that this paragraph is self-explanatory.

Overall comments

Reflection on validity of data: Today, we believe that the IHC data are flawed.

However, the conclusion that exercise regulates IL-6 receptor mRNA expression is solid. The finding that exercise regulates the IL-6 receptor mRNA expression is supported by later studies (Keller C, J Appl Physiol. 2005 Dec;99(6):2075-9. Epub 2005 Aug 11.; Akerstrom TC et al. Exp Physiol. 2009 Nov;94(11):1124-31. Epub 2009 Jul 10.

We have unpublished data showing that human muscle biopsies and human primary muscle cell cultures express IL-6 receptor protein as measured by Western blotting (Scheele et al, unpublished data).

Other studies have shown that the IL-6 receptor protein is expressed by muscle cells, e.g. Weighert C et al. Direct cross-talk of interleukin-6 and insulin signal transduction via insulin receptor substrate-1 in skeletal muscle cells. J Biol Chem. 2006 Mar 17;281(11):7060-7. Epub 2006 Jan 17.

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us, other than those related to the work performed by Milena Penkowa.

Paper 6. Plomgaard P, Penkowa M and Pedersen BK: Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles. Exerc Immunol Rev 11: 53-63, 2005.

HG

Penkowa was second, while Plomgaard was first and corresponding, and BKP senior author on the paper. In this small investigation of seven young healthy subjects, the content of three cytokines was determined by immunohistochemistry in the basal state in three human muscles (triceps, vastus lateralis, soleus) known to have different proportions of type 1 and type 2 fibers. Adjacent muscle sections were stained for cytokines and fiber types, respectively.

Major concerns:

In contrast to Paper 1, in the present study IL-6 was found in muscle fibers at rest. This essential discrepancy between findings in the present and previous study by the group was not even mentioned, and, accordingly, no attempt was made to explain it.

Response

As pointed out before, HG assumes that the IHC technique is quantitative, which is wrong. The strength of the IHC technique is that it provides an expression of the localization of a given protein.

Optimal staining depends on a number of factors including the antibody dilution, the staining chemicals, the preparation and/or fixation of the cells/tissue, and length of incubation with antibody/staining reagents. Immunohistochemistry is not a quantitative method per se and the staining intensity may vary depending on the procedure used. Thus, whether a low expression is visible or not in the image is dependent upon a number of technical issues, also with regard to how the photography is carried out and the following editing of the image. The fact that a protein, evaluated by a qualitative method, is visible in one study performed in 2005 and not in a study performed in 2002 is not an obvious issue for discussion.

With regard to the present study, Peter Plomgaard alone was responsible for the photography.

HG

On the other hand, previous conflicting data regarding IL-6 distribution between fiber types upon contraction were discussed. It was stated that in Paper 1 intense exercise to exhaustion was applied. However, the exercise cannot be considered to be of high intensity, and in the paper it was not described as exhausting either.

Response

Of relevance for the study is the fact that 3 hours of exercise will lead to recruitment of both type 1 and type 2 muscle fibres, whereas bicycle exercise of shorter durations primarily will lead to recruitment of type 1 fibres.

HG

In the Title, Abstract ("TNF-alpha and IL-18 being solely expressed by type 2 fibers") and end of the Discussion ("the novel finding of the present study is that normal resting human skeletal muscles express TNF-alpha, IL-8 and IL-6 in a strict fiber-type specific fashion"), specificity of cytokine expression in different muscle fibers types was emphasized. However, in the first

paragraph of the Discussion the message was more equivocal: “The expression of TNF-alpha and IL-18 is predominantly found in the type 2 fibers whereas IL-6 is most abundant in type 1 fibers”. It is not possible for the reader to evaluate the underlying evidence. Thus, it was not stated how sections were evaluated (how many fibers per section?, by more than one investigator?), and attempts to quantify findings (e.g. simply: per cent of type 1 and 2 fibers, respectively, positive for a given cytokine) were not made.

Response

Parallel sections were evaluated. We made no attempt to quantify how many fibres would express a given cytokine.

HG

Cytokine expression detected by immunohistochemistry was not reflected at the mRNA level measured by rtPCR on muscle biopsies. In fact, the variation in IL-6 mRNA expression between the studied muscles tended to be the inverse of what would be expected from histochemistry data, mean IL-6 mRNA content being highest in triceps (“type 2 muscle”) and lowest in soleus (“type 1 muscle”) muscle (Fig. 3). This discrepancy between contents of cytokine mRNA and protein was suggested to be due to post-transcriptional regulation.

Response

With regard to the lack of mRNA and protein parallelism, we take the liberty to refer to our general comment above.

HG

However, in order to justify the main conclusion about fiber type specificity of cytokine expression as well as to elucidate the discrepancy between cytokine protein and mRNA contents, in situ hybridization should have been carried out and compared with fiber type composition.

Response

Using in situ hybridization would have allowed us to determine if the muscle fibres per se produced/expressed cytokine protein. However, this technique had not allowed us to conclude anything about posttranslational modification.

HG

mRNA for myosin heavy chain (MHC 1 and 2a) was also determined. It was claimed that the MHC 1 mRNA level, as would be expected from previous studies, reflected the corresponding protein level (judged from fiber typing). However, this is not fully correct, because the content of this protein was considerably higher in soleus compared with vastus lateralis muscle, whereas mRNA was not.

Response

We demonstrate that the triceps, quadriceps, and soleus muscles are different with regard to relative distribution of type 1 and 2 fibres. With regard to MHC 2 mRNA we find the expected distribution, whereas we had expected to see a slightly higher level of MHC 1 mRNA in soleus. Such biological variation is not rare and one could argue that it had been out of proportion to discuss this in detail.

HG

Reuse of material and data: See comments to Papers 7 and 10.

Response

We refer to our general comments above, please also see table 1.

HG

Minor concerns:

For immunohistochemistry, sections were incubated with goat serum to block non-specific binding. Some control sections were incubated without the blocking serum. It is not immediately apparent, why immunoreactions "were considered only if this control was negative".

Response

This part of the study was performed by Penkowa.

Overall comments

Reflection on validity of data: Penkowa had previously optimized the antibodies for IHC. A CIM laboratory technician (Ruth Rousing) performed the cutting of the biopsies and the staining under the supervision of Plomgaard in Penkowa`s laboratory. Peter Plomgaard analyzed the slides and made the photographies and compared these images with the fibre type stained slides from serial sections.

Since CIM personnel performed all experiments, we find it unlikely that Penkowa has influenced the conclusion from this study.

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us.

Paper 7. Plomgaard P, Penkowa M, Leick L, Pedersen BK, Saltin B and Pilegaard H: The mRNA expression profile of metabolic genes relative to MHC isoform pattern in human skeletal muscles. J Appl Physiol 101: 817 – 825, 2006.

HG

Plomgaard was first and corresponding, Penkowa second and BKP fourth among six authors of this paper. As in Paper 6 seven young healthy subjects had muscle biopsies (from triceps, vastus lateralis and soleus) taken in the basal state.

Major concerns:

Material: Age, weight and BMI of the subjects in the present paper were identical to corresponding values in Paper 6, and also the experimental protocol was the same. Furthermore, muscle biopsies were analyzed for fiber type distribution and myosin heavy chain (MHC1 and 2a) mRNA content in both papers. Data on these variables, although being presented a little differently in the two papers, appear to be identical, apart from MHC 2a values, which seem to be slightly lower in Paper 6 than in Paper 7. Apparently, subjects and material were the same in Papers 6 and 7 (as well as in Paper 10, see below), respectively, and the same data were included in both (all three) papers. This reuse was reported in none of the papers.

Response

It is obvious that the same subjects were used in the three papers and we had no intention to hide this fact. In addition, it should be obvious that determination of MHC fibre type distribution in the three muscles was not a new finding in any of the papers and only used as confirmatory information in order to demonstrate that the current samples could be used as a model for examining the impact of MHC fibre type distribution on mRNA content of selected metabolic proteins. As stated above, we find the criticism highly peculiar, given that on several occasions Dr. Galbo himself has not only reused biological material without notifying the reader, but has also performed regular double publication of data, even recently (2010 and 2011), please see general comments above and tables 2-4.

HG

Correlation analysis: The paper was widely based on correlation analysis between mRNA content of various metabolic genes determined on muscle biopsies on the one hand and % type 1 fibers in the biopsies on the other. In each analysis measurements from all subjects and the three muscles were included. This is not a correct statistical approach. Each individual can only contribute to the analysis with one pair of measurements. Furthermore, because % type 1 fibers varied considerably and was well separated between the three muscles, significant correlations were found between mRNA and % type 1 fibers, although it is apparent from the data plots that no such correlation existed within the individual muscles. Accordingly, the conclusion drawn i.e. that some metabolic genes are fiber type specific (should be: dependant) is not justified by the correlation analysis performed. Rather, the differences in mRNA between the three muscles reflected other differences between the muscles than fiber type content. Finally, it is of note that data from the soleus muscle of one of the seven subjects were not included in the correlation analyses. The reason was not stated.

Response

We agree that there are many ways whereby data from different subgroups may be visualized. Of relevance is the fact that the data presentation used in this article is highly transparent as every single individual data set is visualized. This data presentation allows the reader to get a detailed impression of the results and how they may be interpreted. Such data presentation also allows the reader to see that one out of the total number of 21 biopsies (7 times three) is not included in the analysis. Thus, we do not hide anything.

Overall comments

Reflection on validity of data: Penkowa has only contributed by lending us equipment and instructed us in how to use it. It is not possible that she could have had any influence on the data.

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us.

Paper 8. Frydelund-Larsen L, Penkowa M, Akerstrom T, Zankari A, Nielsen S and Pedersen BK: Exercise induces interleukin-8 receptor (CXCR2) expression in human skeletal muscle. Exp Physiol 92: 233 – 240, 2007.

HG

Penkowa was second and BKP senior and corresponding among the six authors of this paper. The hypothesis of the authors was that muscle-derived IL-8 plays a role in stimulation of angiogenesis in response to exercise. The aim of this study, then, was to investigate, whether the IL-8 receptor (CXCR2) is expressed in human skeletal muscle, and whether it co-localizes with the TGF-beta receptor (CD105), which was known to be expressed in activated endothelial cells. Healthy young males were studied either before and after 3 h of moderate cycle ergometer exercise or during an identical period of rest.

It is of note that it was explicitly stated that the design was identical to that used in Paper 3 but that different subjects participated in the two studies. A similar statement was not given in Papers 1, 3, 4 and 5, respectively, and neither in Papers 6 and 7, respectively, a fact supporting the supposition that material from the same subjects were used in those papers (see above).

Response

We refer to our general comments above regarding study populations and to table 1.

HG

Major concerns:

Immunohistochemistry: According to the description given in the Methods section, staining for the IL-8 receptor and subsequent examination and recording followed the same procedures used in the former papers (primary antibody against the receptor, detection by secondary biotinylated antibody and streptavidin-biotin-peroxidase complex; examination by light microscopy). Staining for the TGF-beta receptor was not described. Furthermore, in the Results section the stainings mentioned were not reported. On the other hand, results of double immunoflurescense staining for the two receptors were presented (Fig. 2), although neither the fluorescence labeling nor the fluorescence microscopy had been previously described. So, regarding the immunohistochemistry the Results and the Methods sections did not correspond.

In the figure one muscle section from each of six time points before and after exercise was shown. Only sections stained for both receptors, and not sections stained for each alone, were shown. It was also not described, how sections were selected, e.g. whether they were from the same or different subjects, and how they were examined (e.g. by more than one investigator ?, blindfolded ?). It was stated that the IL-8 receptor was “low or absent” in skeletal muscle before and during the first 1.5 h after exercise, but had increased at 3 hours and peaked 6 hours after exercise. However, no attempt was made to quantify the receptor protein content.

Response

Immunohistochemistry (lab work, analysis and interpretation as well as the sections in the article concerning IHC) of skeletal muscle sections for CXCR2 resulting in Figure 2 was performed by Penkowa. We agree that more details could have been provided in the method section.

HG

Western blotting for the receptor protein seems warranted, the more so because of the apparent discrepancy between the absence at rest of immunohistochemically detectable IL-8 receptor protein in the face of expression of IL-8 receptor mRNA.

Response

At that time, our laboratory was not able to carry out the Western blotting technique.

HG

Essential conclusions not supported by data or imprecise: The authors concluded that the IL-8 receptor "is localized primarily to activated microvascular endothelium". This interpretation fits with the authors´ hypothesis on the role of muscle-derived IL-8. However, according to the illustration (Fig. 2), the receptor was primarily localized to the sarcolemma or adjacent cytoplasm (higher magnification than the one presented is warranted). It was also concluded that 21 h after exercise, the expression of receptor protein content had decreased to pre-exercise levels. Again, this is not in agreement with Fig. 2. The statement that the IL-8 receptor was low or absent in muscle before exercise and during the first 1.5 h after (p. 236) disagrees with the statement (p. 236) that the protein was expressed within the muscle fibers and at the sarcolemma at all time points. Another conclusion was that "exercise induces CXCR2 mRNA and protein expression in the vascular endothelial cells of the muscle fibers" (cf. Abstract). However, because in situ hybridization was not carried out, the cellular origin of the observed increase in receptor mRNA is unknown. Finally, in the Discussion, the present findings of increased IL-8 receptor mRNA and protein in muscle was associated with the finding in Paper 3 of a release in IL-8 from muscle, and a local role of IL-8 in muscle was suggested. However, it was not mentioned that a release in IL-8 was only seen in the middle of a 3h exercise session, whereas an increase in IL-8 receptor expression in muscle was not seen until after exercise.

Response

We do not understand the criticism.

HG

mRNA measurements: As judged from the description of the PCR analysis, where undiluted cDNA was used for CXCR2 measurement, the expression of the CXCR2 gene was very low in absolute terms. This is also supported by the high variation (100%) between baseline values in exercising and resting subjects, respectively, (Fig. 1), which is indicative of very low copy numbers in the biopsy. The implication for the interpretation of the claimed protein expression was not discussed. Furthermore, the low CXCR2 RNA level was not readily apparent to the reader, because basal CXCR2/GAPDH (house-keeping gene) mRNA ratios shown in Fig. 1 were close to 1. This could not reflect ratios between measurements in absolute terms, but probably reflected use of some normalization procedure, which, however, was not described to the reader.

Response

Please, see our general comments above about presentation of mRNA expression.

HG

Minor:

It was stated that data were presented as geometric means +/- S.E.M. However, in Fig. 1 (data on IL-8 receptor mRNA) the dispersion was symmetrical around the mean values.

Response

Dr. Galbo is confused and contradicts himself (please see his comments to paper 4). It is clear from Fig. 1 that the dispersion is not symmetrical. This is in agreement with the fact that when data are expressed as geometrical means the dispersion is not symmetrical.

Overall comments

Reflection on validity of data: There is an agreement between PCR and IHC data in the present study. However, given the knowledge we have today, it is uncertain whether we can trust the IHC, which was performed solely by Penkowa.

We are convinced that exercise upregulates the expression of CXCR-2mRNA levels as we have reproduced this finding in murine experiments, demonstrating a robust increase of CXCR2 in murine skeletal muscle (Pedersen L et al, data not published).

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us. We are uncertain as to whether this is also true in regard to the work performed by Milena Penkowa

Paper 9. Petersen AM, Penkowa, Iversen M, Frydelund-Larsen L, Andersen JL, Mortensen J, Lange P and Pedersen BK: Elevated levels of IL-18 in plasma and skeletal muscle in chronic obstructive pulmonary diseases. Lung 185: 161 – 171, 2007.

HG

Penkowa was second and BKP senior and corresponding author on this descriptive study. Plasma and biopsies from the vastus lateralis muscle were sampled from 20 patients with chronic obstructive pulmonary disease and 20 healthy controls.

Critique:

Subjects: It was repeatedly stated (Abstract, Introduction and Discussion) that controls were matched with patients according to age, gender and BMI. However, the statement in the Methods section that "controls were excluded as a result of BMI greater than 30" indicates that no genuine matching procedure was carried out. Still, controls were not randomly picked and, accordingly, the statistical testing for differences in age, gender and BMI carried out between patients and controls was incorrect.

Response

We do not understand this criticism. It is obvious that we chose to exclude patients with obesity. We decided to match the groups for BMI in an attempt to exclude patients with severe end-stage chronic obstructive pulmonary disease (COPD).

HG

For patients an inclusion criterion was smoking abstinence for at least two months. Nevertheless, in the control group, in addition to ten nonsmokers, ten non-symptomatic smokers were included. The rationale for using smoking controls is not clear, and data were not discussed in the context of impact of ongoing smoking.

Response

We find it rather obvious that ongoing smoking is an issue in COPD pathophysiology.

HG

Four patients were on oral steroid therapy, and it was stated in the Results section that differences between groups in leukocytes and CRP were maintained, if these patients were excluded. However, it was not reported, whether cytokine findings, which certainly might be influenced by steroid therapy, differed between these and the other patients.

Response

Exclusion of these four patients had no influence on any signs of inflammation.

HG

Methods: The description of RNA extraction dealt with adipose tissue, which was not studied in the present paper.

Response

Although we did not include data from adipose tissue, we chose to include this information.

HG

It is confusing that ATPase staining for fiber type identification was described in two separate sections. Illustrating the confusion, in the interposed section on immunohistochemistry the authors referred to the subsequent section for ATPase staining, but later it becomes clear that the preceding section on ATPase staining was the relevant one.

It was stated that muscle sections were stained for IL-8 (p. 164). Accordingly, it is surprising that IL-8 results, in contrast to IL-18 results, were not reported and compared with previous IL-8 findings by the group. This the more so, since in Paper 8 the angiogenic potential of IL-8 and its interaction with hypoxia were emphasized, while in the present study patients, in fact, suffered from hypoxia and had increased capillary density in skeletal muscle.

Response

We agree that the information about IL-8 staining is irrelevant.

HG

Histochemistry: Two figures were presented. Fig. 3 served to illustrate that IL-18 and Caspase-1 levels were very low or absent in muscle fibers from healthy subjects, whereas in COPD patients an increase in expression (surprisingly high and, furthermore, increasing with a 2.5 fold fiber magnification) of these peptides was found. From comparison with neighbouring sections stained for fiber typing it was concluded that the peptide expression mainly occured in type 2 fibers.

Fig. 4 served to illustrate that TNF-alpha and IL-6 contents were also very low in controls, whereas in patients an increase in TNF-alpha was found. It is stated that this was located mainly in type 2 fibers, but, somewhat surprisingly, no section stained for fiber typing is included in this figure. IL-1 beta was “hardly expressed” in any of the groups. This is somewhat surprising, because IL-1 beta and IL-18 were stated to be closely related and both activated by Caspase-1, which was increased in COPD patients. This discrepancy was not discussed.

IL-18 mRNA levels in muscle – shown in Fig. 2, but without units – were also higher in patients than in controls. In contrast to this parallelism between mRNA and protein, a significant difference of similar magnitude in IL-18 mRNA between smoking and nonsmoking controls (Fig. 2) was not accompanied by a difference in histochemically determined IL-18 protein between these groups (Fig. 3), a finding which was not discussed. Lack of parallelism between mRNA and protein expression was also observed for TNF-alfa, mRNA levels in muscle being at least as high in controls as in patients (Fig. 2). It was suggested that this might reflect a negative feedback by elevated TNF protein on TNF transcription. However, such a mechanism would not be able to fully inhibit an initial rise in mRNA.

Lack of correspondence between mRNA and protein findings also occurred for IL-6, although apparently this went unnoticed by the authors. Thus, IL-6 mRNA was identical between groups (Fig. 2), whereas histochemically determined IL-6 protein was described as “very low” in controls but, nevertheless, “reduced below the levels of controls” in COPD patients. The depicted findings call for a much more exhaustive description of the evaluation of the immunohistochemical data than

the one presented (e.g. were sections evaluated blindfolded and by more than one investigator? How many fibers were evaluated and were sections from all subjects examined? Were attempts to quantify staining made?). Also, Western blotting should have been carried out to confirm the histochemical findings.

Response

With regard to the lack of correspondence between mRNA and protein, please see our general comments above.

HG

Other findings: Values presented for plasma levels of IL-8 were 2-4 times as high in the Abstract as in Fig. 1.

Response

Plasma-IL-8 was not measured in this study. However, we acknowledge that the IL-18 levels given in the abstract and in fig. 1 do not correspond. We are deeply concerned and apologitic towards the fact that we did not notice this mistake earlier (indeed, neither did the reviewers of the present article or the reviewers of the PhD thesis in which the manuscript was included).

Analyses for plasma IL-18 was carried out twice; the reason being that due to technical errors in the first analysis, values were "missing". When the plasma samples were analysed the second time, we encountered no technical errors. We trusted the data. The data from the "second round" was included in the abstract. However, we must have made a mistake and mixed up the two analyses, so that the data from the "errous first round of analyses" is represented in Fig. 1. We shall write an erratum to the journal. As said, we are deeply sorry about the mistake, however, it has no bearing on the conclusions of this paper.

HG

Conclusion: Because elevated skeletal muscle expression of IL-18 was found in COPD patients with normal body weight, it was concluded (cf. Abstract) that IL-18 potentially may be involved in COPD-associated muscle wasting. However, the opposite conclusion seems more obvious: Despite increased IL-18 expression no wasting had occured, not even in two patients receiving oxygen therapy and six patients waiting for pulmonary transplantation. In this context, it is surprising that no comparisons were made between the observed changes in various cytokine measurements and muscle histology (e.g. capillarization) on the one hand and the clinical condition of the patients (e.g. degree of hypoxia) on the other.

Response

The design of the study allowed us to identify possible candidates to cachexia. We clearly state that the aim of this study was to test the hypothesis that systemic inflammation in patients with COPD is accompanied by enhanced IL-18expression in skeletal muscle, which may precede muscle weight loss.

We conclude that elevated skeletal muscle expression of IL-18 was found in COPD patients with normal body weight, indicating that IL-18 potentially may be involved in the pathogenesis of COPD-associated muscle wasting. We think the conclusion is justified and balanced. As we did not

hypothesize an association between IL-18 and e.g. capillarization, one could also argue that it would have been incorrect to perform such analyses.

HG

Minor:

In addition to mRNA (Fig. 2) (see above), units are also missing for BMI and BMD (Table 1), and for fiber size (Table 2). P-values were not given in Table 1.

Response

P-values are given in the results section.

Overall comments

Reflection on validity of data: In regard to immunohistochemistry, the laboratory work was performed by CIM technician Ruth Rousing in Penkowa's lab. Analysis and interpretation of the IHC was performed by Penkowa. Penkowa was involved in writing IHC sections for IL-18, TNF and IL-6, resulting in Figure 3 and 4. We cannot fully exclude the possibility that the IHC data are flawed.

With regard to IL-18, there is an agreement between PCR and IHC data in the present study. Thus, IL-18 mRNA and IL-18 protein expression were elevated in COPD patients. Moreover, we found elevated plasma-levels of IL-18 in patients with COPD. With regard to TNF, there was an increased expression of TNF protein in type 2 fibres in muscle biopsies. However, TNF mRNA was not elevated. In another set of experiments, we find that TNF protein expression is enhanced in muscle biopsies from patients with type 2 diabetes, however, TNF mRNA is not enhanced (Plomgaard et al. Diabetologia. 2007 Dec;50(12):2562-71. Epub 2007 Oct 10.) Thus, there is consistency across studies. According to available mail correspondence, it appears that there is agreement between the evaluations performed by Penkowa and by Jesper L. Andersen.

I would like to reiterate that we are deeply sorry that we made a mistake by inserting the wrong data set in figure 1. However, we provide the readers of this document with the original data. We think it is obvious that this mistake is unintentional and does not represent "active manipulation".

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us. We are uncertain as to whether this is also true in regard to the work performed by Milena Penkowa.

Paper 10. Nielsen AR, Mounier R, Plomgaard P, Mortensen OH, Penkowa M, Speerschneider T, Pilegaard H and Pedersen BK: Expression of interleukin-15 in human skeletal muscle - effect of exercise and muscle fiber type composition. J Physiol 584: 305 – 312, 2007.

Penkowa was fifth and BKP senior and corresponding among the eight authors. The investigation included two separate studies of healthy young men.

Major concerns:

Muscle biopsies and data from a former study (Paper 6 and 7) seem to have been reused in the present study, but this was not brought to notice. Supporting the suspicion, the protocol used in Study 1 in the present paper was the same as that used in Papers 6 and 7 (biopsies being taken from triceps, vastus lateralis and soleus muscles in the basal state and used for RNA isolation and histochemistry). Furthermore, although it was stated in Methods that 14 subjects took part in this study, it appears from both the legend of Fig. 2 and the section on fiber type composition under Results (p. 310) that histochemistry was only performed in seven subjects, as was the case in Papers 6 and 7. Finally, the range of type 1 fibers in each of the three muscles examined (p. 310) was exactly the same as reported in Paper 7. This reuse must have been apparent to at least those four, who were authors of both papers. Thus, although Myosin Heavy Chain mRNA 2a was not measured in the present study, in the Results section a negative correlation between this variable and occurrence of type 1 fibers was reported (p. 310). The same finding was reported in Paper 7, and Penkowa did only provide fiber type, not MHC data. Whether the original group of seven subjects from Papers 6 and 7 was supplemented with seven new subjects, or whether 14 new subjects did not contribute to the histochemistry, is not clear.

Response

As described in the methods section, we included muscle biopsies from 14 individuals. Seven of these individuals were also included in paper 6 (Plomgaard P, Penkowa M and Pedersen BK: Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles. Exerc Immunol Rev 11: 53-63, 2005). We do not give a reference to the latter study in the methods description, because the n-values were twice as high in the present study and thereby the anthropometric measures differed. The main results were on IL-15 mRNA and IL-15 protein, which was carried out on the total study population. We clearly indicate that supportive data regarding fibre type distribution and IHC were related to analyses carried out on n=7.

Paper 6 is cited in the introduction. We are again surprised that Dr. Galbo finds it proper to critice this procedure as he uses the same procedure himself, even in recent publications, please see table 2 and 3.

HG

Illustration of histochemical findings has flaws and deficiencies (Fig. 2). One section stained for IL-15 was shown for each of the three muscles. It was not described how these sections were selected (e.g. from the same subject). It was stated that neighbouring sections were stained for fiber typing, and identical fibers stained for IL-15 and fiber types, respectively, were allegedly depicted by asterisks. However, in conflict with this the two sections from vastus lateralis muscle (c and d) and the fibers depicted in these were apparently not identical.

Response

As in previous studies, the IHC was carried out to obtain an impression of whether IL-15 was expressed by muscle fibres per se. We acknowledge that the muscle fibres in Fig. 2C are slightly out of position when compared to Fig. 2D. However, this fact does not influence the conclusion that it appears as if IL-15 is equally expressed by fibre types and in the different muscle groups. The latter conclusion is supported by the IL-15 western blotting, carried out in our own laboratory, which demonstrates that the IL-15 protein is expressed to the same extend by the three different muscle groups.

HG

In the figure comparison was also made between a section stained for IL-15 and negative control sections (e.g. incubated without antibody against IL-15). These sections should have been neighbouring, but they obviously were not. In general, the description of the evaluation of the sections was insufficient (e.g. number of fibers evaluated ?, more than one investigator ?).

Response

Dr. Galbo misunderstands these pictures. It is clearly stated that these figures are included to demonstrate an image with and without the IL-15 staining (Ba, Bb, Bc).

HG

Lack of parallelism between IL-15 mRNA and protein measurements. In study 1 IL-15 mRNA was significantly higher in triceps than in soleus muscle, and values in vastus were between these. However, according to both Western blotting and immunohistochemistry IL-15 protein levels did not differ between the three muscles. In study 2 eight subjects carried out knee extensor resistance exercise and had blood and muscle biopsies taken before that and 6, 24 and 48 h later. A significant increase in IL-15 mRNA in muscle was seen at 24 h, whereas no change in IL-15 protein determined by Western blotting was found (histochemistry was not performed). The fact that mRNA increased somewhat late relative to the exercise stimulus was not discussed. The lack of parallelism between mRNA and protein findings in the two experiments was ascribed to existence of transcription without translation, a very unsatisfactory explanation.

Response

We refer to our general comments with regard to mRNA and protein parallelism, please see above.

With regard to the exercise study, the finding that IL-15 mRNA increases late into recovery (24h) suggests that IL-15 may be involved in training adaptation rather than playing a role during the acute exercise. In support of this, we have demonstrated a robust two-fold up-regulation of the IL-15 protein (Western blotting) in resting muscle biopsies, following a 12 weeks training protocol, consisting of high intensity and high amount (Nielsen AR et al, unpublished data). The latter data set is in absolute agreement with the findings of how muscular IL-15 is regulated by exercise.

Overall comments

Reflection of validity of data: Even today we have no specific reason to suspect the IHC data, but Penkowa and/or Penkowa technicians have had "hands on". The finding that IL-15 is expressed by skeletal muscle does not rely on IHC data, alone. In our own laboratory, we have performed IL-15 western blotting, demonstrating that there is no difference between muscle groups.
We think that the present study adds important new information. The finding that skeletal muscles express IL-15 and that muscular IL-15 is regulated by exercise training does not rely on IHC.

Previous studies in humans did not show any change in IL-15 mRNA level following either resistance or endurance exercise (Nieman 2003, 2004). However, the biopsies in these studies were obtained immediately after the end of exercise. We found an elevation of IL-15 mRNA 24 h after resistance exercise, which is not in contrast to previous findings. The finding that the difference in IL-15 mRNA level, both after resistance exercise and between different muscle groups, was not paralleled by similar differences in muscular IL-15 protein expression, visualized by Western blot and immunohistochemistry, suggests that muscular-IL-15 does not play a role during the acute exercise, but may be involved in training adaptation in agreement with unpublished findings by our group, showing a robust up-regulation of muscular-IL-15 protein (Western blotting) as a result of training.

In support of the idea that muscular expression of IL-15 is involved in metabolism, we demonstrated a negative association between plasma IL-15 concentration and body fat mass in humans, independent of the diagnosis of type 2 diabetes. Negative associations were also found between muscle IL-15 mRNA and obesity parameters. Furthermore, IL-15 overexpression in skeletal muscle of mice reduced trunk fat mass, but not subcutaneous fat mass (Nielsen et al J Clin Endocrinol Metab. 2008 Nov;93(11):4486-93.)

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us. We are uncertain as to whether this is also true in regard to the work performed by Milena Penkowa.

Paper 11. Plomgaard P, Nielsen AR, Fischer CP, Mortensen OH, Broholm C, Penkowa M, Krogh-Madsen R, Erikstrup C, Lindegaard B, Petersen AM, Taudorf S and Pedersen BK: Associations between insulin resistance and TNF-alpha in plasma, skeletal muscle and adipose tissue in humans with and without type 2 diabetes. Diabetologia 50: 2562 – 2571, 2007.

HG

Penkowa was the sixth and BKP the senior among 12 authors on this paper. More subjects participated in this than in previous similar studies by other groups. However, some essential variables were only determined in subgroups.

Response

In the present study we included a clinical study material consisting of 199 participants of patients and controls. It is clearly stated that mRNA from muscle biopsies were obtained from 94 controls and 83 patients. Moreover, it is clearly stated that mRNA from adipose tissue was obtained from 84 controls and 83 diabetic patients. This is an unusually large and well-described group of human individuals. In a subgroup of individuals (n=8 in each of four groups), we performed additional analyses: Western blotting and IHC. Thus, the IHC represents a minor part of the study.

The reader is provided with this information.

HG

Critique:

Histochemistry: Previous contrasting findings by the group were not mentioned. Thus, a major conclusion in Paper 6 was that in the basal state immunohistochemically determined TNF-alpha is expressed in skeletal muscle, and, furthermore, only in type 2 fibers. Nevertheless, in the present paper, it appears from Fig. 3 that in control subjects studied in the basal state, no TNF-alpha staining could be demonstrated. This discrepancy was not discussed. In fact, Paper 6 was not quoted at all. This is the more surprising because, in Paper 6, the authors quoted a study, in which it was found that TNF-alpha mRNA and protein in humans increased in elderly subjects. In the present study subjects were, in fact, elderly, whereas young subjects were studied in Paper 6. The conclusion from the histochemistry findings of the present study was that in type 2 diabetics TNF-alpha is increased in skeletal muscle, in particular in an -undefined- subgroup of type 2 fibers.

Response

The IHC image was included in order to get an impression of whether muscle cells expressed TNF protein or not. Given the many other results performed on this large study material, the focus of the discussion did not include the data from the IHC.

HG

Again, considering the conclusion in Paper 6, it is surprising that the authors emphasized the occurrence of TNF-alpha in type 2 fibers rather than the fact that the presence of TNF-alpha in type 1 fibers also seen in the type 2 diabetic subjects was in contrast to their previous finding that these fibers contained no TNF-alpha in healthy subjects. Finally, the marked difference in TNF-alpha staining of muscle fibers between diabetics and controls shown in Fig. 3 contrasted with the presented Western blot data, according to which TNF-alpha protein content in muscle was on

average at most only twice as high in diabetics compared with controls (Fig. 2). This fact was not noted.

Response

Once again, the IHC technique is not quantitative and therefore it is not correct to discuss discrepancies between findings obtained by the quantitative Western blotting method and those obtained with the IHC technique.

HG

As in the other papers the description of the evaluation of the histochemical data was insufficient (e.g. in the present paper: How many fibers were evaluated per section, and what was the percentage of type 2 fibers showing increased TNF-alpha in the diabetics, i.e. the size of the putative subgroup. Were sections evaluated by more than one investigator, were they evaluated blindfolded?).

Response

The purpose was not to describe how many fibres that were expressed by the cytokine, but to get an impression of whether cytokines were expressed by muscle cells. The IHC figure represents supportive data.

The allocated space did not allow us to give an in-depth description of the subgroups that underwent further analysis.

HG

In this as in several previous papers negative control sections were incubated without the goat serum used to block potential nonspecific binding of the primary and secondary antibodies. However, as mentioned in the review of Paper 6, the relevance of this procedure is not clear, and it was not used in the immediately preceding Paper 10. So, it is surprising that it was taken up again here.

Western blotting: A “cleaved” form of TNF-alpha protein in addition to the membrane-bound proTNF-alpha was found in muscle. According to the text and the blots shown (Fig. 2) the amounts of proTNF were higher than those of the cleaved form. However, this is in contrast to Fig. 2, panels b and c, where values were expressed relative to beta-actin protein, and the reverse relationship seems to hold. It was also stated in the Results section that levels of both forms were higher in diabetics than in controls, and in the summary of findings in the beginning of the Discussion that TNF-alpha was elevated in muscle of diabetics compared with controls regardless of the degree of obesity. However, according to Fig. 2b and the Results section, the difference was not significant for proTNF in non-obese subjects. The legend of the figure states that a one-way ANOVA was used, but this contrasts with the outcome described in the legend as well as with the Statistics section.

Response

When summarizing the data in the beginning of the discussion, we did not give all the details. However, we find that Fig. 2 provides the reader with a clear overview.

HG

No differences between patients and controls in TNF-alpha mRNA in muscle (and in adipose tissue) were found. In the present paper the discrepancy between TNF-alpha mRNA and protein levels was tentatively ascribed to posttranscriptional regulation of the protein content, whereas, in Paper 10, a similar discrepancy was ascribed to negative feedback inhibition of the mRNA.

Response

There are several possible explanations as to why protein and mRNA levels do not correspond, please see our general comment above.

HG

Material and other methods: It was stated in the Introduction as well as in Methods that a case-control design was used. However, this is not correct as controls were dealt with as a group and not matched individually with patients. V02 max as well as fat mass values were reported, but the methods used to analyze these variables were not.

Response

We do not agree. “A case-control study is an analytical study which compares individuals who have a specific disease ("cases") with a group of individuals without the disease ("controls")” – http://www.ehib.org/faqlist.jsp; http://www.newsroom.heart.org/index.php?s=43&item=1315; http://healthinformatics.wikispaces.com/Case+Control+Study

HG

Main conclusion: Based mainly on correlation analysis between plasma concentrations of TNF-alpha and a simple index related to insulin resistance (HOMA2-IR) it was concluded that plasma TNF-alpha is associated with insulin resistance and may play a role in the pathogenesis of chronic insulin resistance. This conclusion seems daring in light of the fact that the correlation was not significant in control subjects. Furthermore, in diabetics the correlation coefficient was only 0.3 and, judged from presented p-values, considerably lower when adjusted for various confounders.

Response

This is not correct. A multivariate analysis was performed and five different models were presented. Plasma-TNF is significantly correlated with a measure of insulin sensitivity in all models. The control group did not include individuals with impaired glucose tolerance test and thus represents a homogenous group with regard to insulin sensitivity. The finding of any correlational relationships between plasma-TNF and measures of insulin sensitivity can therefore not be expected.

HG

Central unintelligible/ illogical statements; examples: At the end of the Abstract (and similar sentence at the end of Discussion): “However, findings on the TNF-alpha protein level in plasma and skeletal muscle indicate that measurement of TNF mRNA content in adipose or muscle tissue provides no information with regard to the degree of insulin resistance”. In the Discussion, in the context of the finding that TNF-alpha mRNA in adipose tissue did not differ between patients and controls: “Visceral fat in particular has been suggested to have a high level of TNF-alpha (27,35), which is a possible explanation for the lack of difference in subcutaneous adipose tissue between

controls and diabetic patients in the present study". In the Discussion, in the context of the finding of no difference between groups in skeletal muscle TNF-alpha mRNA: "However, patients with type 2 diabetes who performed a resistance exercise demonstrated improved insulin sensitivity despite an increase in TNF mRNA transcripts (37), suggesting no association of insulin sensitivity with TNF mRNA content in skeletal muscle".

Response

We are unable to follow the arguments and the criticism.

HG

Data dispersion: For several variables this was surprisingly small. E.g. SD can be calculated to be 1-2% for e.g. plasma TNF-alpha, plasma glucose and diastolic blood pressure in healthy subjects. Considering the small dispersions it may also be surprising that it was possible to establish significant correlations, e.g. the 95% confidence interval for sTNFR2 (soluble TNF receptor) was less than 2% in healthy subjects and, nevertheless, this variable correlated significantly with HOMA2-IR.

Response

Dr. Galbo is confused. The data are expressed as means and SEM. Original data are included.

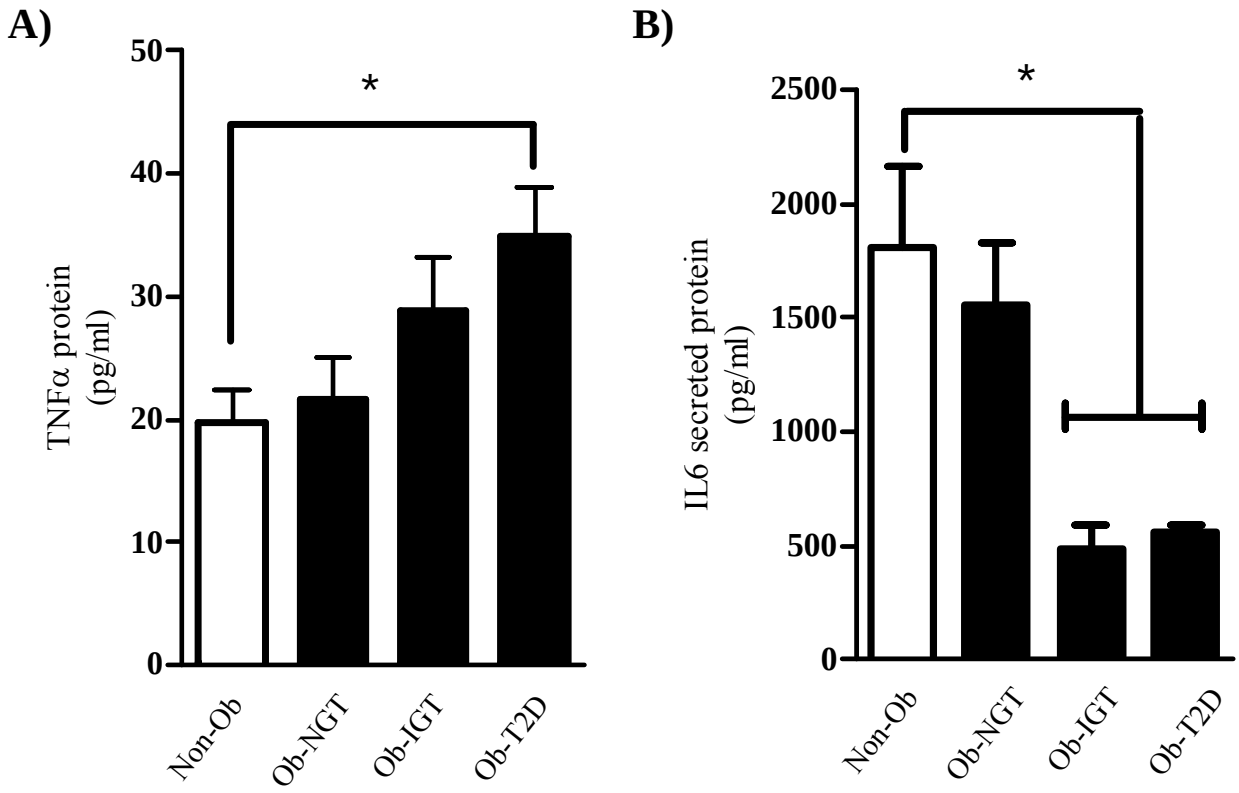
Overall comments

Reflection on validity of data: There is no conflict between Western and IHC-data with regard to muscular expression of TNF in muscle biopsies from controls and patients with type 2 diabetes. However, today we do not know if we can trust the IHC work, which was performed by Penkowa.

In general, we think that the present study is of high quality. The study population (n=199) is large for a study involving analysis of both muscle and adipose tissue biopsies.

We demonstrate that the plasma TNF-α level is associated with insulin resistance even after adjustment for multiple confounders, and that the level of TNF-α protein in muscle is elevated in patients with type 2 diabetes. Our findings with regard to TNF-α protein levels in plasma and skeletal muscle indicate that measurement of the TNF mRNA content in adipose or muscle tissue provides no information with regard to the degree of insulin resistance. We think this conclusion is justified by data.

With regard to the TNF-protein content, we show (by Western) that there is an increase in TNF protein content in patients with type 2 diabetes. The IHC image suggests that diabetic muscle fibres per se express TNF protein. To back up these data, we have recently shown that primary human cell cultures from obese patients with type 2 diabetes express and release TNF protein to the media (Green et al Diabetes 2011, in press; the relevant figure is inserted below for your information).



In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us. We are uncertain as to whether this is also true in regard to the work performed by Milena Penkowa.

Paper 12. Matthews VB, Astrøm MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek T, Åkerstrøm T, Yfanti C, Broholm C, Mortensen OH, Penkowa M, Hojman P, Zankari A, Watt MJ, Bruunsgaard H, Pedersen BK and Febbraio MA: Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. Diabetologia 52: 1409 – 1418, 2009.

HG

Penkowa was the 11th among 17 authors of this study, which resulted from collaboration between BKP´s group and Australian scientists, and which was co-directed by BKP and Mark Febbraio, who has had a number of papers retracted in recent years. The study consisted in both experiments on humans, electroporation experiments in mice, and in vitro studies on myotubes and rat muscle. It is most likely that the human experiments were conducted in Copenhagen.

Response

Mark Febbraio (MF) is a highly distinguished and well-reputed researcher. It is correct that he has retracted two papers (not several papers as stated). Once he received information that there may have been some misrepresentation of data, his organisation initiated an inquiry under the National Health and Medical Research Council of Australia Code of Research Conduct. It was found that one of MF's PhD students had inappropriately represented data and MF deemed it appropriate to retract the data. Importantly, however, neither MF nor any other author was found to be incriminated in any way.

HG

Major concerns:

Study population: According to the Methods section eight young men performed two hours bicycle exercise and had blood and vastus muscle biopsies sampled before as well as at multiple time points up to 72 h after exercise. No resting controls were mentioned. In conflict with these informations, in the Results section and legends of Figs. 1 and 2, a resting cohort/ resting controls were mentioned. Analyses from the resting cohort were presented, but the origin and composition of the cohort was not described.

The uncertainty as regards the origin of the study populations is supported by the fact that in Fig. 2 platelet counts were not given in absolute numbers but as percentage of baseline values. Furthermore, while in Fig. 1 the number of subjects was eight, in Fig. 2 the number of subjects was ten, and accordingly higher than the number of subjects included in the study according to the Methods section. It is remarkable that age, weight and height given for the eight subjects included in the present paper were the same as given for the 15 men included in Paper 8.

Response

We accept the criticism that the first part of the method section does not include a precise description of the study material. In the final version of the manuscript, we had limited space. During our attempt to reduce the text length, we by mistake left out the description of one of the two studies. One study was made only for the present BDNF-article and has not been used in any other publications. The study included 10 subjects, who exercised and another 10 subjects who

rested. Muscle biopsy samples were obtained from *vastus lateralis* at time points 0, 2, 3, 5, 8, 24, 48 and 72 h. This material led to the data included in fig. 1.a, 1b, 1d and fig. 2. Due to lack of sufficient muscle protein (freezer break-down), we included material from a former study (n=8, only exercise) for the Western. The description in the Method section refers to the latter material. The description of the 10+10 was unfortunately not included in the method section.

From a draft of this article, we have copied the following description of the study material:

Twenty healthy, physically active but untrained men (mean ±SD age: 25.6± 3.5 years, weight: 78.9 ± 9 kg, height: 185 ± 6.5 cm, BMI: 21.3 ±2.11 kg/ m^2) with no prior medical illnesses were recruited to participate in the study which was approved by the Ethical Committee of Copenhagen and Frederiksberg Council, Denmark. Before the experimental day, all subjects underwent a thorough clinical examination and blood samples for evaluation of renal, hepatic and thyroid function, hemoglobin, white blood cells counts, thrombocytes, electrolytes, plasma coagulation factors and plasma glucose were obtained. All tests were normal. The subjects were given both written and oral information about the experimental procedures before they gave their written informed consent. The subjects were randomised to either exercise (n=10) or control (n=10). There was no difference between the two groups with regard to age, weight, height or VO_2max. Prior to the day of the experimental tria,l all subjects performed two maximal incremental exercise tests to measure maximal oxygen uptake tests (VO_2max test) on an electrically braked cycle ergometer (Monark 839E, Monark Ltd, Varberg, Sweden). The first of these served as a familiarization trial, conducted 10-14 d before the experiment. The second test, upon which the workload during the experimental trial was based, was performed 4 d before the trial. VO_2max averaged 52 ml/ kg/ min (range 37-60). On the day of the experiment, the subjects arrived ~0700 after an overnight fast. A catheter was placed in an antecubital vein for blood sampling. Subjects performed either 120 min of bicycle exercise at 60% VO_2max followed by a 6 h recovery period (exercise) or rested in bed for 8 h (control). Subjects also reported to the laboratory after an overnight fast 24, 48 and 72 h after the commencement of the eexperimental trial.

Blood was obtained at time points 0, 2, 3, 5, 8, 24, 48 and 72 h. Muscle biopsy samples were obtained from vastus lateralis at time points 0, 2, 3, 5, 8, 24, 48 and 72 h, using the percutaneous needle biopsy technique with suction. Blood was drawn into serum tubes and tubes containing EDTA for plasma isolation. Plasma was immediately separated from blood cells by centrifugation at 3500 rpm for 15 min at 4 ℃. Serum tubes were left at room temperature for 1 h and then centrifuged at 3500 rpm for 15 min at 4 ℃: Both serum and plasma were stored at –20 ° C until assayed. BDNF is stored in platelets and BDNF is released in vitro from platelets when measured in serum. The in vitro release of BDNF from platelets is limited when BDNF is measured in plasma that has been separated according the above-mentioned procedure. Plasma and serum levels of BDNF were measured by ELISA (R&D systems GmbH Wiesbaden-Nordenstadt, Germany, detection limit 31.25pg/mL; intra- and interassay CVs <5%). P-selectin was measured by ELISA (R&D systems Minneapolis, USA, detection limit 0,5 ng/mL). All samples from a given subject were analysed in duplicate in the same microtitre plate, and for the exercise study both exercise and rest groups were equally represented on each plate. Plasma BDNF and P-selectin were measured at baseline, at 2 h (corresponding to immediately post exercise) and at 5 h. Cell counts were determined by standard laboratory procedures at the department of clinical chemistry of Rigshospitalet, Copenhagen, Denmark. Muscle tissue was immediately separated into two samples. One was snap frozen in liquid nitrogen for RNA and protein extraction and stored at –80 ℃. The

second was sealed in Tissue-tek and snap frozen for immunohistochemistry analysis and stored at –80 ℃.

We are very sorry that a short summary of the above method section by a mistake was not included and we will notify the journal in question.

HG

Brain-derived neurotrophic factor (BDNF) protein: In human vastus muscle, based on Western blotting a significant 50% increase in BDNF expressed relative to beta-actin was found 24 h after exercise compared with pre-exercise values. BDNF mRNA was measured at multiple time points in both exercising subjects and resting controls and peaked 5-8 h after exercise. In light of these facts, it is surprising that BDNF protein in exercising subjects was not measured more frequently before and after the 24 h time point, and that it was not measured in resting controls. Furthermore, it can be doubted, whether the observed 1.5 (-3) -fold increase in BDNF mRNA could account for a 50% increase in BDNF protein, because in previous studies of rat muscle, a 5 fold increase in BDNF mRNA was not accompanied by any increase in protein (ref. 24 in the paper. See also comments on BDNF mRNA below).

Response:

Dr. Galbo describes himself as a researcher who has worked "in endocrinology as it relates to muscle". Again, it appears that cell biology and cell signalling are not areas of his expertise when one considers his assertions with respect to findings in this paper. Differences in fold changes between mRNA levels and protein levels are common since protein expression involves a balance between translational efficiency and protein turnover and stability. Please also see the general comments above.

<u>mRNA</u>
We report that there were no differences in BDNF mRNA levels in the exercise cohort compared with the resting cohort (BDNF paper Fig. 1a). This was likely due to the fact that the BDNF mRNA levels demonstrated a marked inter-individual variation with peak mRNA levels between 5 and 8 h for the majority of the subjects. Advised by our statistician, we calculated the area under the curve over time for both groups and showed a difference ($P<0.05$) for the AUC when comparing the two groups. This method of statistical analysis was not questioned in the peer review process of the manuscript. Thus, the data show a low to moderate, but robust expression of BDNF mRNA in human skeletal muscle, which is induced from app. 33 Ct values to 30 Ct values. We think we describe the data as they are without any over-interpretation.

<u>Western</u>

The antibody against BDNF protein recognises both the pro- and mature-form of BDNF. In all Western blot experiments, rhBDNF was loaded into one lane as a positive control. In the human samples, the mature form of BDNF was recognised and the expression appeared to increase progressively following exercise. At 24 h into recovery from exercise, BDNF protein expression was increased (~50%; $P<0.05$) in the muscle homogenates (Fig. 1b).

IHC

The quantification of the BDNF protein was performed by Western. The immune-histochemistry data (Fig 1d in the publication) were accumulated to support data generated by two independent scientists (KS Krabbe and C Broholm; Fig 1a-c) in CIM and two independent scientists (VB Matthews and MHS Chan; Fig 2 d-f) in the Febbraio laboratory.

The IHC was also performed to obtain some indications of whether the BDNF expression might be increased within muscle fibres. We chose to show the IHC image from the person, who had the most mRNA response (id. CH). This person appeared also to have the most pronounced BDNF protein response. Thus, we show the IHC image that best supported the notion that the increased BDNF expression 24 h post-exercise was indeed intramyocellular, Fig.1d.

The IHC was not performed by Penkowa, but by Maj-Brit Åstrom (second author on the paper), with the help from a student, Dan Sonne Pedersen, in the Penkowa laboratory. Penkowa was involved in supervising the IHC technique and in the composition and description of Fig.1d.

The studies presented in Fig.1 showed that there was a small increase in BDNF mRNA and protein (Western) in response to exercise. The IHC image suggested that muscle cells might express the BDNF protein.

We state "that the kinetics of the exercise-induced elevation in serum BDNF levels failed to correlate with the increased expression of BDNF in muscle 24h post exercise suggesting that the skeletal muscle is not a source of the increase in serum BDNF observed early into recovery from exercise".

We state "Our IHC results suggested that the contraction-induced increase in BDNF protein expression was increased in skeletal muscle cells". Since it was possible that BDNF was produced by other cell types within the skeletal muscle bed before being taken up by muscle cells, it was necessary to move to a cell culture model to determine whether skeletal muscle cells *per se* can increase BDNF expression when contracted." Thus, we did not just conclude based on IHC that muscle cells express BDNF, but established a cell culture system to contract differentiated C2C12 myotubes *in vitro*.

Dr. Galbo insinuates that we ought to have questioned the above-mentioned findings. However, other scientists disagree with him as they regarded our data as being not novel. When the original manuscript was submitted to FASEB Journal, which did not include extensive cell and animal work that was performed and eventually published in Diabetologia, we received the comments below (13-10-2007), which provide an excellent summary of the literature and highlight the fact that BDNF is expressed by skeletal muscle and can be induced by contractions. The reviewer actually expresses concern about the novelty of the data. In other terms, he/she finds that it is well accepted that contracting skeletal muscle expresses BDNF.

The reviewer states:

"There are major concerns about the novelty of the data presented here. Several studies in rodents have demonstrated that exercise, electrical stimulation (and contraction) of skeletal muscle leads to an induction of BNDF expression in muscle. (Copray et al. (2000), Experimental Neurobiology, 161: 597-608, Gomez-Pinilla et al. (2002), J. Neurophysiol, 88: 2187-2195' Park et al. (2003), Neuroscience Research Communications, 34:10-19, Seidl et al. (1998), J. Cellular Physiol, 176:

10-21). Moreover, recently in Med Sci Sports Exerc 2007 April; 39, 728-34 Ferris et al demonstrated that exercise induced BNDF levels in humans, they also showed that cognitive functions increased (although they did not correlate with BNDF changes). In the opinion of this reviewer this manuscript requires new additional functional insights into the role of BNDF function in skeletal muscle metabolism prior to publication. The authors should consider BNDF siRNA and/or ectopic overexpression experiments in the C2C12 in vitro cell culture system to ascertain the effect on several pertinent aspects of metabolism. The present version of the manuscript provides information that is largely descriptive (with incremental insights) rather than significant new insights.

For example, the authors suggest that the novelty of this data presented is due to the increased expression of BDNF from contracting skeletal muscle. Several studies have also reported these or similar findings. For example, Copray et al. (2000), Experimental Neurobiology, 161: 597-608, found intense contraction of soleus muscle in both normal and diabetic rats caused an increase in the expression of BDNF. Moreover, ultrastructural studies from these same authors found BDNF expression was localized within muscle fibers and activated satellite cells. Furthermore, no expression of BDNF was observed in Schwann cells or fibroblasts, suggesting that the localization of BDNF was defined within the muscle fibers.

In other studies, Gomez-Pinilla et al. (2002), J. Neurophysiol, 88: 2187-2195, found BDNF mRNA and protein levels in rodents increased in soleus muscle after 3 and 7 days of exercise. Moreover, following paralysis of the soleus muscle, BDNF mRNA levels were reduced, demonstrating that active muscle contraction is important in modulating BDNF levels in muscle. Moreover, Park et al. (2003), Neuroscience Research Communications, 34:10-19, also identified electrical stimulation of rat soleus and medial gastocnemius muscles".

HG

The immunoblot selected to illustrate the findings does not strengthen the confidence in the measurements as beta-actin contents and image contrast varied considerably between lanes/time points of tissue sampling.

Response

It is unclear what Dr. Galbo means by "image contrast" since the representative blot has not been spliced. It is a single image and, therefore, each lane cannot be manipulated relative to another. The fact that the beta actin controls may be different in expression is precisely why loading controls are necessary when quantifying Western blot measures.

HG

In C2C12 myotubes stimulated electrically to contract in vitro for 2 h, a 70% increase in both BDNF mRNA and protein was found at the end of contractions. The authors did not discuss the observed difference between myotubes and human muscle in the time course of these effects of contractions. In the discussion they concluded that it is likely that muscle-derived BDNF "works in an autocrine and/or paracrine manner". They did not mention that this view was in conflict with the fact that they did not find any difference in release of BDNF to the medium in contraction-treated versus control C2C12 cells. Because data were not shown, it cannot be evaluated, whether a release occurred at all.

Response:

Again, it appears that Dr Galbo is confused. Autocrine signalling is a form of signalling in which a cell secretes a hormone or chemical messenger (called the autocrine agent) that binds to autocrine receptors on the same cell, leading to changes in the cell. Paracrine signalling is a form of cell signalling in which the target cell is near the signal-releasing cell. The fact that the BDNF receptor within the skeletal muscle was activated when we overexpressed BDNF in that muscle and the fact that we found no increase in BDNF either in the medium of the cells or in the circulation of the animals, is precisely why we concluded that the effects was either paracrine or autocrine and not endocrine (which is occurs when the tissue releases the protein to act upon another tissue). The data were not shown for the cell media experiments simply because we didn't detect any changes in BDNF in the medium.

HG

Immunohistochemistry for BDNF protein was performed on muscle samples taken pre-exercise and 24 h post-exercise as well as at identical time points in non-exercising subjects. One muscle section was shown from each of these four categories. It was not reported how sections were selected, how many fibers they represented, and how they were evaluated. An increase in staining, non-uniformly distributed among fibers and appearing gritty, was found 24 h after exercise. The staining looks peculiar and appears to exceed the 50% increase in BDNF found by Western blotting. No comments were made on these features.

Response

As said, the IHC was only performed to obtain some indications of whether the BDNF expression might be increased within muscle fibres. We chose to show the IHC image from the person, who had the most remarkable mRNA response (id. CH). This person appeared also to have the most pronounced BDNF protein response. Thus, we show the IHC image that best supported the notion that the increased BDNF expression 24 h post-exercise was indeed intramyocellular, Fig.1d.

We do not understand what is meant by "gritty". The non-uniform staining is characteristic of immunohistochemical detection within fibres and suggests that the present IHC was performed correctly.

HG

BDNF mRNA: The variation between baseline values in exercising compared with resting subjects was high, a fact indicative of low mRNA copy numbers. In agreement with this view a critical threshold value for BDNF mRNA of above 37, corresponding with mRNA presence in only a small percentage of cells in muscle tissue, has been found in other studies, and also in situ hybridization studies of various skeletal muscles have failed to demonstrate significant BDNF expression within mature myofibers (J Neurosci. 26, 5739, 2006). However, the fact that BDNF mRNA levels in vastus muscle in the present study probably were very low was not readily apparent to the reader, because levels were expressed by some normalization procedure, which was not described. In response to exercise a significant increase in mRNA levels (expressed as fold changes from basal) was not immediately apparent, but could be demonstrated in comparisons of AUCs (area under curve) over time between exercising and resting subjects, respectively. However, the calculation procedure and calculated numbers were not reported, and the procedure would for instance favor a difference, if incremental areas above the differing basal values were used. Anyhow, the, at most

small, numerical increase in BDNF mRNA (judged from Fig. 1) above very low basal levels would not be able to account for the widespread increase in intramyocellular BDNF indicated by the immunohistochemical findings.

Response

We refer to our general statement with regard to regulation of mRNA and protein and with regard to expression of mRNA levels as fold changes. The view that CT of 37 is a critical threshold value is not shared by all molecular biologists (e.g. Peter Schjerling, personal communication). Our data show a low to moderate, but robust expression of BDNF mRNA in human skeletal muscle, which is induced from app. 33 Ct values to 30 Ct values. Again, we think we describe the data as they are without any over-interpretation.

One problem with assessing BDNF at the mRNA level is the unusually high amount of splice variants (Pruunsild P et al., Genomics, 2007 Sep;90(3):397-406. Epub 2007 Jul 12). In our study, published in Diabetologia, we therefore choose to use a pre-developed and optimized primer assay from Applied Biosystems, the leading company for quantitative real-time PCR.

Copray et al demonstrate that intense contraction of soleus muscle in both normal and diabetic rats caused an increase in the expression of BDNF (Copray et al. (2000), Experimental Neurobiology, 161: 597-608). The finding that there was also a small increase in creatine kinase, indicating muscle damage, does not confirm that BDNF was stimulated by damage and does not exclude the possibility that muscle contractions per se might enhance BDNF mRNA expression. Moreover, ultrastructural studies from these same authors found BDNF expression to be localized within muscle fibres and activated satellite cells. Furthermore, no expression of BDNF was observed in Schwann cells or fibroblasts, suggesting that the localization of BDNF was defined within the muscle fibres. These findings could easily be interpreted as a support for our findings.

Another study by Liem (Liem et al Histochem Cell Biol. 2001 Dec;116(6):545-51. Epub 2001 Nov 27) demonstrates that in adult rat muscle the constitutive expression of muscular BDNF is confined to the myofibres. However, they state that satellite cells, Schwann cells, endothelial cells, fibroblasts or axons do not appear to contribute to BDNF production in normal muscle. These authors state that although muscular BDNF is a neurotrophic factor for innervating motoneurons and supposedly released only at the motor endplates, the production of BDNF mRNA appears to occur along the entire length of the myofibres and is not confined to nuclei in the postsynaptic regions. This study further supports the idea that BDNF is expressed by myofibres.

In other studies, Gomez-Pinilla (Gomez-Pinilla et al. (2002), J. Neurophysiol, 88: 2187-2195) found that BDNF mRNA and protein levels in rodents increased in soleus muscle after 3 and 7 days of voluntary wheel running exercise. Moreover, following paralysis of the soleus muscle, BDNF mRNA levels were reduced. These results indicate that basal levels of neuromuscular activity are required to maintain normal levels of BDNF in the neuromuscular system.

Park et al (Park et al. (2003), Neuroscience Research Communications, 34:10-19) also identified that electrical stimulation of rat soleus and medial gastocnemius muscles resulted in a significant induction of both BDNF mRNA and protein.

Cuppini et al (Arch Ital Biol 2007 May;145(2):99-110) reported that BDNF mRNA increased over the second day after acute exercise. BDNF protein level progressively increased also after the mRNA went back to the basal level, so suggesting that it cumulates within the cell after acute

exercise, whereas it followed the mRNA level time course after repetitive exercise. These results point to the following conclusions: BDNF mRNA is up-regulated by activity, but this response is delayed to the second day after acute exercise. The finding that the BDNF protein was not up-regulated until the second day after an acute bout of exercise is totally in agreement with our findings that the BDNF protein peaks on the second day after acute exercise.

Ogborn et al (Muscle Nerve 2010 Mar;41(3):385-91) reported that BDNF expression was elevated in rat soleus following 5 days, but not 10 days of exercise. They conclude that BDNF may be a protein with an uncharacterized contribution to the acute adaptation of skeletal muscle to exercise.

Thus, there are many indications in the literature that rodent skeletal muscle expresses BDNF and that contractions stimulate an up-regulation of this expression. We find that it is most likely that human skeletal muscle would respond in a similar way. Therefore, we conclude that our data are consistent with the published literature.

HG

Other concerns:

A significant increase in palmitate oxidation, albeit less than 10%, was found in L6 myotubes stimulated with BDNF (Fig. 4). However, the illustration of the increase was misleading, because the y-axis did not start from zero, a fact not indicated. Furthermore, dispersions were surprisingly small and differed between similar experiments (SEM being about 1.4% in panel e and up to about 4.3% in panel d).

Response:

We find the statement ”the illustration of the increase was misleading, because the y-axis did not start from zero, a fact not indicated” unintelligible. There is nothing misleading about the y axis not starting at 0. We did not try to hide the fact that the axis ranged from 2-2.6 mg/protein/ per hour. This is clearly “indicated” in the figure. The fact that the measure is a “rate” and not an accumulation implies that the changes are subtle, but consistent and, importantly, statistically significant.

HG

The authors proposed that BDNF enhanced fat oxidation by stimulating AMPK and, in turn, its downstream target Acetyl coenzyme A carboxylase (ACC). This view was maintained, even though in L6 myotubes ACC was phosphorylated at a lower dose of BDNF than AMPK (Fig. 4), while in isolated rat muscle ACC was phosphorylated earlier than AMPK during treatment with BDNF (Fig. 5).

Response

One experiment was conducted in whole muscle ex vivo and one experiment in a muscle cell line in vitro. The fact that the kinetics of the response in two very different model systems is somewhat different should not be surprising. What is important, at least in our opinion, is that when either muscle cells or whole muscle strips were stimulated with BDNF, AMPK/ACC was phosphorylated and palmitate oxidation was increased in both independent model systems. When AMPK was blocked, not by one, but by two very different interventions (pharmacological blockade and genetic

adenovirus), the effect of BDNF on fat oxidation was eliminated. Together, the combined data clearly indicate that BDNF increases fat oxidation in muscle cells via the activation of AMPK.

Overall comments

Reflection on validity of data: This is a sound paper. Penkowa's contribution to this manuscript was minor. We do not believe that there is any flawed data in this paper. The reason why Penkowa was included as an author is that she performed preliminary experiments showing that BDNF appeared to be up-regulated at time point 24 h. These pilot experiments stimulated us to perform a new exercise study in which we obtained multiple muscle biopsies up to time point 72 h. The pilot study was not included in the present manuscript and hence Penkowa has not had "hands on".

In short, there is an agreement between Western and IHC data in the present study with regard to BDNF protein detection in human samples. The human studies are highly supported by studies performed in vivo in mice as well as in cell cultures, including electrical stimulation of human primary cells. Later studies performed in our laboratory confirm that human primary muscle expresses BDNF (Western).

In conclusion, based on the critique raised against the present publication, we do not find that Dr. Galbo is able to provide any valid support for his serious accusations against us.

TABLE 1

Exercise study conducted by Sune Jauffred and associates	Faseb J 2003	J Phys 2005	Exp. Physiology 2005	Faseb J 2005
Article no.	Article 1	Article 3	Article 4	Article 5
Last author	Pedersen BK	Pedersen BK	Pedersen BK	Pedersen BK
Main aim(s)	Protein expression of IL-6 by immunohistochemistry in human muscle tissue after exercise	Is there a release of IL-8 from exercising muscle?	Protein expression of MT by immunohistochemistry in human muscle tissue after exercise	IL-6 receptor regulation in response to exercise and IL-6 infusion in humans
• N • Age • Weight • Height • VO_2 max	18 27 (20-30) median 80 (64-96) median 189 (164-197) median 46,7 (41-68) median	11 26 (21-28) median 77,5 (65-96) median 186 (164-197) median 50,4 (45,9-58,4) median	18 27 (20-30) median 80 (64-96) median 189 (164-197) median 46,7 (41-68) median	11 25 (1) mean 79 (3) 184 (3)
Protocol • Pre Tests • Fasting • Catheters • Muscle Biopsies • Intervention:	VO_2max test on bicycle Overnight **Not reported** Vastus lateralis 0, 3, 4.5, 6 9 and 24 hours post exercise 3 hours of cycling at 60 % of VO_2max followed by 6 hours of recovery: 12 subjects Control (resting): 6 subjects	VO_2max test on bicycle Antecubital vein Vastus lateralis 0, 3, 4.5, 6, 9 and 24 hours post exercise **Study 1** 3 hours of cycling at 60 % of VO_2max followed by 6 hours of recovery: 6 subjects Control (resting): 5 subjects **Study 2** Two legged knee extensor exercise	VO_2max test on bicycle Overnight Vastus lateralis 0, 3, 4.5, 6, 9 and 24 hours post exercise 3 hours of cycling at 60 % of VO_2max followed by 6 hours of recovery: 12 subjects Control (resting): 6 subjects	VO_2max test on bicycle Overnight Antecubital vein Vastus lateralis 0, 3, 4.5, 6, 9 and 24 hours post exercise 3 hours of cycling at 60 % of VO_2max followed by 6 hours of recovery: 6 subjects Control (resting): 5 subjects
Analysis	Muscle fiber type IL-6 mRNA Plasma IL-6	 IL-8 mRNA	MTI+II protein expression, NITT, MDA MTII mRNA	IL-6 receptor, gp130, GAPDH and 18S Plasma IL-6 and IL-6 receptor
Cross references	Not relevant	No references in methods or discussion	No references in methods. Reference to Penkowa et al 2003 (Article 1) in discussion.	No references in methods or discussion

Exercise study conducted by Fischer and associates	J Physiol 2004
Article no	2
Last Author	Pedersen BK
Aim	To study the effect of supplementations with antioxidants on the release of IL-6 from contracting human skeletal muscle
Subjects • N • Age • Height • Weight • BMI	 14 males 25.6(0.4) (mean(SEM)) 177.3(0.7) 23.5(0.3)
Protocol • Pre Tests • Fasting • Catheters • Muscle Biopsies • Intervention	 Maximal power output during two-legged exercise Overnight Femoral artery and vein Vastus lateralis 0, 3, and 6h Treatment group: Oral supplementation with Vitamin C and E (n=7) or placebo (n=7) 29 days prior to experimental exercise protocol.
Analysis	Plasma vitamin C and E, plasma glucose Plasma 8-iso-prostaglandin F2alpha Plasma IL-6 and IL-1ra and cortisol Muscle-IL-6mRNA (PCR)
Cross reference	Not relevant

Exercise study conducted by Alaa Zankari and associates	Experimental Physiology 2007 Jan
Article no	8
Last Author	Pedersen BK
Aim	Expression of the interleukin-8 receptor CXCR2 in human skeletal muscle biopsies after exercise
Subjects • N • Age • Height • Weight • BMI	 15 males 24,9 (4) mean 180,9 (1) mean 82 (8) mean 24,9 (2) mean
Protocol • Pre Tests • Fasting • Catheters • Muscle Biopsies • Intervention	 VO_2max test on bicycle Overnight Antecubital vein Vastus lateralis 0, 3, 4.5, 6, 9 and 24 hours post exercise 3 hours of cycling at 60 % of VO_2max followed by 6 hours of recovery: 8 subjects Control (resting): 7 subjects
Analysis	CXCR2 mRNA in skeletal muscle TGF-β receptor
Cross reference	Not relevant. Reference to study design used in Article 1 in methods. Same protocol as Article 3, but different study.

Fiber type study conducted by Peter Plomgaard and associates	Exercise immunology review 2005	J Appl Phys 2006	J Phys 2008 Nov
Article no	6	7	10
Last Author	Pedersen BK	Pilegaard H	Pedersen BK
Aim	To obtain information on possible fibre type specific expression of cytokines.	To test whether or not metabolic gene expression is tightly coupled with MHC isoform pattern for all genes in human skeletal muscle.	To study the regulation and expression of IL-15 I human skeletal muscle.
Subjects • N • Age • Weight • BMI	 7 26 (1) mean 84 (3) mean 24,7 (0,1) mean	 7 26(1) mean 84 (3) mean 24,7 (0,1) mean	 14 24 (1) mean 79 (2) 24 (1)
Protocol • Fasting • Catheters • Muscle Biopsies • Intervention	 Overnight, no exercise for 24 hours prior Triceps brachii caput medialis, triceps surae pars soleus, quadriceps pars vastus lateralis Biopsies in fasting, resting state	 Overnight, no exercise for 24 hour prior Triceps brachii caput medialis, triceps surae pars soleus, quadriceps pars vastus lateralis	**Study 1:** Overnight, no exercise for 24 hour prior Triceps brachii caput medialis, triceps surae pars soleus, quadriceps pars vastus lateralis **Study 2** Strength training study 4 sets of 6-8, 6-8, 10-14, 10-14 legpress and knee extensor machine. Each set to exhaustion. This was carried out on 8 different human subjects.
Analysis •	Fiber type composition IL-6 mRNA, MCH-I mRNA, MCH-IIa mRNA, IL-18 mRNA, TNFα mRNA	Fiber type composition is given in the text to tell the reader that the muscle groups differed as expected. Enzyme activity (CS, HAD, LDH). mRNA GLUT-4, HKII, GS, PFK, LDH A, LDH B, LPL, HSL, FABP, CD36, CPT1, HAD, CS, αKCDH, Cyt c, PGC-1α, Fox01, PPARα, PPARσ	Fiber type composition is given in the text to tell the reader that the muscle groups differed as expected. IL-15 mRNA and protein
Cross references	Not relevant	No references in methods or discussion to paper 6	No.

Glycogen depletion study conducted by Adam Steensberg and associates. (Steensberg J Physiol. 2001 Dec 1;537(Pt 2):633-9.)	J Phys 2005
Article no	3
Last Author	Pedersen BK
Aim	Is IL-8 protein expressed in contracting muscle fibres and and is there a release of IL-8 from exercising muscle?
Subjects • N • Age • Weight • Height	 6 26 (22-33) 78,1 (70-93) 187 (175-193)
Protocol • Fasting • Catheters • Muscle Biopsies • Intervention	 Overnight Femoral artery and femoral vein 30 min, 1.5 and 3 hours of exercise. 2 hours recovery Vastus lateralis 30 min, 1.5 and 3 hours of exercise. 2 hours recovery (5 hour) Two legged knee extensor exercise at 60 % of maximal workload for 3 hours: 6 subjects
Analysis • Femoral arterial blood flow • Plasma	 IL-8 release IL-8
Cross reference	No reference was given in methods.

IL-6 infusion study conducted by Adam Steensberg and associates (van Hall J Clin Endocrinol Metab. 2003 Jul;88(7):3005-10).	Faseb J 2005
Article no	5
Last Author	Pedersen BK
Aim	IL-6 receptor regulation in response to exercise and IL-6 infusion in humans
Subjects • N • Age • Weight • Height	 12 23,5 (0,5) mean 79 (2) 184 (2) mean
Protocol • Fasting • Catheters • Muscle Biopsies • Intervention	 Overnight Femoral artery Vastus lateralis. Before infusion and at the end of 3 hour infusion, at 6 hour and 24 hour Infused with rhIL-6 for 3 hours: 6 subjects Controls infused with saline for 3 hours: 6 subjects
Analysis • RNA • Plasma	 IL-6 receptor, gp130, GAPDH and 18S IL-6 and IL-6 receptor
Cross reference	No reference is given in the methods as it was only the low dose rhIL-6 that was included in the present study. The relevant reference is given in the discussion.

Exercise study conducted by MajBritt Aström and associates	Diabetologia 2009 July
Article no	12
Last Author	Febbraio MA
Aim	We aimed to determine the signalling processes and metabolic actions of BDNF in muscle cells
Subjects • N • Age • Height • Weight • BMI	 8 males 25 (4) mean 181 (1) 82 (8) 25 (2)
Protocol • Fasting • Muscle Biopsies • Intervention	 Overnight Vastus lateralis immediately after exercise (0), 3, 5, 8, 24, 48 and 72 h after exercise 120 min bicycling at 60 % of VO_2max followed by 24 hour recovery period
Analysis	mRNA and protein content of BDNF LDH release, Phosforylation of AMPK, ACCβ, ERK, TrkB,
Cross reference	Not relevant

Diabetes Cohort Study conducted by Anders Rinnov and associates	Diabetologia 2007 dec
Article no	11
Last Author	Pedersen BK
Aim	To test the hypothesis that elevated levels of TNF-α in plasma, muscle and adipose tissue and plasma levels of sTNFR2 are associated with insulin resistance independently of obesity
Subjects • N • Age • BMI • VO2 max • Age • BMI • VO2max	 199 *103 healthy control:* 52,9 30,06 31 *96 patients with T2D:* 58,2 30,9 24,4
Protocol • Tests • Fasting • Catheters	 OGTT Overnight Antecubital vein
Analysis • RNA • Plasma	 mRNA TNFα in muscle TNFα, IL-6, sTNFR2, LDL, HDL, TG, CRP, glucose and insulin
Cross references	Not relevant

COPD study conducted by Anne Marie W. Petersen and associates	Lung 2007 May-June
Article no	9
Last Author	Pedersen BK
Aim	To test whether systemic inflammation in patients with chronic obstructive pulmonary disease (COPD) is accompanied by enhanced interleukin 18 (IL-18) expression in skeletal muscle, which may precede muscle weight loss
Subjects • N • Age • BMI • N • Age • BMI	*20 patients with severe COPD:* 66 (9,4) 24,9 (5,5) *20 controls*: 64 (8,3) 24,9 (3,1)
Protocol • Tests • Muscle Biopsies	Pulmonary function DXA scanning Vastus lateralis
Analysis	mRNA IL-18 and IL-6, TNFα in muscle Fiber type composition
Cross references	Not relevant

Table 2

1. Elevated muscle interstitial levels of pain-inducing substances in symptomatic muscles in patients with polymyalgia rheumatica. Kreiner F, Galbo H. Pain. 2011 May;152(5):1127-32. Epub 2011 Mar 8.

2. Increased muscle interstitial levels of inflammatory cytokines in polymyalgia rheumatica. Kreiner F, Langberg H, Galbo H. Arthritis Rheum. 2010 Dec;62(12):3768-75. doi: 10.1002/art.27728

3. Insulin sensitivity and related cytokines, chemokines, and adipokines in polymyalgia rheumatica. Kreiner F, Galbo H. Scand J Rheumatol. 2010;39(5):402-8. Jan

Journal	Scand J Rheumatol 2010	Arthritis & Rheumatism 2010	Pain 2011
Last Author	Galbo H	Galbo H	Galbo H
Article no.	3	2	1
Aim	To evaluate the insulin sensitivity (IS) and levels of peptides with impact on IS in polymyalgia rheumatica (PMR) before and after prednisolone treatment	To elucidate the disease mechanisms in symptomatic muscles by measuring interstitial levels of cytokines before and after prednisolone treatment	To explore pain mechanisms in affected muscles of patients with PMR
Subjects • N • Age • BMI • Age • BMI	15 patients with recent onset of PMR and 15 controls without PMR *Patients:* 73 (58-86) 24.1 (16.4-30-6) *Controls:* 72 (61-85) 26.3 (21.6-31-4)	20 Patients with recent onset of PMR 20 matched controls *Patients:* 75.3 (60.5-87.2) 24.6 (16.5-30.9) *Controls:* 73.8 (59.3-85.2) 25.7 (21.6-32.1)	20 Patients with recent onset of PMR 20 matched controls *Patients:* 75.3 (60.5-87.2) 24.6 (16.5-30.9) *Controls:* 73.8 (59.3-85.2) 25.7 (21.6-32.1) **Table 1 is exactly the same as Table 1 in Article no. 2 (reuse).** **Method and material description is exactly the same as in Article no. 2.**

Protocol • Fasting • Catheters; blood sampling • Catheters; blood flow • Intervention	Overnight Not described Not described 14 days of prednisolone treatment (20mg/day)	Overnight **Retrograde direction in dorsal hand vein** Upper Trapezius muscle (20 subjects), middle part of vastus lateralis (20 subjects) Dialysate collected at 60, 100 and 140 min 14 days of prednisolone treatment (20mg/day)	Overnight **Retrograde direction in dorsal hand vein** Upper Trapezius muscle (20 subjects), middle part of vastus lateralis (20 subjects) Dialysate collected at 60, 100 and 140 min 14 days of prednisolone treatment (20mg/day)
Analysis	ESR and CRP TNF, IL-6, IL-8, and MCP-1, and in 10 patients and 10 controls for plasma levels of leptin, adiponectin, and resistin.	ESR and CRP Blood flow (outflow/inflow ratio), IL-1α, IL-1β, IL-1Ra, IL-6, IL-8, TNF-α, MCP-1	**ESR and CRP (same as Article 2)** **Blood flow (outflow/inflow ratio) (same as in Article 2**) Glutatamate, PGE_2, 5-HT, ATP, Lactate, Pyruvate
Cross reference	Could potentially be the same subjects as Articles 1 and 2	No reference in Methods. Reference in discussion to Article 3.	No reference in Methods. Ref. to Article 2 in introduction and discussion (“recently presented view”). Table 1 is exactly the same as in Article 2 without reference.

Table 3

1. Muscle metabolism during graded quadriceps exercise in man. Helge JW, Stallknecht B, Richter EA, Galbo H, Kiens B. J Physiol. 2007 Jun 15;581(Pt 3):1247-58. Epub 2007 Mar 22.

2. Interstitial glycerol concentrations in human skeletal muscle and adipose tissue during graded exercise. Stallknecht B, Kiens B, Helge JW, Richter EA, Galbo H. Acta Physiol Scand. 2004 Apr;180(4):367-77.

3. The effect of graded exercise on IL-6 release and glucose uptake in human skeletal muscle. Helge JW, Stallknecht B, Pedersen BK, Galbo H, Kiens B, Richter EA. J Physiol. 2003 Jan 1;546(Pt 1):299-305.

Journal	J Phys 2003	Acta Phys 2004	J Phys 2007
Last Author	Richter EA	**Galbo H**	Kiens B
Article no.	3	2	1
Subjects • N • Age • Weight • Height • VO2 peak	 7 25(1) 77(3) 183(2) 3.9(0.2)	 9 (2 did not have last blood sample) 25(1) 76(3) 182(2) 3.9(0.2)	 9 25(1) 76(3) 182(2) 3.9(0.2)
Protocol • Fasting • Catheters • Microdialysis • Muscle Biopsies • Intervention 45 min at 25% 35 min at 65% one leg 35 min at 85% other leg	 12 hours Femoral vein and artery Not reported? Vastus lateralis Two-legged knee extension exercise 13(1)W, 23(1))%VO2 peak, heart rate 95(2)bpm 32(3)W, 40(1))%VO2 peak, heart rate 141(4)bpm 44(4)W, 40(1))%VO2 peak, heart rate 141(4)bpm	 12 hours Femoral vein and artery Muscle and Abd sucutis Vastus lateralis Two-legged knee extension exercise 13(3)W, 23(1)%VO2 peak, heart rate 96(2)bpm 32(3)W, 40(1)%VO2peak, heart rate 141(4)bpm 44(3)W, 40(1)%VO2peak, heart rate 141(4)bpm	 10 hours? Femoral vein and artery Not reported? Vastus lateralis Two-legged knee extension exercise 13(1)W, 23(1)%VO2 peak, heart rate 95(2)bpm 32(3)W, 40(1)%VO2peak, heart rate 141(4)bpm 44(3)W, 40(1)%VO2peak, heart rate 141(4)bpm
Cross reference	Not relevant	No cross references	Reference to Acta Phys 2004 (Article 2) in discussion, but not in methods. No reference to J Phys 2003 (Article 3)

Table 4

1. Myosin heavy chain isoform transformation in single fibres from m. vastus lateralis in spinal cord injured individuals: effects of long-term functional electrical stimulation (FES). Andersen JL, Mohr T, Biering-Sørensen F, **Galbo H**, Kjaer M. Pflugers Arch. 1996 Feb;431(4):513-8.

2. Long-term adaptation to electrically induced cycle training in severe spinal cord injured individuals. Mohr T, Andersen JL, Biering-Sørensen F, **Galbo H**, Bangsbo J, Wagner A, Kjaer M. Spinal Cord. 1997 Jan;35(1):1-16. Erratum in: Spinal Cord 1997 Apr;35(4):262.

3. Increased bone mineral density after prolonged electrically induced cycle training of paralyzed limbs in spinal cord injured man. Mohr T, Podenphant J, Biering-Sorensen F, **Galbo H**, Thamsborg G, Kjaer M. Calcif Tissue Int. 1997 Jul;61(1):22-5.

4. Insulin action and long-term electrically induced training in individuals with spinal cord injuries. Mohr T, Dela F, Handberg A, Biering-Sørensen F, **Galbo H**, Kjaer M. Med Sci Sports Exerc. 2001 Aug;33(8):1247-52.

Journal	Pflugers Arch. 1996	Spinal Cord. 1997 jan	Calcif Tissue Int. 1997 july	Med Sci Sports Exerc. 2001
Last Author	Kjaer M	Kjaer M	Kjaer M	Kjaer M
Article no	1	2	3	4
Aim	The myosin heavy chain (MHC) composition of single fibres from m. vastus lateralis of five spinal- cord-injured (SCI) individuals was analysed	To investigate the reversibility with training of inactivity related changes in muscle and in maximal cardiopulmonary activation in individuals with SCI	To evaluate if this (which?) inactivity-associated condition in the SCI population can be reversed with prolonged physical training	To examine whether regular electrically induced cycle training increases insulin sensitivity, oral glucose tolerance, and the GLUT 4 content in the stimulated muscles in individuals with SCI

Subjects • N	5 spinal- cord-injured (SCI) individuals; level of injury between C6 and T4	10 SCI individuals; 8 males and 2 females 6 were tetraplegic (injury level: C6) and 4 paraplegic (injury level: T4); 7 resulting from traffic accidents, 2 due to accidents during sporting activities and 1 from shallow water diving	10 SCI individuals; 8 males and 2 females 6 were tetraplegic (injury level: C6) and 4 paraplegic (injury level: T4)	10 subjects with SCI, 8 males and 2 females 6 subjects were tetraplegic (injury level C6) and 4 were paraplegic (injury level T4); 7 due to traffic accidents, 2 due to accidents in sporting activities, and 1 due to shallow water diving
• Age • Weight	35.6 + 6.2 (27-44) years 70.0 + 13.3 kg (range 54-93 kg)	35 (27–45) years	35 (27–45) years 78.0 ± 3.8	35 +/- 2 y 73 +/- 5 kg
• Time since injury	11.2 ±7.0 years	12.5 (range 3 - 23)	12.5 (2–24) 5 able bodied individuals (four males, one female), at similar ages, served as controls.	12 yr (range 3–23 yr)
Protocol • Biopsy	Vastus lateralis. Before training as well as after 6 and 12 months of training.	Vastus lateralis. Before and after training		Vastus lateralis. Before training as well as after 6 and 12 months
• Electrodes placed	Quadriceps, hamstrings and gluteal muscle groups of both legs	Quadriceps, hamstrings, and gluteal muscle groups	Quadriceps, hamstrings, and gluteal muscle groups	Quadriceps, hamstrings, and gluteal muscle groups
• Intervention	30 Hz, contraction (18–40 mA) up to a maximum of 130 mA.	30 Hz, contraction (18–40 mA) up to a maximum of 130 mA.	30 Hz, contraction (18–40 mA) up to a maximum of 130 mA.	30 Hz, contraction (18–40 mA) up to a maximum of 130 mA.

	Functional electrical stimulation of the legs for 30 min, three times a week for 12 months, followed by one time a week for 6 months	Functional electrical stimulation of the legs for 30 min, three times a week for 12 months, followed by one time a week for 6 months	Functional electrical stimulation of the legs for 30 min, three times a week for 12 months, followed by one time a week for 6 months	Functional electrical stimulation of the legs for 30 min, three times a week for 12 months, followed by one time a week for 6 months
Analysis	• Fiber type composition	• MR of thigh • Fiber type composition • CS activity • Exercise tolerance • Training duration • Total work output • VO2max • Muscular microstructure	• Bone mineral density (BMD) was measured in the lumbar spine • (L2–L4), femoral neck, and proximal tibia • Urine, deoxypyridinoline by high performance liquid chromatography and was corrected for urine creatinine excretion	• Hyperinsulinemic, euglycemic clamp • OGTT • Determination of GLUT 4 transporter protein in muscle
Cross references	No references	No references in introduction, methods or discussion	No reference in introduction, method or discussion	Reference to exercise ergometer (Article 1) in method. Reference to Article 2 in discussion (it is mentioned that it is the same study and same subjects)