RESEARCH ARTICLE

REVISED Association of fat mass profile with natriuretic peptide receptor alpha in subcutaneous adipose tissue of medication-free healthy men: A cross-sectional study [version 2; peer review: 2 approved]

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Abstract

Background: Atrial natriuretic peptide increases lipolysis in human adipocytes by binding to natriuretic peptide receptor-A (NPRA). The aim of the current study was to examine the associations of NPRA mRNA of subcutaneous adipose tissue with fat mass, fat-free mass, body mass index (BMI) and arterial blood pressure in medication-free healthy men.
Method: Thirty-two volunteers [age (years): 36.06±7.36, BMI: 27.60±4.63 (kg/m^2)] underwent assessments of body height/weight, % fat mass, fat-free mass (kg), blood pressure, and a subcutaneous adipose tissue biopsy via a surgical technique.
Results: We found that NPRA mRNA was negatively associated with % fat mass (r=-0.40, R^2=0.16, p=0.03) and BMI (r=-0.45, R^2=0.20, p=0.01). Cohen's f^2 effect size analyses showed a small effect size between NPRA mRNA and BMI (f^2=0.25). One-way analysis of variance with Bonferroni post-hoc tests showed a tendency for mean differences of NPRA mRNA across BMI categories (p=0.06). This was confirmed by Cohen's *d* effect size analyses revealing a large effect size of NPRA mRNA between obese individuals (BMI≥30 kg/m^2) and either normal weight (BMI=19-25 kg/m^2; *d* =0.94) or overweight (BMI=25-30 kg/m^2; *d*=1.12) individuals.
Conclusions: NPRA mRNA is negatively associated with % fat mass and BMI in medication-free healthy men, suggesting a possible role of NPRA in the control of fat mass accumulation.

Keywords

NPRA, atrial natriuretic peptide, lipolysis, BMI, obesity

Open Peer Review

Reviewer Status ✓ ✓

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	1	**2**
REVISED **version 2** published 18 Jul 2018	✓ report	✓ report
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Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: Dinas PC: Conceptualization, Data Curation, Formal Analysis, Investigation, Resources, Writing – Original Draft Preparation; **Nintou E**: Conceptualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Psychou D**: Investigation, Resources, Writing – Review & Editing; **Granzotto M**: Methodology, Resources, Writing – Review & Editing; **Rossato M**: Methodology, Resources, Writing – Review & Editing; **Vettor R**: Methodology, Resources, Writing – Review & Editing; **Jamurtas AZ**: Conceptualization, Resources, Writing – Review & Editing; **Koutedakis Y**: Formal Analysis, Supervision, Writing – Review & Editing; **Metsios GS**: Formal Analysis, Supervision, Writing – Review & Editing; **Flouris AD**: Conceptualization, Funding Acquisition, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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REVISED **Amendments from Version 1**

We have added in the limitations section information regarding the link between NPRA and levels of triglycerides, cholesterol, ANP and BNP. Similarly, we have included in our limitations and discussion section possible connections between NPRA mRNA and hormone sensitive lipase, adipocytes' size as well as the ANP-BNP/NPRA signalling axis. We put all the above information in context with the obesity phenomenon, while we have listed all the necessary references. Finally, we have added further details about blood pressure registers, providing a relevant reference, while we acknowledge the relevance of our results in context with the ethnicity and the gender of the participants in our study.

See referee reports

Introduction

Atrial natriuretic peptide (ANP) lowers arterial pressure to maintain fluid volume homeostasis, thus protecting against renal and cardiac pathogenesis[1]. ANP also increases lipolysis in human adipocytes[2] by binding to natriuretic peptide receptor-A (NPRA)[3]. NPRA is less expressed in subcutaneous adipose tissue (SAT) in obese individuals and type 2 diabetes patients than in normal glucose tolerant individuals[4]. Also, NPRA signalling in skeletal muscle may enhance long-term insulin sensitivity[5]. Collectively, NPRA may potentially treat obesity-related disorders while ANP may play a role in the therapeutic mechanisms of beta-adrenoceptor antagonists in the mitigation of heart dysfunction and utilization of lipid mobilization[6]. However, the role of ANP in lipolysis has been primarily investigated mainly *in vitro* models[7–9], in human blood cells from individuals under medication treatment[10], and in animal models[9]. To our knowledge, no such information is currently available in relation to the role of its receptor (NPRA) on the adipocytes of healthy individuals. Therefore, the aim of the current study was to examine the associations of NPRA mRNA of SAT with fat mass, fat-free mass (FFM), body mass index (BMI) and arterial blood pressure (BP) in medication-free healthy men.

Methods

The study was approved by the Ethics Committee of the University of Thessaly (protocol no. 698/2013). The inclusion criteria were: healthy adult men, non-smokers, no chronic disease and/or being under medication treatment. The participants were recruited by advertisements in a local newspaper in Trikala, Thessaly, Greece and the data collection started in July 2013 and ended in June 2014. Written consent was obtained from the 32 healthy men recruited [age (years): 36.06±7.36, BMI: 27.60±4.63 (kg/m^2)].

To avoid misleading results, the participants refrained from exercise, alcohol, and passive smoking 72h prior the measurements, while they followed an overnight fast before they visit the physiology laboratory in the School of Exercise Science between 07:00–09:00 am. PCD and DP performed the following measurements: body height using a Seca 220 (Hamburg, Germany) stadiometer, body weight using a precision scale (KERN & Sohn GmbH, Version 5.3, Germany) and blood pressure (BP) using a Standard Aneroid sphygmomanometer (Medisave, UK) according to standard guidelines[11]. Briefly, participants were instructed to empty their bladders and sit for five minutes in a relaxed back rest position without talking. BP readings were taken twice, each two minutes apart, while the mean of the two BP readings was considered as the final BP values. Fat mass percentage (%FM) and FFM were measured via bioelectrical impedance using a body composition monitor (Fresenius Medical Care AG & Co. KGaA D-61346 Bad Hamburg, Germany).

Following the aforementioned measurements, the participants underwent a SAT biopsy in the physiology laboratory by a trained physician, as previously described[12]. Briefly, the site of the incision was disinfected and a 10 ml of 2% xylocaine (no adrenaline) was injected for local anaesthesia. An incision of 2–2.5 cm was made 3–5 cm to the left of the navel. Nearly 500 mg of subcutaneous adipose tissue was captured and removed. The NPRA mRNA analysis of SAT samples is described elsewhere[13]. Briefly, total RNA was extracted using RNeasy Lipid Tissue mini kit (QIAGEN). First-strand cDNAs were synthesized from equal amounts of total RNA using random primers and M-MLV reverse transcriptase (Promega). Quantitative real-time polymerase chain reaction was performed using Sybr Green fluorophore. 18S rRNA gene was used as a reference for normalization.

Following previous methodology, we removed two mean values (i.e. outliers) of NPRA mRNA that were at a distance of more than two standard deviations from the mean of the distribution[14,15]. Also, there were three missing values in the NPRA mRNA analysis of SAT samples due to failure to extract RNA from adipose tissue. Eventually, 27 NPRA mRNA values were included in the statistical analysis using SPSS (version 24; SPSS Inc., Chicago, IL, USA). Normal distribution was determined using Shapiro-Wilk test, whereas Pearson's correlation coefficient, linear regression, and Cohen's f^2 effect size ($R^2/1\text{-}R^2$)[16] were used to detect associations between NPRA mRNA, %FM, FFM (kg), BMI, and BP. We also used one-way analysis of variance (ANOVA) with Bonferroni post-hoc tests, and Cohen's *d* effect size analyses to explore the mean differences of NPRA mRNA across different BMI categories [normal weight <25 kg/m^2 (n=9); overweight 25–30 kg/m^2 (n=9); obese >30 kg/m^2 (n=9)]. The level of significance was set at $p \leq 0.05$.

Results

The participants' characteristics are provided in Table 1. The NPRA mRNA was negatively correlated with %FM (r=-0.40, p=0.03) (Figure 1) and BMI (r=-0.45, p=0.01) (Figure 2). No associations were found between NPRA mRNA and FFM, systolic or diastolic BP (p>0.05).

Table 1. Characteristics of the participants.

Age (years) (n=32)	36.06±7.36
BMI (kg/m^2) (n=32)	27.60±4.62
Fat mass (%) (n=32)	28.32±8.87
Fat free mass (kg) (n=32)	52.90±5.02
Systolic blood pressure (mmHg) (n=32)	124.28±10.09
Diastolic blood pressure (mmHg) (n=32)	84.28±6.91

BMI: Body mass index

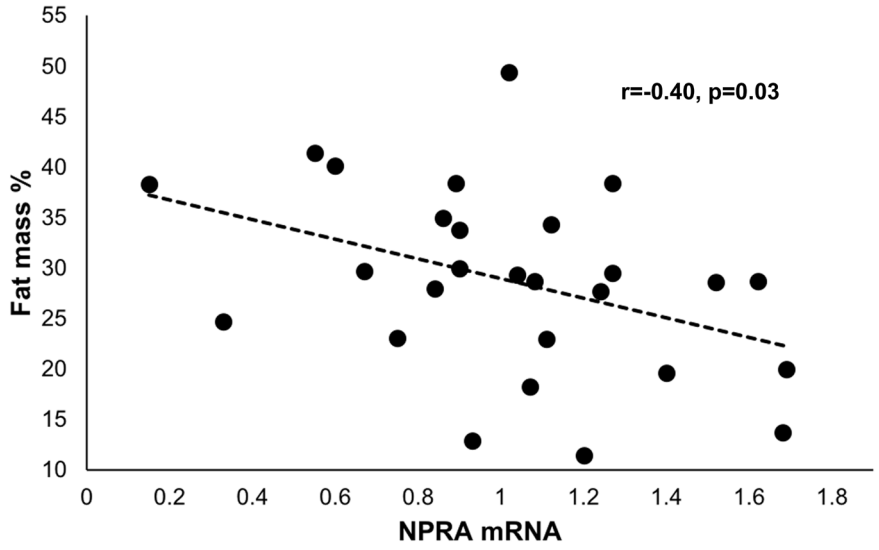

Figure 1. Association of NPRA mRNA with fat mass %.

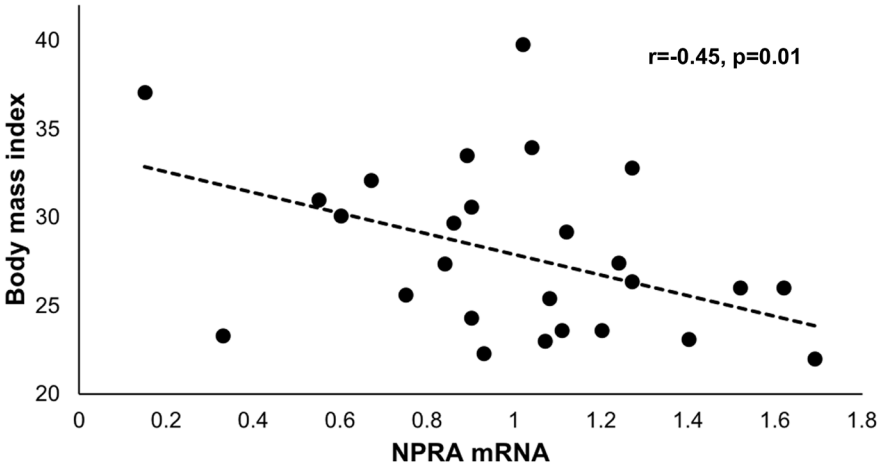

Figure 2. Association of NPRA mRNA with body mass index.

Linear regression analyses confirmed the associations between NPRA mRNA and %FM (R^2=0.16, p=0.03) as well as BMI (R^2=0.20, p=0.01). Cohen's f^2 effect size analyses showed a small effect size between NPRA mRNA and BMI (f^2=0.25), however, no effect size was detected between NPRA mRNA and %FM (f^2<0.20). ANOVA demonstrated a strong tendency for mean differences in NPRA mRNA across BMI categories (p=0.06). This was confirmed by Cohen's *d* effect size analyses in NPRA mRNA, revealing large effect sizes between obese individuals (BMI≥30 kg/m^2) and either normal weight (BMI <25 kg/m^2; *d*=0.94) or overweight (BMI=25–30 kg/m^2; *d*=1.12) individuals.

Dataset 1. Subcutaneous adipose tissue NPRA mRNA of medication-free healthy men

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BMI=Body mass index; SBP=Systolic blood pressure; DBP=Diastolic blood pressure; FFM=Fat-free mass; NPRA= Natriuretic peptide receptor-A. BMI categories= 1. <25 kg/m^2, 2. 25–30 kg/m^2, 3. >30 kg/m^2.

Discussion and conclusions

We have shown that the NPRA mRNA is negatively associated with %FM and BMI in medication-free healthy men and that it is expressed less in obese compared to lean individuals. Previous evidence showed that NPRA mRNA is expressed less in normal glucose tolerant individuals than in type 2 diabetes patients[4], while it is positively associated with insulin sensitivity[4]. Given that insulin sensitivity is negatively associated with excessive FM in humans[17,18] the inverse association of NPRA mRNA with %FM and BMI observed in the current study suggests a possible role of NPRA in lowering FM in humans. Indeed, it has been established that natriuretic peptides by binding to NPRA, increase cyclic guanosine monophosphate – a well-known intracellular second messenger – which phosphorylates protein kinase G leading to activation of hormone sensitive lipase[19,20]. This process mediates triglyceride degradation (i.e. lipolysis), which subsequently increases fatty acid availability[19]. This lipolytic effect of NPRA however, appears to be supressed by NPRC action in obese individuals and clearance of NPR3 gene – coding for NPRC – in animals showed to restore NPRA action[9], which

should be considered in future studies. Furthermore, findings in mice showed that the lack of NPRA gene may increase FM[9]. Finally, NPRA signalling as part of ANP/NPRA axis may induce a browning of white adipocytes, indicating increased energy expenditure and thus, a potential to mitigate obesity[21].

The current study may be affected by methodological limitations such as the lack of insulin sensitivity measurements and *a priori* power calculation to determine the sample size. However, a post-measurements power calculation was conducted using an online software (DSS Research) to test statistical power. This revealed 89% of statistical power for the 27 available samples, based on the NPRA mRNA value (1.02±0.38) we detected in our study and expected NPRA mRNA value (0.81±0.08) from a previous similar study that examined NPRA in SAT in humans[4]. Another limitation was the lack of triglyceride, cholesterol and ANP plasma levels, to determine whether there is a link with NPRA mRNA, given the association of the aforementioned factors with lipolysis[2,19]. Previous evidence also suggests that large adipocytes express higher NPRA mRNA than small adipocytes, indicating enhanced ANP-stimulated lipolysis in large adipocytes[22]. However, we did not determine the size of the examined adipocytes and its possible association with NPRA mRNA. Furthermore, brain natriuretic peptide (BNP) may alter expression of NPRA to release free fatty acids from adipose tissue, while obesity is inversely associated with circulating BNP, a situation known as "natriuretic handicap"[23,24]. Circulating BNP was not measured in our study to examine whether there is an association with NPRA mRNA. Also, ANP may inhibit the secretion of adipokines and cytokines from adipose tissue and, thus, may attenuate chronic inflammation and insulin resistance[25,26], a mechanism that was not investigated in our study. Given the action of ANP/BNP on lipolytic hormones, it would have been interesting to determine hormone sensitive lipase in the current study[2,6]. Finally, it is important to note that the current results are limited to the Greek population and male participants, therefore, they should be treated with caution when applied to other ethnicities and females.

In conclusion, NPRA mRNA is negatively associated with %FM and BMI in medication-free healthy men, suggesting a possible role of ANP/NPRA axis in the control of FM accumulation. To date, the investigation of NPRA has mainly focused either on circulating and muscle NPRA[27–29] or on medication-dependent NPRA measurements[4,10]. Our study indicates that NPRA may also play role in FM profile of healthy individuals, which should be further explored in a cause-and-effect research setting.

Data availability

Dataset 1: Subcutaneous adipose tissue NPRA mRNA of medication-free healthy men 10.5256/f1000research.14198.d197694[30]

BMI=Body mass index; SBP=Systolic blood pressure; DBP=Diastolic blood pressure; FFM=Fat-free mass; NPRA=Natriuretic peptide receptor-A.

BMI categories= 1. <25 kg/m^2, 2. 25–30 kg/m^2, 3. >30 kg/m^2.

Competing interests

No competing interests were disclosed.

Grant information

This study was supported by funding from the European Union 7th Framework Program (FP7-PEOPLE-2012-IRSES grant no. 319010; FP7-PEOPLE-2013-IRSES grant no. 612547).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Data Source

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Version 2

Reviewer Report 24 July 2018

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✔ **Kailash N. Pandey**
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The manuscript has been revised according to the previous comments and there is no further concerns with current manuscript.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 19 July 2018

https://doi.org/10.5256/f1000research.17059.r36192

✔ **Marcelo Roberto Choi**
School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

The manuscript has been revised according to the reviewers' comments. I have no further concerns.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 25 June 2018

https://doi.org/10.5256/f1000research.15445.r34721

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The current manuscript by P.C. Dinas et al., describes the association of fat mass profile with the mRNA levels of natriuretic peptide receptor-A (NPRA) in the subcutaneous adipose tissues (SAT) of medication-free healthy individuals. The major goal of the present study was to determine the association of NPRA mRNA in SAT with fat mass (FM), fat-free mass (FFM), body mass index (BMI), and arterial blood pressure in 32 volunteers with average age of 36 years. The subcutaneous adipose tissues biopsy was done by surgical procedures. The authors report that NPRA mRNA was negatively associated with % FM as well as BMI. Based on the present findings, the authors suggest that NPRA signaling mechanism might play a critical role in the control of fat mass accumulation. The results of the current study are interesting and critical in understanding the versatility of the NPRA signaling mechanisms in the health and disease; however, there are some concerns, which need to be considered to improve the contents of this manuscript.

1. The present study establishes a link between NPRA mRNA, % FM, and BMI. The results indicate that atrial and brain natriuretic peptides (ANP, BNP) and their cognate receptor NPRA might play a vital role in lipolysis. Thus, it could have been a logical extension to determine the hormone-sensitive lipases. Although, ANP and BNP have been shown to act as the lipolytic hormones and also to affect energy use and metabolism in adipocytes, thus their actions seem to be associated with hormonal stimulation of lipases (Sengenes et al., 2003[1]; Lafontan et al., 2008[2]). It would be helpful that these issues and limitation might be discussed in the manuscript.

2. The ethnicity of the Greek subjects under the study should be indicated and if there is any limitation that could be mentioned. The study is based on the small number of subjects and the limitations might also be indicated.

3. The cGMP- dependent protein kinase 1 (CGK1) phosphorylates perilipin-1, a hormonal-regulated lipase that initiates lipolysis (Sengenes et al., 2003; Lafontan et al., 2005[3]). Nonetheless, it has been suggested that the potent lipolytic function of ANP and BNP seems to be restricted to primates (Sengenes et al., 2002[4]; Engeli et al., 2012[5]). Intriguing was the finding that a lower concentrations of ANP and BNP seem to be associated with obesity, insulin resistance, and metabolic consequences, thereby, ANP-BNP/NPRA/cGMP axis might regulate fat oxidation to prevent obesity and glucose intolerance (Wang et al., 2004[6]; Wang et al., 2007[7]; Mitsuishi et al., 2008[8]; Miyashita et al., 2009[9]). These paradigms and issues of the ANP/NPRA/cGMP signaling could be discussed.

4. Interestingly, ANP-BNP/NPRA signaling axis has been shown to induce a browning of white adipocytes in humans, which seems to be physiologically significant (Enerback, 2010[10]; Collins et al., 2014[11]; Liu et al., 2018[12]). A brief discussion on this aspect of ANP/NPRA signaling mechanisms might be helpful.

5. The previous findings have suggested that NPRA acts as a determinant of insulin sensitivity; however, the upregulation of natriuretic peptide receptor-C (NPRC) decreases the glucose tolerance in obase subjects, which seems to repress ANP-BNP/NPRA signaling axis and thereby the lipolytic effects of ANP was completely rescued in *Npr3* (coding for NPRC) gene-knockout mice (Bordicchia et al., 2012[13]). The significance of these previous findings and the relationship to the current work could be discussed in the current manuscript.

6. The conclusions of the present study are based on the findings in male gender. The limitations on the inclusion of female gender should be mentioned.

I have read and reviewed the manuscript and believe that I have the expertise to evaluate and state that the current work is of an acceptable scientific quality. Indeed, there are some concerns in the manuscript, which are summarized above.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 14 Jul 2018

Petros Dinas, University of Wolverhampton, Walsall, UK

We thank you very much for your comments. Please see below our responses to your comments:

Comment 1: Thank you for this suggestion. Indeed, this would have been an interesting analysis to perform. Unfortunately, there are no samples available at this stage to perform the suggested work. Therefore, we have included in the limitations (Page 6, paragraph 2) the lack of hormone sensitive lipase measurements.

Comment 2: We have added this information in the limitations (Page 6, paragraph 2).

Comment 3: The issue regarding the obesity prevention via ANP/BNP axis has been added in the Discussion section (Page 6, paragraph 1). Insulin sensitivity measurements in the current study has also been indicated as a limitation. (Page 6, paragraph 2).

Comment 4: We have added this information at the end of the first paragraph of the Discussion section (Page 6, paragraph 1).

Comment 5: We have included a brief discussion on these topics in the first paragraph of the Discussion section (Page 6, paragraph 1).

Comment 6: We have added this information in the limitations (Page 6, paragraph 2).

Competing Interests: No competing interests were disclosed.

Reviewer Report 24 April 2018

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Marcelo Roberto Choi
School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

The present work by Dinas et al. is a descriptive study that investigated the association of NPRA mRNA of subcutaneous adipose tissue (SAT) with fat mass, fat-free mass, body mass index (BMI) and arterial blood pressure in thirty-two medication-free healthy volunteers. The authors observed a negative association between NPRA mRNA with %fat mass and BMI. Based on these results the authors suggest a possible role of ANP-NPRA axis in the control of FM accumulation. Although the topic is of interest, some issues need to be addressed in order to improve the quality of the manuscript.

1. Given that NPRA plays a role in lipolysis, it would have been interesting if the authors could determine the levels of triglycerides and cholesterol as well as ANP plasma levels in order to verify a possible correlation between these parameters and the percentage of fat and/or NPRA mRNA levels in SAT. This issue should be considered in the discussion section and mentioned as a limitation of this study.

2. Please provide more details about blood pressure registers (for example: number of measures, guideline used to define hypertension, brand of the device used).

3. Yu et al.[1] suggested that adipocyte size is an important determinant of ANP-stimulated lipolysis. In this way, expressed higher mRNA levels of receptor (NPR)-A and hormone sensitive lipase as well as more NPR-A on the membrane than small It would have been interesting if the authors had determined the NPRA mRNA according to the size of the adipocyte. This issue should be considered in the discussion section and mentioned as a limitation of this study. (see also Laurencikiene et al.[2])

4. It has been reported that obesity is associated with low circulating levels of BNP and recent evidences suggest an altered expression of BNP receptors-both the signaling receptors (NPR)-A

and the clearance NPR-C receptor-in Pivovarova et al.[3]; Gentili et al.[4]). Moreover, increased tissue secretion of adipokines and cytokines has been implicated in the chronic low-grade state associated with obesity (Schindler et al.[5]; Moro et al.[6]). This issue should be considered in the discussion section and mentioned as a limitation of this study.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Jul 2018

Petros Dinas, University of Wolverhampton, Walsall, UK

We thank you very much for your comments. Please see below our responses to your comments:

Comment 1: Thank you for this suggestion. Indeed, this would have been a very interesting analysis to perform. Unfortunately, there are no samples available at this stage to perform the suggested work. Therefore, we have included this information in the limitations of the Discussion section (Page 6, paragraph 2).

Comment 2: We have added further details about blood pressure registers and we provide a relevant reference (24) in methods section (Page 4, paragraph 1).

Comment 3: As suggested, we have included this information in the limitations of the Discussion section and we provide the relevant reference (25). (Page 6, paragraph 2).

Comment 4: As suggested, we have added this information in the limitations along with the relevant references (26,27, 28,29). (Page 6, paragraph 2).

Competing Interests: No competing interests were disclosed.