CIM
Centre of Inflammation and Metabolism

Udvalgene vedr. Videnskabelig Uredelighed
Ministeriet for Videnskab, Teknologi og Udvikling
Forsknings- og Innovationsstyrelsen
Bredgade 40
1260 Copenhagen K

Copenhagen, October 28, 2011

Dear Beatrice Lamberget Sloth,

I acknowledge receipt of your letter of consultation of October 10, 2011, including e-mail correspondences between UVVU and Prof. James A. Timmons (JT).

I (BKP), Matthew Laye (ML), Camilla Scheele (CS) and Søren Nielsen (SN) have read the response by JT. We will not respond to UVVU with regard to the issues concerning the media and personal issues, as these issues clearly fall outside the responsibilities of UVVU.

When reading the additional material (10 pages), we do not find any statements that relate to specific potential scientific dishonesty other than those already mentioned in JT's report of April 18, 2011. In fact, we do not find that JT raises any new concerns or that he has issued any rebuttals to our response. However, we will briefly respond to the report from JT, which we received on October 10, 2011.

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

Sincerely

Bente Klarlund Pedersen MD DMSc
Professor of Integrative Medicine
Director of Centre of Inflammation and Metabolism (CIM)
Rigshospitalet 7641
University of Copenhagen, Faculty of Health Sciences
DK-2100 Copenhagen, Denmark
Phone: + 45 35 45 77 97 ; + 45 26 17 05 24 (mobile)
bkp@rh.dk
http://www.inflammation-metabolism.dk

H:S Rigshospitalet – 7641
Blegdamsvej 9 2100 Copenhagen Denmark
Tel +45 35457797 Fax+45 35457644 bkp@rh.hosp.dk

The Genome Medicine microRNA paper, JT's reply page 3 to 8:

We (BKP and CS) take the liberty to refer to our report of August 22, 2011, page 5 to 24. In addition, we provide below a brief comment with regard to "the BDNF idea" and "the expression of BDNF in human muscle samples".

The BDNF idea

We have provided a highly detailed explanation to the BDNF-story and collaboration in our initial report (Aug 22, 2011), which starts at page 5. I (BKP) point to the fact that JT appears to conduct active manipulation with regard to e-mail addresses (p.7) resulting in the fact that I (BKP) did not receive the final version of the manuscript sent to Genome Medicine. JT replies in his report to the matter of his active manipulation by referring to a previous version of the manuscript, the one submitted to JCI.

JT writes (p.3) "BKP states that she sent the Diabetologia paper for scientific discussion in Feb 19th 2010." This is, however, wrong. The year is 2009. JT seems not to remember the course of events correctly. We have e-mail documentation in regard to the following:

February 19-21, 2009: CS forwards (upon BKPs request) the BDNF Diabetologia paper to JT. In his reply he expresses an interest in further measurements of BDNF in satellite cells. BKP replies positively to this and CS therefore moves on with western blots of BDNF for the new version of the Genome Medicine paper (H_1)

May 27, 2009: CS sends the finalized western blots for the Genome Medicine paper to JT, including the blots on BDNF (H_2_1 and H_2_2).

The BDNF expression in human muscle samples

There are multiple examples in the literature of human studies demonstrating different results, also with regard to differences in gene expression regulation. This is normally accounted for as being due to biological and physiological variation and does not make the scientific community cry "fraud"!

However, below we summarize, clarify and provide explanations to JTs concerns regarding discrepancies in his and our data on BDNF.

BDNF expression: Transcripts per cell
First of all, despite all of JTs efforts to calculate how many mRNA transcripts per cell a certain Ct value corresponds to, having not done any functional studies of BDNF himself, it is impossible for JT to know how many mRNA transcripts that is needed to make the expression "physiologically relevant".

In addition, different mRNA transcripts has different stabilities (depending on the composition of the 3'UTR), and a stable mRNA transcript can produce more protein (i.e. can

H:S Rigshospitalet – 7641
Blegdamsvej 9 2100 Copenhagen Denmark
Tel +45 35457797 Fax+45 35457644 bkp@rh.hosp.dk

be translated several times) than a less stable mRNA transcript.

Differences in mRNA levels (qPCR Ct values) / adjusting the Ct threshold
If JT would adjust the Ct threshold in the qPCR analysis software for his unpublished samples, it appears (by looking at the screenshot) that his Ct values would not differ much from the ones we obtained in our study. Basing his argumentation on differences in Ct values that may in fact not differ at all doesn't make sense and indicates that he has a lack of knowledge in interpreting qPCR raw data.

Degraded cDNA
We suggested in our previous reply that degraded cDNA could explain the somewhat divergent findings and it remains a possibility. The other gene presented by JT (anti-BDNF), which is measured in the same cDNA, can of course not be used as a control for degradation as different mRNAs have different stabilities and thus, the other transcript (anti-BDNF) might be more resistant to degradation. JT's argumentation reveals severe lack of knowledge in basic molecular biology.

Use of different PCR assay
As an answer to our original response in which we inform that BDNF have multiple splice variants and that therefore the primer design would influence the result, JT replies *"The majority of genes have splice variants, many at the '3UTR. BDNF has only a small number of exons and in fact the variants are almost all 3'UTR variants (http://genome.ucsc.edu). As the PCR primers target a common exon region this 3' UTR variance is irrelevant for the real-time PCR. "* Again, JT's argumentation indicates a lack of knowledge about qPCR and basic molecular biology. The company Applied Biosystems has 22 different pre-designed primer assays, making it possible to detect every single splice variant. We made sure to use the assay recommended by Applied Biosystems for "Best coverage", detecting as many splice variants as possible. It is still unclear which primer assay JT utilized in his BDNF study (unpublished).

Differences in protein levels (western blot data)
In collaboration with BKP and CS, JT has found that BDNF is difficult to detect in muscle tissue, using western blot. This analysis was performed on subjects of around 60 years of age. In the BDNF Diabetologia western blot, muscle biopsies from young healthy men were analyzed, using the same antibody, and a clear band for BDNF could be demonstrated. As said, there are multiple examples in the literature of human studies demonstrating different results, and indeed, differences in gene expression regulation occurring with aging is a huge and exciting field. Since the publication of the Genome Medicine paper, we have used another BDNF-antibody and we have been able to identify BDNF protein also in skeletal muscle biopsies from middle-aged humans. In our previous report, we included an example of this, a western blot, which clearly demonstrates BDNF protein expression in healthy controls and patients with type 2 diabetes (F_2).

JT appears to believe that since a protein is not detectable in 60-year-old men in one study, it must be false that it is detected in young healthy men in another study. This unbalanced assumption would probably disappoint many researchers in the field of aging.

H:S Rigshospitalet – 7641
Blegdamsvej 9 2100 Copenhagen Denmark
Tel +45 35457797 Fax+45 35457644 bkp@rh.hosp.dk

MiRNA fraud, JT's reply page 8 to 9:

Here I (BKP) include a response formulated by Matthew Laye:

In response to the "miRNA Fraud" case Dr. Timmons has provided no evidence contrary to our evidence from our initial submission. He has not disputed his limited actual contribution to the paper, but disputed my (ML) contributions to the paper. While Dr. Timmons has said that, "I also stated to Soren that I would prefer my data to be published first as that was fair. Soren sent me a manuscript with no authors listed. The project was discussed with Soren many times in person and also by email," he has not provided any evidence of discussing this "project" via email nor disputed the instant messenger exchange with Søren stating that we should go ahead and submit first and that he would prefer to submit at the same time. Dr. Timmons has tried to use his influence as a more senior researcher and friend to receive a false authorship when in fact he contributed very little to the actual manuscript. I (ML) stand by my role as being both ethical and appropriate and believe Dr. Timmons has provided no evidence to the contrary.

Here I (BKP) include a statement formulated by Camilla Scheele:

It is surprising that JT has forgotten that CS has a PhD in Functional Genomics (with the title "Functional genomics studies of PINK1"; attached please find PhD diploma from Karolinska Institutet (H_3)). The cell work that JT refers to as molecular biology work for the Genome Medicine paper was only included as supplementary figures, while the analysis of RNA (isolated by CS) and the protein (isolated by CS and analyzed by western blots by CS) are all included in the main text.

Here I (BKP) include a response formulated by Søren Nielsen:

JT:
Matt Layes version of the miRNA events demonstrates he had no idea where the ideas came from or the fact that I met with the student Soren Nielsen over 20 times to discuss miRNA research prior to his arrival, during and after ML took over as SN "boss".

Reply:
At no point did I (SN) have a meeting about miRNAs with JT. SN had one meeting with JT about his Master thesis defense in the summer of 2009. SN's master thesis was about the effects of antioxidants on training adaptation and certainly not about miRNAs. JT is an exercise physiologist and SN was happy, at that time, to get feedback on the physiological challenges in his thesis.
Importantly, JT stayed in our (SN and CS) apartment for approximately one month after his relation to his former girlfriend (Pernille Keller) abruptly stopped in 2009. It would be a major exaggeration to call the mornings and evenings, before and after work, for miRNA meetings. Emotional support and psychological help is a much more appropriate description.
We do not understand why JT thinks it is important to discuss ML's job title within CIM. But I am happy to clarify that ML is the daily supervisor for SN.

H:S Rigshospitalet – 7641
Blegdamsvej 9 2100 Copenhagen Denmark
Tel +45 35457797 Fax+45 35457644 bkp@rh.hosp.dk

JT:
This long term interaction with SN on miRNA was precisely why I was sent the manuscript and why Soren was apprehensive about what I might think. I am also clearly the person that introduced CIM to the miRNA field.

Reply:
JT did not invent miRNAs (Ambros discovered the first miRNA in 1993) and he was not the first to measure muscle specific miRNAs in vivo. Chen et al. published that work in Nature Genetics in 2006. Neither was he the first exercise physiologist to measure muscle specific miRNAs in response to exercise in humans. Drummond et al. published an article about that in 2008 and several papers about the topic were published during the following years. JT´s first senior author article about measured miRNAs was published last year. To call himself a miRNA expert within the field is surely an overstatement.

JT:
The SN miRNA data are a replication of the data generated in 2007 that I shared with the BKP group, and I also stated to Soren that I would prefer my data to be published first as that was fair. Soren sent me a manuscript with no authors listed. The project was discussed with Soren many times in person and also by email.

Reply
It is very important to state that JT has edited a major part out of the email correspondance, which he uses as proof in his report. We refer to the unedited version (B_9), submitted with our first response. SN clearly offers JT to read the manuscript so that he can see if there are any conflicts (19:30) and not as JT claims, "to get comments". Hereafter, SN clearly asks JT if he thinks there is too much overlap and if SN can submit the paper. JT replies: "the only overlap is the training part - we have only down but 80% of our paper is about the mRNA changes... would be ideal if we submit at the same time but mine is not ready with other authors for ~3-4wks". He continues 19:44: "so best you submit". In our opinion that is a very clear answer and thus, 4 days later we submitted the manuscript.

JT:
At no stage did I imagine that I would not be an author – even though I did wish for my labs study to be published first. It was actually under review at the same time at the same journal but mysteriously got rejected.

Reply
We have never discussed the possibility of JT being an author on the paper and we do not believe that 23 minutes of reading and 5 minor comments (mainly advice about citing his own papers) is enough to get an authorship.

We can only guess as to why JT´s paper was rejected by the Journal of Physiology.

H:S Rigshospitalet – 7641
Blegdamsvej 9 2100 Copenhagen Denmark
Tel +45 35457797 Fax+45 35457644 bkp@rh.hosp.dk

JT:
When I finally saw the version of the article that now had an author list – I was very unhappy. It was only later that I reflected on the fact that the version Soren sent me had no authors listed – a situation that now appears to me to be very odd. Most people assign the author list at the start of the process – but then I now appreciate it was to hide that Matt was senior author and I would not be on the paper because BKP insisted on this to be the case.

Reply:
SN sent the manuscript to ensure that the there was no data overlap (at 19:30, please see B_9 in our first reply) and not for proofreading, as JT claims. In that context the author list is without relevance. In our previous report (page 24-26) we have provided a thorough account of ML's contribution to the paper and discussed in-depth why he is last author.

I (BKP) have one more comment to make:

JT:
BKP claims not to know the reviewers - yet BKP was an editor at that very same journal (although she has now been removed from the editorial board) and the paper was reviewed by her friend Matthew Watt and she knows this. In fact, BKP was accused of irregular behaviours in peer review duties by senior editors during her time at the journal so she was asked to leave for more than one reason.

Reply (BKP)
For the information of UVVU I enclose a letter from my lawyer Hanne Rahbæk to the Editor-in-chief Professor David Patterson at the Journal of Physiology (H_4).

Myokines, JT's reply page 9

JT:
I will not comment in detail on the myokine work as others will in more detail than I can.

Reply:
We assume that with "others", JT refers to his interaction with Professor Henrik Galbo and I take the liberty to refer to my response (dated 12 August 2011) to the report formulated by the latter person and to our previous response regarding reports from JT. Specifically regarding IL-8, please see page 31 in our response regarding the report from JT.

Anyone who follows the literature will be well aware of the fact that several independent research groups have found that muscles produce and secrete IL-6. Studies using microdialysis indicate that the concentration of IL-6 within the contracting skeletal muscle may be 5- to 100-fold higher than the levels found in the circulation and that IL-6 appears to accumulate within the contracting muscle fibres as well as in the interstitium during exercise

Rosendal,L. et al. Increase in interstitial interleukin-6 of human skeletal muscle with repetitive low-force exercise. J Appl. Physiol. ***98****, 477-481 (2005).*

H:S Rigshospitalet – 7641
Blegdamsvej 9 2100 Copenhagen Denmark
Tel +45 35457797 Fax+45 35457644 bkp@rh.hosp.dk

IL-6 has been shown to be expressed by human myoblasts

De Rossi,M., Bernasconi,P., Baggi,F., de Waal,M.R., & Mantegazza,R. Cytokines andchemokines are both expressed by human myoblasts: possible relevance for the immune pathogenesis of muscle inflammation. Int. Immunol. ***12****, 1329-1335 (2000) and Bartoccioni,E., Michaelis,D., & Hohlfeld,R. Constitutive and cytokine-induced production of interleukin-6 by human myoblasts. Immunol. Lett.* ***42****, 135-138 (1994).*

Moreover, IL-6 is locally and transiently produced by growing murine myofibers and associated muscle stem cells (satellite cells)

Serrano,A.L., Baeza-Raja,B., Perdiguero,E., Jardi,M., & Munoz-Canoves,P. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Cell Metab ***7****, 33-44 (2008).*

In addition, IL-6 is released from human primary muscle cell cultures from healthy individuals and from patients with type 2 diabetes

Haugen,F. et al. IL-7 is expressed and secreted by human skeletal muscle cells. Am. J. Physiol Cell Physiol. ***298****, C807-C816 (2010) and Green,C.J., Pedersen,M., Pedersen,B.K., & Scheele,C. Elevated NFkappaB Activation is Conserved in Human Myocytes Cultured from Obese Type 2 Diabetics and Attenuated by AMP-activated Protein Kinase. Diabetes In press (2011).*

Moreover, a number of research groups have contributed to the identification of the muscle cell secretome. In a study conducted by Schiaffino and his group, a computational approach was applied. They screened 6255 products of genes expressed in normal human skeletal muscle. They reported that the resulting putative skeletal muscle secretome consisted of 319 proteins, including 78 still uncharacterized proteins

Bortoluzzi,S., Scannapieco,P., Cestaro,A., Danieli,G.A., & Schiaffino,S. Computational reconstruction of the human skeletal muscle secretome. Proteins. ***62****, 776-792 (2006).*

Yoon et al incubated differentiated IL-6 rat skeletal muscle cells with or without insulin and comparatively analyzed the proteins secreted into the media, using offline RP HPLC, digested the fractionated proteins, and analyzed the resulting peptides with LC-ESI-MS/MS. They identified a total of 254 proteins, among which 153 were classified as secretory proteins. Fourteen proteins were secreted at higher levels during insulin stimulation, including several proteins known to be highly secreted in metabolic diseases

Yoon,J.H. et al. Comparative proteomic analysis of the insulin-induced L6 myotube secretome. Proteomics. ***9****, 51-60 (2009).*

H:S Rigshospitalet – 7641
Blegdamsvej 9 2100 Copenhagen Denmark
Tel +45 35457797 Fax+45 35457644 bkp@rh.hosp.dk

I (BKP) coauthored a study led by Kratchmarova, in which we used a quantitative proteomics platform to investigate the factors secreted during the differentiation of murine C2C12 skeletal muscle cells. We identified and quantitatively analyzed 635 secreted proteins, including 35 growth factors, 40 cytokines and 36 metallopeptidases. In addition to molecules reported earlier, we identified many secreted proteins that have not previously been shown to be released from skeletal muscle cells

*Henningsen,J., Rigbolt,K.T., Blagoev,B., Pedersen,B.K., & Kratchmarova,I. Dynamics of the skeletal muscle secretome during myoblast differentiation. Mol. Cell Proteomics. **9**, 2482-96 (2010).*

Most recently, Norheim et al

Norheim F, Raastad T, Thiede B, Rustan AC, Drevon CA, Haugen F. Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. Am J Physiol Endocrinol Metab. Nov;301(5):E1013-21. Epub 2011 Aug 9.(2011)

demonstrated that a total of 236 proteins were detected by proteome analysis in medium conditioned by cultured human myotubes. RT-PCR analyses showed that 15 of the secreted muscle proteins had significantly enhanced mRNA expression in m. vastus lateralis and/or m. trapezius after 11 wk of strength training among healthy volunteers.

JT:
The arguments BKP make about the general acceptance of the Myokine work reflects the fact that she has produced an enormous number of articles and reviews over-stating their role.

Reply:
To let others speak on our behalf, we take the liberty to insert a few sentences from the peer-review of an article entitled “Skeletal muscle as a secretory organ”, which will soon be published in Nature Reviews Endocrinology:

Peer-Reviewer #1 (Remarks to the Author):

“The review is written by two outstanding experts in the field of myokines. Bente Pedersen and Mark Febbraio made the term “myokine" popular in the scientific world. Based on numerous own studies in exercising humans, rodent models and in vitro cell culture experiments, they developed the concept of the exercising skeletal muscle as an endocrine organ which by releasing several cytokines and other peptides is responsible for the health benefits of regular physical activity.”

Peer-Reviewer #2 (Remarks to the Author):

This is an excellent and very timely review that will attract considerable attention. The field of myokines is in its infancy, and the review by Pedersen and Febbraio will provide a broad audience with an excellent overview of this exciting field.

H:S Rigshospitalet – 7641
Blegdamsvej 9 2100 Copenhagen Denmark
Tel +45 35457797 Fax+45 35457644 bkp@rh.hosp.dk