

Artificial intelligence-enabled clinical phenotyping of human brown fat

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

Summary

In this study, Gomez-Banoy et al. explore the association between the presence/activity of brown adipose tissue (BAT) and clinical parameters/health record data in humans. Based on their previously published work (Becher et al. Nat Med 2021) the authors extended their initial patient cohort and eventually combined both cohorts to more than 100.000 patients of which almost 10% of patients were classified as BAT+. In line with previous knowledge, BAT prevalence decreased with age, higher ambient temperature, and increasing BMI. Using propensity score matching (PSM), BAT+ and BAT- cohorts were defined, matching for age, sex, BMI and ambient temperature. In this cohort, the association between BAT positivity and decreased prevalence of cardiometabolic diseases was confirmed and psychiatric diagnoses were also enriched in their association with BAT. BAT+ status was associated with higher incidence of skin diagnoses and osteoporosis. Next, the authors employed a new TriDFusion machine-learning algorithm to semi-automatically define total and depot-specific BAT metabolic activity. Whereas total BAT activity associated with reduced odds of type 2 diabetes, depot-specific BAT showed variable associations with diabetes and cardiometabolic parameters. Inverse associations between total BAT activity and glucose and systemic blood pressure were detected, whereas total BAT activity did not correlate with lipids, but BAT positivity associated with decreased markers of left ventricular remodeling. Assessing medications across the patient cohort, almost 40 medications associated with BAT status, including beta-blockers, glucocorticoids, and angiotensin receptor blockers (ARBs), commonly used drugs to lower blood pressure. ARB-treatment of primary brown adipocytes and wild-type mice led to an upregulation of UCP1 under these conditions, the effect of which appeared to be independent from angiotensin receptor 1 activation. Overall, the authors conclude that their AI and big data platforms may serve as tools for drug repurposing efforts in BAT activation in humans.

General Comments

Despite the current success of incretin-based weight loss drugs, the identification of efficient and sustainable measures against obesity remains a major challenge in biomedical research. While the efficacy of BAT activation to improve metabolism in rodent models has been well established, its relevance for human physiology and as target in anti-obesity efforts is still under debate. In this respect, Gomez-Banoy et al. explore an interesting and relevant field of research. The study benefits from the use of big data and machine learning tools in extensive human cohorts. The manuscript is well written and structured, and the conclusions are supported by experimental data. However, several major concerns remain: While the experimental approaches are state-of-the-art, the manuscript essentially remains a refinement/evolution of the author's previous study with many confirmatory data and approaches included (eg. main correlation between BAT and CVD, correlation with glucose/lipid parameters etc) so that the conceptual advance appears limited. In fact, the main advance seems to reside in the development of a semi-automatic method to determine BAT activity and the discovery of ARBs as potential upstream regulators of BAT activity in humans. These latter findings remain at a very preliminary level so that the manuscript appears more like an interesting resource rather than a major research advance. A further and in-depth mechanistic characterization of the impact of ARBs on whole body energy expenditure, futile cycling, and metabolite handling in animal models would significantly strengthen the case for publication by providing PoC for the discovery power of this human cohort approach beyond simple description. This holds particularly true for the fact that ARBs do not seem to work through the default ATR1 but via a completely obscure ATR1-independent mechanisms (even synergizing with beta

adrenergic signaling).

Referee #2

(Remarks to the Author)

Summary of the key results

The manuscript "Systems-level analysis of human brown fat: clinical phenotypes and pharmacological determinants" by Gómez-Banoy et al. describes the clinical impact of functionally active brown adipose tissue in a large cohort of patients, using big data and artificial intelligence tools. They were able to confirm the earlier findings that higher BAT activity is associated with lower odds of cardiometabolic diseases. Further, treatment with angiotensin receptor blocker losartan associated with BAT presence and losartan activated brown adipocytes.

Originality and significance: if not novel, please include reference

Majority of the extensive results are confirmatory to previous results by the same group, which slightly weakens the novelty of the findings in the manuscript.

Data & methodology: validity of approach, quality of data, quality of presentation

An important question regarding the data is, whether BAT activity is either positive, existing BAT (BAT+) or negative, nonexisting BAT (BAT-). More advanced approach would be to handle BAT activity as a continuous variable without a cut point or visibility vs. nonvisibility in PET scan.

It is mentioned on page 8, line 172 that "we utilized quantitative BAT data from our AI tool". It is noteworthy that SUV data is semiquantitative and does not convert to quantitative by multiplying with tissue mass or with the use of AI tool.

Throughout the manuscript, it is not constantly clear whether the authors mean with "BAT" a mass of BAT or the activity based on FDG uptake. The assumption is, however, that it means "activity" since there is no accurate tool to measure the mass of BAT.

In vitro experiments: Differentiated brown adipocytes were used for the experiments of losartan effect. In addition, immortalized mature brown adipocytes and primary mature brown adipocytes are mentioned on page 11. Do the authors mean the same cell types with all of these? Are the primary cells from known cell lines, or from animal or human origin? Rodent mature brown adipocytes may differ significantly from the human brown/beige adipocytes. While vehicle was used along with losartan, it would be good to show also the same effects of losartan and vehicle in white adipocytes, and if possible with beige adipocytes.

Appropriate use of statistics and treatment of uncertainties

Statistics are appropriately conducted. For cancer, the site, stage and treatment were included to the statistical analysis, but could disease duration have impact on BAT function?

Conclusions: robustness, validity, reliability

In order to understand the importance of the impact of BAT to clinical outcomes, associations may present somewhat misleading interpretations in some cases. For example, earlier preclinical studies have shown that brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development (Berbée JF et al. Nature Commun 2015 PMID: 25754609). In the current study BAT+ patients had higher total cholesterol and low-density lipoprotein cholesterol (LDL-C). Do the authors have any explanation for this? This question would require more specific and targeted approach to understand the controversy to earlier findings.

BAT activity is reported to associate with lower rates of psychiatric diseases, depression and anxiety. However, olanzapine, an antipsychotic drug, had a positive association with BAT presence, and serotonin re-uptake inhibitors used for depressive symptoms, negatively regulate BAT function. These controversies require clarification.

Suggested improvements: experiments, data for possible revision

It remains unclear to this reviewer whether the authors wish to emphasize AI to promote the extensive findings or the proof-of-concept experiments. Please clarify. Maybe the approaches could be divided into two separate stories.

Use of antibiotics was excluded because of typical short-term use. Since antibiotics may have more profound and longer term effects on microbiota, which in turn may have effects on BAT activity, it would be important to reanalyze the data with the use of antimicrobials.

References: appropriate credit to previous work?

The authors have meritoriously given credit and referred to previous work. Perhaps the only work that would have also earned the credit in Introduction, "Since the landmark reports that adult humans have inducible BAT in 2009 8-10", is the report by Saito et al. Diabetes 2009, PMID: 19401428. Practically, the studies were carried out at the same time with the other three publications in NEJM.

Referee #3

(Remarks to the Author)

This retrospective study in a very large cohort of patients investigates the role of brown adipose tissue (BAT) in human health, leveraging big data and AI to investigate associations of BAT with a wide range of diseases, metabolic parameters, and drug intake. Based on the analysis of FDG-PET/CT scans, the authors quantified BAT activity and examined its links to cardiometabolic diseases, psychiatric conditions, and liver function. The authors report that individuals with detectable BAT on PET showed lower prevalence of type 2 diabetes, ischemic heart disease, hypertension, and psychiatric disorders like anxiety and depression. These associations persisted even after adjusting for age, sex, BMI, and ambient temperature.

Several prior studies (including animal studies) have already shown that BAT may have a protective function in terms of development of obesity and metabolic diseases – thus, the topic is clearly of clinical relevance.

The main strengths of the current study are

(a) its very large cohort size of >100,000, compared to the largest comparable BAT study in human adults published in NEJM in 2009, which had <10,000.

(b) the use of a previously published, dedicated nnU-Net for BAT quantification on PET, which allowed investigation of depot-specific effects, revealing for example that higher BAT activity in the axillary and mediastinal regions was linked to improved metabolic outcomes.

(c) strong supporting evidence for the previously suggested activation of BAT by drugs, in particular angiotensin receptor blockers, which may enable fat repurposing. The caveat here is the trend towards higher osteoporosis prevalence in BAT+ individuals in this study.

There are very few actual drawbacks if the study - the retrospective design, for example, is difficult to avoid in studies of that size, but the authors are clearly aware that some of their residents require prospective validation.

My main suggestions would be to:

1. Focus a bit more on the potential bias introduced by using exclusively data from a cancer center, where many patients received systemic drugs - what data are currently available for BAT in cancer patients, as compared to BAT in healthy individuals and patients without cancer? Also discuss the observed differences between BAT+ and BAT- rates (Table 1) for the most prominent examples, e.g. patients with breast cancer and patients treated with mitotic inhibitors.
2. Discuss the potential link between osteoporosis and BAT in more detail - what is the weighing hypothesis here? Could this be a cancer population-specific finding?

Referee #4

(Remarks to the Author)

GENERAL COMMENTS :

An interesting paper reporting the results of big data and artificial intelligence (AI) analyses conducted on a very large convenience sample of patients followed at a well-known academic cancer center subjected to FDG-PET/CT imaging. Analyses are state-of-the-art and have been produced by a competent academic team. The paper reports a considerable amount of work. As mentioned by the authors themselves, the paper is an extension of their previous work in going beyond the report of associations between the presence or absence of brown adipose tissue (BAT) and cardiometabolic risk variables (their previous paper published in Nature Medicine in 2021).

In the section below, this reviewer has raised several questions and comments with the hope of being helpful to this qualified team of investigators. My main criticism is causality when the relationships between BAT activity and clinical outcomes are reported. As this is a convenience cohort of patients followed at a cancer center, I respectfully consider that it is premature to consider pharmacological targets to improve BAT volume and activity with the hope of generating clinical benefits. The preliminary work on ARBs is not at par with the nice and novel AI-derived analyses reported in the present study. I would strongly recommend to publish that part of the paper (in vitro studies) elsewhere and focus on novel associations (and interpret them as associations).

SPECIFIC COMMENTS:

1. TITLE: Please modify the title to be clear on the type of cohort from which results were derived (patients screened for cancer). This is not a population-based study. There is nothing wrong with that but it should be stated clearly.
2. SUMMARY: ... "full clinical impact of BAT"... be careful with that type of statement. The section relevant to ARBs is premature (see general comments).
3. INTRODUCTION: Obesity is heterogeneous. You should refer to obesity phenotypes. Your second statement about adipose tissue dysfunction is more appropriate.
4. Because of its heterogeneity, would it be possible for this competent team to use AI to identify obesity phenotypes (i.e. subcutaneous vs visceral obesity) that could possibly be related to different BAT phenotypes? Would this be possible to generate that type of additional data from the already collected images?
5. Since sex is an important determinant of differences in both white adipose tissue (WAT) distribution and BAT mass/activity, it would be important to conduct the analyses by sex rather than adjusting for sex. Indeed, pooling men and women is questionable for that type of analyses considering the sex dimorphism in WAT and BAT distribution, as well as in

cardiometabolic risk.

6. L.119: replace "non-insulin dependent diabetes » by type 2 diabetes.

7. WAT distribution is related to depression and anxiety disorders, idem for sleep apnea. It is therefore of no surprise that individuals with type 2 diabetes with less BAT (and more abdominal visceral WAT) would show a greater prevalence of these conditions. The link between type 2 diabetes and these disorders is well established.

8. The findings on the distribution of the six BAT depots and clinical outcomes are quite novel and interesting. Considering the previous comments, it would be very interesting to probe the possible associations of these BAT depots with WAT distribution.

9. L.160: "... specific benefits derived from this depot..." Please, rephrase. No evidence of a causal relationship.

10. The section on AI-derived BAT activity vs. cardiometabolic risk variables is very interesting and novel and should be the main focus of the paper.

11. Pharmacological regulators of BAT: Please keep in mind that that, although large, this cohort is a convenience sample of patients followed at a cancer center. If you want to keep this section included the paper, you should remain very prudent in reporting these exploratory analyses. For instance, dexamethasone is positively associated with BAT presence!

12. DISCUSSION: The discussion is too speculative and sometimes falls in the trap of suggesting causality. I would respectfully suggest to these competent investigators to focus on the original, novel and interesting findings derived from their AI-generated analyses. If the heterogeneity in BAT activity (the 6 depots) could be related to the heterogeneity in WAT phenotypes and linked to health outcomes, this paper would make a very novel contribution to the already available literature on the topic.

13. This reviewer has tremendous respect for this established team of investigators and the above comments are made in a constructive spirit with the hope of improving the quality of this paper.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

In their revised version, the authors have improved the manuscript by the addition of new data. However, I am still concerned about the degree of conceptual advance as compared with their original publication in Nat. Med.

In addition, while they added new data on the mechanism of Losartan, there are still some inconsistencies in these experiments that require attention:

a) Reviewer Fig 1: There is inconsistent color coding of bars with respect to Losartan/NE treatments. Pls double check.

b) In contrast to Reviewer Fig 4, Losartan treatment in Reviewer Fig 1 triggers aP2 expression as a single treatment while Losartan exposure in vivo leaves aP2 levels unaffected. This also contrasts its effect on UCP1 expression which is also induced by single Losartan exposure in isolated cells (Reviewer Fig 3). Thus, there is inconsistency between in vitro and in vivo data.

c) Reviewer Fig 4: The impact of Losartan treatment on BAT gene programs is rather unimpressive at the RNA level. Please provide WB to assess Losartan effects on protein expression levels.

d) Reviewer Fig 5: Please also show body weight development upon Losartan exposure. If EE is elevated with no effects on food intake and locomotor activity, it would be interesting to see the actual impact on body weight.

e) Reviewer Fig 6: Here Losartan only shows an effect upon elevated beta adrenergic signaling (= cold) which again is in contrast to Reviewer Fig. 1.

While the new data are interesting, they currently still do not provide PoC for the suggested ability of this AI-based approach to identify novel targets for BAT activation but rather remain rather preliminary in nature.

Referee #2

(Remarks to the Author)

The authors have responded properly to the concerns raised by this reviewer. Additional work load by the group to improve the manuscript is greatly appreciated.

Referee #3

(Remarks to the Author)

Thank you for your revisions. I have no further comments.

Referee #4

(Remarks to the Author)

The authors have adequately addressed all my specific questions. The limitations of the study are better addressed and some of the added results have increased the level of originality of the paper.

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have adequately addressed all remaining questions.

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Summary of authors' response:

We respectfully submit this response to the reviewers' critiques of our manuscript. We appreciate their thorough evaluation and recognize the constructive spirit in which the comments were provided. Importantly, none of the reviewers raised concerns about the quality of the experimental design and data or the core claims we made. On the contrary, there was broad acknowledgment of the rigor and comprehensiveness of our approach.

The primary point of discussion centered on the novelty of our findings. While this study benefits from the expansion of a previously published cohort, we report multiple, key novel findings that we strongly feel will change how the field thinks about human brown fat:

1. This is the first study that examines associations of brown fat with a comprehensive spectrum of adult diseases. This allowed us to confirm in an unbiased manner that diagnoses in the category of cardiovascular disease are significantly associated with BAT, while also uncovering a completely novel link with psychiatric disorders. Moreover, we report associations between > 500 individual diagnostic codes and BAT status.
2. We report for the first time the utilization of an AI-based tool to semi-automatically measure BAT metabolic activity across each described depot in thousands of patients. This allowed us to extensively describe depot-specific associations with diagnostic codes and a variety of clinical traits.
3. While previous studies (including ours) reported exploratory (uncontrolled) associations between a handful of laboratory studies and BAT, here we perform rigorously controlled statistical analyses on a wide variety of laboratory tests, clinical indices, and imaging studies. These include common laboratory and diagnostic tests used to assess metabolic, immune, cardiac, liver, and bone pathology.
4. We report associations between > 120 individual medications and BAT status with the goal of discovering approved medications that could be repurposed to activate BAT in humans. We are not aware of any prior study in our field that has applied such a comprehensive approach, with previous studies examining at most a handful of general medication classes. Our findings led us to postulate that angiotensin receptor blockers (ARBs) are potential BAT activators, which is supported by our proof-of-concept and mechanistic experiments in brown adipocytes and mice.

In response to specific reviewer feedback:

- **Reviewer 1** requested further characterization of ARBs on whole body energy expenditure, as well as exploring a potential angiotensin receptor 1 (AT1R) independent mechanism. We now present extensive, new experimental data demonstrating that ARBs activate brown adipocytes in a PPAR γ -dependent manner. We have also done detailed metabolic phenotyping of mice treated with losartan. We feel the inclusion of these data, as suggested by the reviewer, greatly strengthens the manuscript.
- **Reviewer 2** requested clarification on the definition of BAT activity and its use as a quantitative or semiquantitative variable. The reviewer also wanted further

analyses/discussion on some controversial findings regarding BAT status, cholesterol levels, and psychiatric diseases. We have performed extensive additional analyses to address these key points.

- **Reviewer 3** requested additional discussion on the fact that our patients come from a cancer center, as well as our finding that BAT associates with higher rates of osteoporosis. We have revised our text to address these questions and have proposed potential mechanisms underlying these findings.
- **Reviewer 4** offered extensive comments rooted in the fact that our patients come from a convenience cohort followed at a cancer center and it is therefore premature to propose our medication analyses as a potential tool for drug repurposing. Reviewer 4 considered the proof-of-concept experiments on ARBs to be preliminary and not at par to the rest of the work. We agree with the reviewer that our cohort represents a convenience cohort but have also explained why large prospective studies of BAT in humans are currently not feasible. Having said that, our medication analyses match and confirm findings from multiple published prospective human studies. This provides proof that our studies offer the opportunity to identify clinically approved drugs that activate BAT. While reviewer 4 did not propose additional experiments, we have significantly complemented our ARB studies to demonstrate that these drugs do indeed activate BAT, highlighting the value of our medication analysis as a means to potentially repurpose drugs to activate BAT. We have also performed extensive new analyses and established novel associations between BAT activity and WAT distribution and phenotypes.

For these reasons, we believe our study makes a much needed and substantial contribution and warrants publication. The brown fat field is lacking large studies done in humans, and our work provides a firm foundation anchored in human biology that we believe will open up many new areas of investigation for both basic and translational researchers.

Below you will find a point-by-point response to the reviewers' concerns. Reviewers' comments are displayed in italics, followed by our response in standard font.

Referee #1 (Remarks to the Author):

Summary

In this study, Gomez-Banoy et al. explore the association between the presence/activity of brown adipose tissue (BAT) and clinical parameters/health record data in humans. Based on their previously published work (Becher et al. Nat Med 2021) the authors extended their initial patient cohort and eventually combined both cohorts to more than 100.000 patients of which almost 10% of patients were classified as BAT+. In line with previous knowledge, BAT prevalence decreased with age, higher ambient temperature, and increasing BMI. Using propensity score matching (PSM), BAT+ and BAT- cohorts were defined, matching for age, sex, BMI and ambient temperature. In this cohort, the

association between BAT positivity and decreased prevalence of cardiometabolic diseases was confirmed and psychiatric diagnoses were also enriched in their association with BAT. BAT+ status was associated with higher incidence of skin diagnoses and osteoporosis. Next, the authors employed a new TriDFusion machine-learning algorithm to semi-automatically define total and depot-specific BAT metabolic activity. Whereas total BAT activity associated with reduced odds of type 2 diabetes, depot-specific BAT showed variable associations with diabetes and cardiometabolic parameters. Inverse associations between total BAT activity and glucose and systemic blood pressure were detected, whereas total BAT activity did not correlate with lipids, but BAT positivity associated with decreased markers of left ventricular remodeling. Assessing medications across the patient cohort, almost 40 medications associated with BAT status, including beta-blockers, glucocorticoids, and angiotensin receptor blockers (ARBs), commonly used drugs to lower blood pressure. ARB-treatment of primary brown adipocytes and wild-type mice led to an upregulation of UCP1 under these conditions, the effect of which appeared to be independent from angiotensin receptor 1 activation. Overall, the authors conclude that their AI and big data platforms may serve as tools for drug repurposing efforts in BAT activation in humans.

General Comments

Despite the current success of incretin-based weight loss drugs, the identification of efficient and sustainable measures against obesity remains a major challenge in biomedical research. While the efficacy of BAT activation to improve metabolism in rodent models has been well established, its relevance for human physiology and as target in anti-obesity efforts is still under debate. In this respect, Gomez-Banoy et al. explore an interesting and relevant field of research. The study benefits from the use of big data and machine learning tools in extensive human cohorts. The manuscript is well written and structured, and the conclusions are supported by experimental data. However, several major concerns remain: While the experimental approaches are state-of-the-art, the manuscript essentially remains a refinement/evolvment of the author's previous study with many confirmatory data and approaches included (eg. main correlation between BAT and CVD, correlation with glucose/lipid parameters etc) so that the conceptual advance appears limited. In fact, the main advance seems to reside in the development of a semi-automatic method to determine BAT activity and the discovery of ARBs as potential upstream regulators of BAT activity in humans. These latter findings remain at a very preliminary level so that the manuscript appears more like an interesting resource rather than a major research advance. A further and in-depth mechanistic characterization of the impact of ARBs on whole body energy expenditure, futile cycling, and metabolite handling in animal models would significantly strengthen the case for publication by providing PoC for the discovery power of this human cohort approach beyond simple description. This holds particularly true for the fact that ARBs do not seem to work through the default ATR1 but via a completely obscure ATR1-independent mechanisms (even synergizing with beta adrenergic signaling).

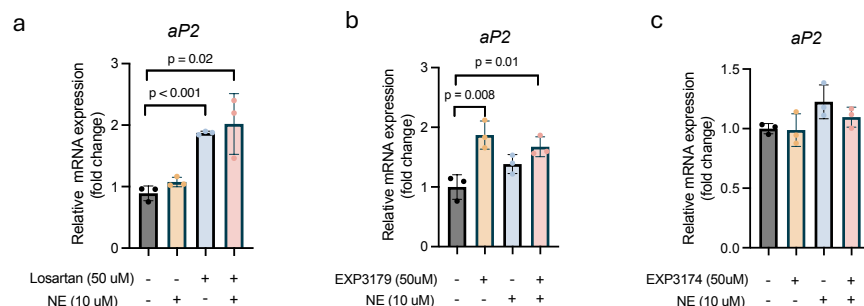
Author response:

We thank the reviewer for acknowledging our experimental approaches as “state of the art” and that the conclusions are supported by the experimental data. We respectfully disagree with the reviewer that the main advance of the manuscript is limited to our AI tool and the discovery of ARBs as potential regulators of BAT. There are several substantial advances to the field that are included in the manuscript as mentioned below:

1. This is the first study that examines associations of brown fat with a comprehensive spectrum of adult diseases. This allowed us to confirm in an unbiased manner that diagnoses in the category of cardiovascular disease are significantly associated with BAT, while also uncovering a completely novel link with psychiatric disorders. Moreover, we report associations between > 500 individual diagnostic codes and BAT status.
2. More than just the development of the AI tool to measure BAT activity, it is really the analyses that this tool allowed us to do that constitute a significant advance for the field. The depot-specific analyses demonstrate that specific locations of BAT are differentially important for individual health outcomes. It also demonstrates that the degree of BAT metabolic activity is important, as it correlates with health outcomes (e.g. diabetes diagnosis) and clinical traits (blood pressure measurements, echocardiographic measurements).
3. Our systems approach allowed us to analyze associations between BAT and > 120 medications, as well as >30 commonly assessed clinical parameters, laboratory values, and imaging studies. This uncovered multiple novel associations, including the observation that ARB usage is associated with the presence of BAT.

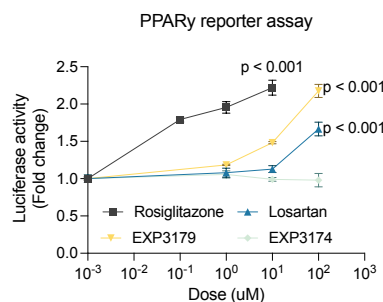
We agree with the reviewer that further experiments in animal models and the assessment of the mechanism behind the activation of BAT by ARBs will significantly increase the scope of our manuscript and provide additional novelty. To that point, we now include extensive new experiments in brown adipocytes to demonstrate that losartan and its ‘inactive metabolite’, EXP3179, promote *Ucp1* expression via PPAR γ . We also add data from comprehensive metabolic phenotyping of animals treated with losartan vs. vehicle control in the context of cold exposure.

Mechanism of action. To directly address the reviewer’s comment regarding the mechanism through which losartan and its metabolite EXP3179 affect *Ucp1* levels, we reviewed available literature and found work in 3T3-L1 adipocytes suggesting that some ARBs could function as partial PPAR γ agonists. We therefore hypothesized that losartan and its inactive metabolite EXP3179 could promote *Ucp1* transcription through a PPAR γ dependent mechanism in mature brown adipocytes. We found that losartan and its inactive metabolite EXP3179, but not the active metabolite EXP3174, increased the transcription of the classic PPAR γ target *aP2* with and without norepinephrine stimulation (Reviewer Response, Figure 1). These data are included in Extended Data Figure 14.



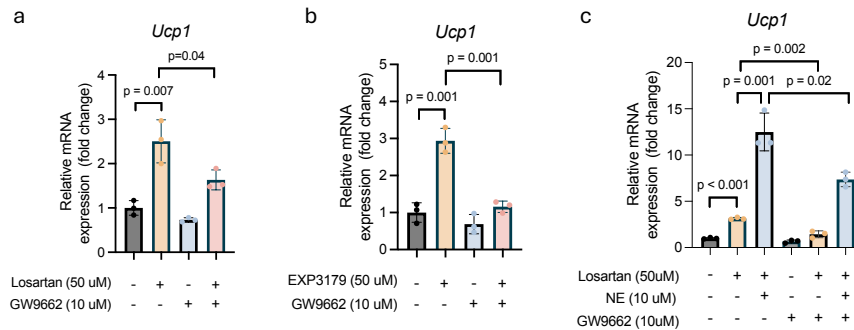
Reviewer Figure 1. a-c *aP2* mRNA levels quantified by qPCR in mature immortalized brown adipocytes pre-treated with a) losartan b) EXP3179 c) EXP3174 versus vehicle for 24 hours and then subjected to norepinephrine (NE) vs vehicle stimulation for 6 hours.

To assess whether losartan and its inactive metabolite EXP3179 activate PPAR γ transcriptional activity directly, we transfected HEK293T cells (low PPAR γ expression at baseline) with PPAR γ and a PPAR γ response element (PPRE) luciferase reporter construct. We then treated cells with increasing concentrations of losartan, EXP3179, EXP3174 and rosiglitazone as a positive control. As expected, we saw a significant increase in reporter activity with rosiglitazone even at minimal doses (100 nM), reaching a 2.2-fold increase at 10 uM. Moreover, we saw a significant, dose-dependent increase in reporter activity following EXP3179 (1.5-fold at 10 uM, 2.2-fold increase at 100 uM). Treatment with losartan also resulted in a significant increase in reporter activity, with a minimal increase (1.1-fold) at 10 uM, followed by a 1.7-fold increase with 100 uM dose. On the other hand, treatment with EXP3174 resulted in no measurable reporter activity (Reviewer Response, Figure 2). These data are included in Figure 4.



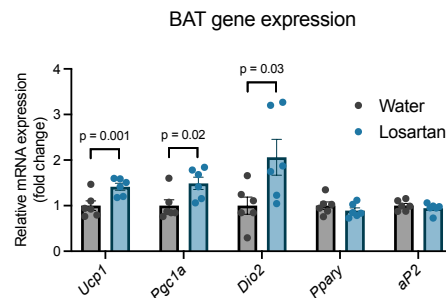
Reviewer Figure 2. Transcriptional activity of PPRE was measured via luciferase assays in HEK293T cells following treatment with increasing concentrations of Rosiglitazone, Losartan, EXP3179, and EXP3174.

To directly test whether PPAR γ activation mediates the increase in *Ucp1* expression observed with losartan and EXP3179 in brown adipocytes, we co-treated these cells with our compounds and a pharmacological inhibitor of PPAR γ , GW9662, which binds the ligand binding domain preventing agonist-induced activation. Blocking PPAR γ significantly attenuated the observed effects of losartan and its inactive metabolite on *Ucp1* mRNA induction at baseline and upon NE stimulation (Reviewer Figure 3). These data are included in Figure 4 and Extended Data Figure 14.



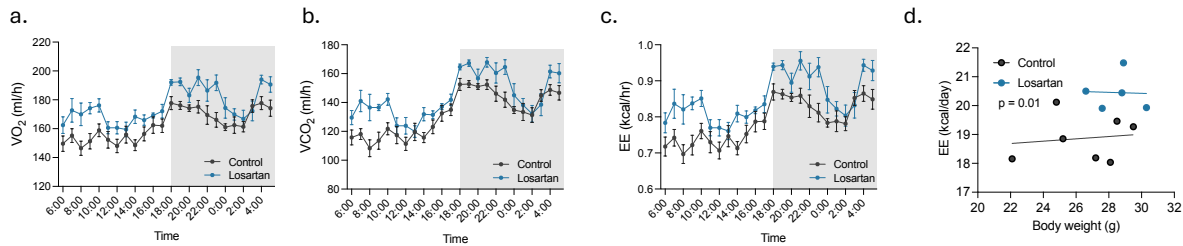
Reviewer Figure 3. a-c *Ucp1* mRNA levels quantified by qPCR in mature brown adipocytes pre-treated with GW9662 or vehicle for 24 hours and then stimulated with a) losartan, b) EXP3179, or c) losartan +/- NE.

Treatment with losartan *in vivo*. To perform a deeper characterization of the effects of losartan in animal models, we performed new *in vivo* studies in which we provided the medication in drinking water to WT mice and performed longer term experiments, which better model chronic drug exposure in humans. We confirmed that providing the medication for 2 weeks at room temperature significantly increased the expression of *Ucp1* and other thermogenic markers in BAT of WT mice (Reviewer Figure 4).



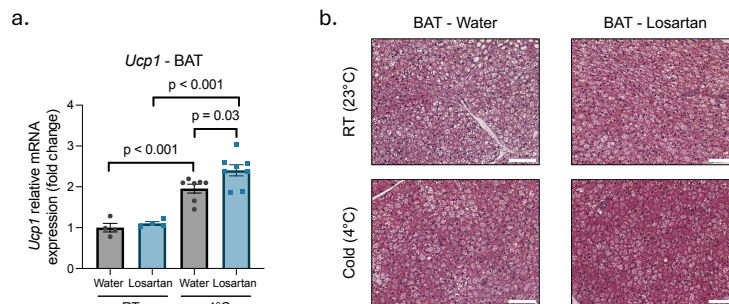
Reviewer Figure 4. mRNA levels of thermogenic/adipogenic markers in BAT from mice treated with losartan in drinking water (100 mg/kg/day) or control for 2 weeks.

We then investigated the effects of losartan treatment on BAT activity *in vivo*. WT mice pre-treated with losartan in drinking water for 10 days were challenged with cold exposure for 5 days and energy expenditure (EE) was assessed via indirect calorimetry. Losartan-treated mice exhibited increased O₂ consumption, CO₂ production, and EE compared to control mice (Reviewer Figure 5). This was independent of body weight, as assayed by ANCOVA analysis (Reviewer Figure 5). There were no differences in food intake, water consumption, RER, and locomotor activity between losartan-treated and control mice (Not shown here, in Extended Data Fig. 16).



Reviewer Figure 5. a-d. 10-week-old B6J WT mice were treated with losartan in their drinking water (100 mg/kg/day) or regular water for 10 days at room temperature (RT) and then challenged with cold temperature (4°C) for 5 days. Representative 24-hour periods during the cold challenge are shown. a) Oxygen consumption (VO₂). b) Carbon dioxide production (CO₂). c) Energy expenditure (EE). d) Regression analysis of EE vs body weight.

Moreover, cold exposure in mice treated with losartan resulted in a significant increase in their BAT *Ucp1* expression (Fig. 4I), as well as smaller lipid droplets (Reviewer Figure 6). We observed similar histologic findings in the inguinal fat of losartan-treated versus control animals, however there was no statistical increase in *Ucp1* expression (Not shown here, in Extended Data Fig. 16). Altogether, these new data confirm that treatment with losartan results in BAT activation and an increase in whole-body EE, and they have been included in Figure 4 and Extended Data Figure 16.



Reviewer Figure 6. a-b. WT mice treated with losartan or water at basal room temperature (RT) or after 5-day cold exposure were sacrificed and tissues harvested a) *Ucp1* mRNA levels quantified by qPCR in whole BAT. b) Representative images of H&E staining of BAT.

Referee #2 (Remarks to the Author):

Summary of the key results

The manuscript "Systems-level analysis of human brown fat: clinical phenotypes and pharmacological determinants" by Gómez-Banoy et al. describes the clinical impact of functionally active brown adipose tissue in a large cohort of patients, using big data and artificial intelligence tools. They were able to confirm the earlier findings that higher BAT activity is associated with lower odds of cardiometabolic diseases. Further, treatment with angiotensin receptor blocker losartan associated with BAT presence and losartan activated brown adipocytes.

Originality and significance: if not novel, please include reference

Majority of the extensive results are confirmatory to previous results by the same group, which slightly weakens the novelty of the findings in the manuscript.

Author response:

We thank the reviewer for the constructive comments. We respectfully disagree that the majority of the results are confirmatory, and below we include a list of completely novel findings not reported in our previous publication:

1. This is the first study that examines associations of brown fat with a comprehensive spectrum of adult diseases. This allowed us to confirm in an unbiased manner that diagnoses in the category of cardiovascular disease are significantly associated with BAT, while also uncovering a completely novel link with psychiatric disorders. Moreover, we report associations between > 500 individual diagnostic codes and BAT status.
2. We report for the first time the utilization of an AI-based tool to semi-automatically measure BAT metabolic activity across described depots in thousands of patients. This allowed us to extensively describe depot-specific associations with diagnostic codes and a variety of clinical traits. We demonstrate that specific locations of BAT could be differentially important for individual health outcomes. We also show that the degree of BAT metabolic activity is important, and it correlates with health outcomes (e.g. diabetes diagnosis) and clinical traits (blood pressure measurements, echocardiographic measurements).
3. While previous studies (including ours) reported exploratory (uncontrolled) associations between a handful of laboratory studies and BAT, here we perform rigorously controlled statistical analyses on a wide variety (>30) of laboratory tests, clinical indices, and imaging studies. These cover common clinical parameters to assess and diagnose metabolic, immune, cardiac, liver, and bone pathology.
4. We report associations between > 120 individual medications and BAT status with the hopes of discovering approved medications that could be repurposed to activate BAT in humans. We are not aware of any other studies using such a comprehensive approach to screen for candidate BAT modulators, with previous studies at most examining a handful of general medication classes. Our findings led us to postulate that angiotensin receptor blockers (ARBs) are potential BAT activators, which is supported by our proof-of-concept experiments in brown adipocytes and mice.

Data & methodology: validity of approach, quality of data, quality of presentation

An important question regarding the data is, whether BAT activity is either positive, existing BAT (BAT+) or negative, nonexisting BAT (BAT-). More advanced approach would be to handle BAT activity as a continuous variable without a cut point or visibility vs. nonvisibility in PET scan.

Author response: We thank the reviewer for this comment and opportunity to clarify. We measured the level of BAT metabolic activity using our AI tool in patients that were initially classified as BAT positive based their PET scan read. Once we established BAT metabolic activity in these >8,700 patients, we treated it as a continuous variable and divided activity into tertiles for some analyses (e.g. association with diagnostic codes) or compared it to other continuous variables (e.g. blood pressure readings).

It is mentioned on page 8, line 172 that “we utilized quantitative BAT data from our AI tool”. It is noteworthy that SUV data is semiquantitative and does not convert to quantitative by multiplying with tissue mass or with the use of AI tool.

Author response: We thank the reviewer for this comment and agree SUV data is not quantitative but rather semiquantitative. We have changed the terminology throughout the manuscript.

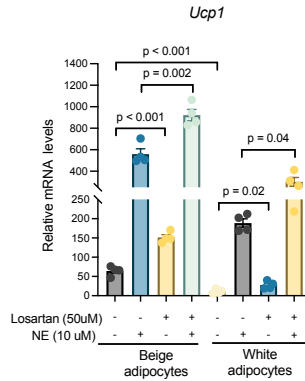
Throughout the manuscript, it is not constantly clear whether the authors mean with “BAT” a mass of BAT or the activity based on FDG uptake. The assumption is, however, that it means “activity” since there is no accurate tool to measure the mass of BAT.

Author response: We thank the reviewer for this comment and opportunity to clarify. The reviewer is correct that when we refer to BAT, we are referring to BAT activity based on FDG uptake. We have clarified this throughout the manuscript.

In vitro experiments: Differentiated brown adipocytes were used for the experiments of losartan effect. In addition, immortalized mature brown adipocytes and primary mature brown adipocytes are mentioned on page 11. Do the authors mean the same cell types with all of these? Are the primary cells from known cell lines, or from animal or human origin? Rodent mature brown adipocytes may differ significantly from the human brown/beige adipocytes. While vehicle was used along with losartan, it would be good to show also the same effects of losartan and vehicle in white adipocytes, and if possible, with beige adipocytes.

Author response: We thank the reviewer for this comment and suggestions. The cells utilized in this study are all murine, and we utilize either an immortalized brown adipocyte cell line or primary *in vitro* differentiated brown adipocytes from 4–5-week-old mice. We have now made sure to clarify which cells were utilized for specific experiments.

We agree with the reviewer that it would be interesting to assess the effects of losartan on white/beige adipocytes. To do so, we isolated the stromal vascular fraction (SVF) from the inguinal region (enriched in beige adipocyte progenitor cells) and the dorsolumbar region (enriched in white adipocyte progenitor cells) from the inguinal subcutaneous fat depot of 4-week-old mice. We then differentiated these cells into mature adipocytes and treated them with losartan and control, with and without NE stimulation, and measured *Ucp1* transcription. As expected, baseline *Ucp1* expression was higher in cells derived from the inguinal region compared to the dorsolumbar region, confirming beige adipocyte enrichment. We found that losartan treatment stimulated *Ucp1* expression in both beige and white adipocytes (Reviewer Figure 7). Interestingly, the effects of losartan on *Ucp1* expression were more significant in beige compared to white adipocytes. These data were included in Extended Data Figure 13.



Reviewer Figure 7. *Ucp1* mRNA levels quantified by qPCR in white and beige adipocytes treated with losartan and control, in the presence or absence of NE.

Appropriate use of statistics and treatment of uncertainties

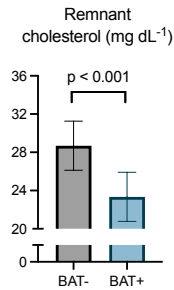
Statistics are appropriately conducted. For cancer, the site, stage and treatment were included to the statistical analysis, but could disease duration have impact on BAT function?

Author response: We thank the reviewer for this comment. Unfortunately, our current data extraction approach from the electronic health record does not allow us to accurately extract data pinpointing disease duration.

Conclusions: robustness, validity, reliability

In order to understand the importance of the impact of BAT to clinical outcomes, associations may present somewhat misleading interpretations in some cases. For example, earlier preclinical studies have shown that brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development (Berbée JF et al. Nature Commun 2015 PMID: 25754609). In the current study BAT+ patients had higher total cholesterol and low-density lipoprotein cholesterol (LDL-C). Do the authors have any explanation for this? This question would require more specific and targeted approach to understand the controversy to earlier findings.

Author response: We agree with the reviewer that the finding of higher LDL-c in BAT positive patients is paradoxical, given their lower odds of CAD. To address this, we calculated the remnant cholesterol value (Total Cholesterol – LDL – HDL), which has been shown to be an independent risk factor for atherosclerotic cardiovascular disease (PMID: 37439258, 41260759). After adjusting for age, sex, BMI, race, ethnicity, ambient temperature, cancer site and stage, cancer treatment, and outpatient lipid lowering medication usage, we found remnant cholesterol is significantly lower in BAT+ patients (23.34 ± 2.57 mg/dL) when compared to BAT- patients (28.69 ± 2.57 mg/dL). These data are included in Extended Figure 7.



Reviewer Figure 8. Comparison of calculated remnant cholesterol in BAT negative (BAT-) versus BAT positive (BAT+) patients.

BAT activity is reported to associate with lower rates of psychiatric diseases, depression and anxiety. However, olanzapine, an antipsychotic drug, had a positive association with BAT presence, and serotonin re-uptake inhibitors used for depressive symptoms, negatively regulate BAT function. These controversies require clarification.

Author response: We thank the reviewer for this observation. To address this, we performed additional medication-specific analyses focusing on psychiatric medications, adjusting for pertinent diagnoses such as depression, anxiety, and psychosis, in addition to the covariates adjusted for in our initial analyses.

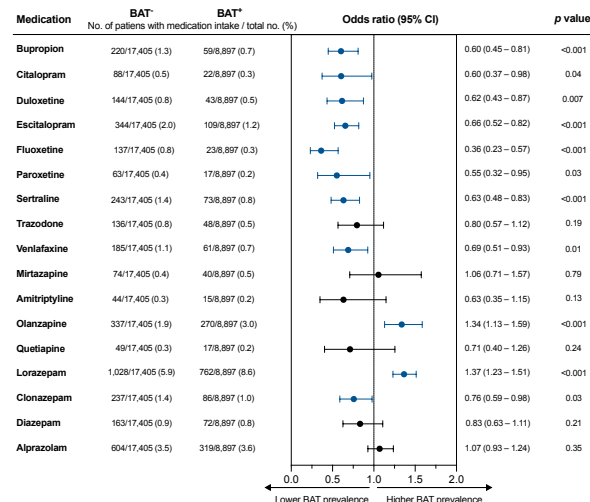
Psychiatric diagnoses were organized as follows (Included as Supplementary Table 7):

Diagnosis category	Diagnoses (ICD-10)
Depression	Depressive episode (F32) Recurrent depressive disorder (F33)
Anxiety	Other anxiety disorders (F41)
Psychosis	Acute and transient psychotic disorder (F23)

We found that even after controlling for psychiatric diagnoses, most psychiatric medications remain significantly associated with lower BAT prevalence (Reviewer Figure 9). Specifically, selective serotonin reuptake inhibitors (SSRIs) were consistently associated with reduced BAT prevalence, including citalopram (OR 0.60; 95% CI 0.37-0.98; $p = 0.04$), escitalopram (OR 0.66; 95% CI 0.52-0.82; $p < 0.001$), fluoxetine (OR 0.36; 95% CI 0.23-0.57; $p < 0.001$), paroxetine (OR 0.55; 95% CI 0.32-0.95; $p = 0.03$), and sertraline (OR 0.63; 95% CI 0.48-0.83; $p < 0.001$). Similarly, serotonin-norepinephrine reuptake inhibitors (SNRIs) showed inverse associations with BAT prevalence, including venlafaxine (OR 0.69; 95% CI 0.51-0.93; $p = 0.01$) and duloxetine (OR 0.62; 95% CI 0.43-0.87; $p = 0.007$). The norepinephrine/dopamine reuptake inhibitor (NDRI) bupropion was also associated with lower BAT prevalence (OR 0.60; 95% CI 0.45-0.81; $p < 0.001$).

In contrast, among antipsychotic medications, the dopamine D2/serotonin 5-HT_{2A} receptor antagonist olanzapine remained significantly associated with higher BAT prevalence even after adjustment for the pertinent diagnosis (OR 1.34; 95% CI 1.13-1.59;

$p < 0.001$), whereas quetiapine showed no significant association. We did not observe a uniform direction of effect across benzodiazepines; lorazepam was associated with higher BAT prevalence (OR 1.37; 95% CI 1.23-1.51; $p < 0.001$), while clonazepam was associated with lower BAT prevalence (OR 0.76; 95% CI 0.59-0.98; $p = 0.03$). We have included these data in Extended Data Figure 12.



Reviewer Figure 9. Forest plot of odds ratios for the association between antidepressants, anxiolytics, and antipsychotics usage and BAT status in a propensity score matched (PSM) cohort. The odds ratios were obtained with the use of a logistic regression after adjustment for age, sex, BMI, temperature, ethnicity, race, smoking history, family history of cardiovascular disease, cancer sites, cancer stage, cancer treatment, cardiovascular diagnoses, and psychiatric diagnoses.

Suggested improvements: experiments, data for possible revision

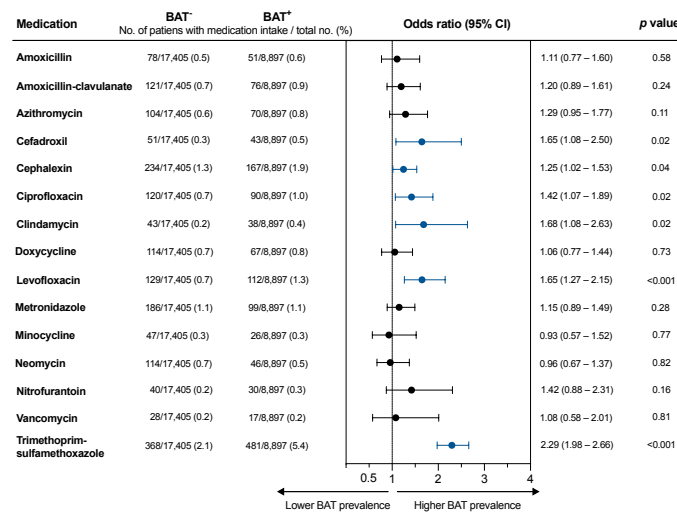
It remains unclear to this reviewer whether the authors wish to emphasize AI to promote the extensive findings or the proof-of-concept experiments. Please clarify. Maybe the approaches could be divided into two separate stories.

Author response: We thank the reviewer for this suggestion. To clarify, our goal is to use a systems level approach to analyze the full extent of clinical outcomes associated with BAT. Since our cohort is retrospective and therefore establishing causality is not possible, we further investigated the association between BAT presence and ARB use to demonstrate that our platform can yield biologically valid observations. We then performed additional experiments on ARBs to further demonstrate these medications could be repurposed to activate BAT. In accordance with Reviewers #1 and #4 we have performed additional mechanistic and *in vivo* experiments to complement this proof-of-concept.

Use of antibiotics was excluded because of typical short-term use. Since antibiotics may have more profound and longer-term effects on microbiota, which in turn may have effects on BAT activity, it would be important to reanalyze the data with the use of antimicrobials.

Author response: We thank the reviewer for this suggestion. To address this, we performed a multivariable logistic regression analysis to assess the association between individual antibiotic usage up to 1 month before each patient's index scan and their BAT

status, adjusting for age, sex, BMI, temperature, ethnicity, race, smoking history, family history of cardiovascular disease, cancer site, cancer stage, cancer treatment, and cardiovascular diagnoses. We found that several cephalosporins were associated with higher BAT prevalence, including cefadroxil (OR 1.65; 95% CI 1.08-2.50; $p = 0.02$) and cephalexin (OR 1.25; 95% CI 1.02-1.53; $p = 0.04$). In terms of fluoroquinolones, ciprofloxacin (OR 1.42; 95% CI 1.07-1.89; $p = 0.02$) and levofloxacin (OR 1.65; 95% CI 1.27-2.15; $p < 0.001$), were also associated with higher BAT prevalence. In addition, clindamycin (OR 1.68; 95% CI 1.08-2.63; $p = 0.02$) and trimethoprim-sulfamethoxazole (OR 2.29; 95% CI 1.98-2.66; $p < 0.001$) showed a significant association with higher BAT prevalence (Reviewer Figure 10). We have included these data in Extended Data Figure 10.



Reviewer Figure 10. Forest plot of odds ratios for the association between antibiotic usage and BAT status in a propensity score matched (PSM) cohort. The odds ratios were obtained with the use of a logistic regression after adjustment for age, sex, BMI, temperature, ethnicity, race, smoking history, family history of cardiovascular disease, cancer sites, cancer stage, cancer treatment, and cardiovascular diagnoses.

References: appropriate credit to previous work?

The authors have meritoriously given credit and referred to previous work. Perhaps the only work that would have also earned the credit in Introduction, “Since the landmark reports that adult humans have inducible BAT in 2009 8-10”, is the report by Saito et al. Diabetes 2009, PMID: 19401428. Practically, the studies were carried out at the same time with the other three publications in NEJM.

Author response: Thank you for this point. We have included this additional reference as suggested.

Referee #3 (Remarks to the Author):

This retrospective study in a very large cohort of patients investigates the role of brown adipose tissue (BAT) in human health, leveraging big data and AI to investigate associations of BAT with a wide range of diseases, metabolic parameters, and drug intake. Based on the analysis of FDG-PET/CT scans, the authors quantified BAT activity and examined its links to cardiometabolic diseases, psychiatric conditions, and liver function. The authors report that individuals with detectable BAT on PET showed lower prevalence of type 2 diabetes, ischemic heart disease, hypertension, and psychiatric disorders like anxiety and depression. These associations persisted even after adjusting for age, sex, BMI, and ambient temperature.

Several prior studies (including animal studies) have already shown that BAT may have a protective function in terms of development of obesity and metabolic diseases – thus, the topic is clearly of clinical relevance.

The main strengths of the current study are

(a) its very large cohort size of >100,000, compared to the largest comparable BAT study in human adults published in NEJM in 2009, which had <10,000.

(b) the use of a previously published, dedicated nnU-Net for BAT quantification on PET, which allowed investigation of depot-specific effects, revealing for example that higher BAT activity in the axillary and mediastinal regions was linked to improved metabolic outcomes.

(c) strong supporting evidence for the previously suggested activation of BAT by drugs, in particular angiotensin receptor blockers, which may enable fat repurposing. The caveat here is the trend towards higher osteoporosis prevalence in BAT+ individuals in this study.

There are very few actual drawbacks if the study - the retrospective design, for example, is difficult to avoid in studies of that size, but the authors are clearly aware that some of their residents require prospective validation.

My main suggestions would be to:

1. Focus a bit more on the potential bias introduced by using exclusively data from a cancer center, where many patients received systemic drugs - what data are currently available for BAT in cancer patients, as compared to BAT in healthy individuals and patients without cancer? Also discuss the observed differences between BAT+ and BAT- rates (Table 1) for the most prominent examples, e.g. patients with breast cancer and patients treated with mitotic inhibitors.

Author response: We thank the reviewer for your encouraging comments and for noting that “there are very few actual drawbacks.” We appreciate your suggestions. With regard to your first question, since FDG-PET/CT remains the gold standard for imaging BAT, these scans are most commonly performed in patients with known or suspected cancer. We are not aware of any large whole body FDG-PET/CT dataset that only includes healthy individuals. We have acknowledged this as a limitation of our work and elaborated further in the discussion. Regarding the second question, we have now discussed these observations extensively in the discussion, especially focusing on patients with breast cancer treated with mitotic inhibitors.

2. Discuss the potential link between osteoporosis and BAT in more detail - what is the weighing hypothesis here? Could this be a cancer population-specific finding?

Author response: We thank the reviewer for this comment. We have discussed this observation more extensively and detailed that it could be a cancer-population specific finding.

Referee #4 (Remarks to the Author):

GENERAL COMMENTS

An interesting paper reporting the results of big data and artificial intelligence (AI) analyses conducted on a very large convenience sample of patients followed at a well-known academic cancer center subjected to FDG-PET/CT imaging. Analyses are state-of-the-art and have been produced by a competent academic team. The paper reports a considerable amount of work. As mentioned by the authors themselves, the paper is an extension of their previous work in going beyond the report of associations between the presence or absence of brown adipose tissue (BAT) and cardiometabolic risk variables (their previous paper published in Nature Medicine in 2021).

In the section below, this reviewer has raised several questions and comments with the hope of being helpful to this qualified team of investigators. My main criticism is causality when the relationships between BAT activity and clinical outcomes are reported. As this is a convenience cohort of patients followed at a cancer center, I respectfully consider that it is premature to consider pharmacological targets to improve BAT volume and activity with the hope of generating clinical benefits. The preliminary work on ARBs is not at par with the nice and novel AI-derived analyses reported in the present study. I would strongly recommend to publish that part of the paper (in vitro studies) elsewhere and focus on novel associations (and interpret them as associations).

Author response: We thank the reviewer for acknowledging our analyses as “state-of-the art”. We agree with the reviewer that our cohort constitutes a convenience cohort. However, our medication analyses match and confirm findings from multiple human, prospective studies. This provides proof that our studies offer the opportunity to identify previously unstudied pharmacologic agents that activate BAT. We agree that this part of the manuscript was originally underdeveloped, so we have now added substantial additional *in vitro* and *in vivo* experiments with ARBs to make this portion of the manuscript as compelling as our human findings.

SPECIFIC COMMENTS:

1. *TITLE: Please modify the title to be clear on the type of cohort from which results were derived (patients screened for cancer). This is not a population-based study. There is nothing wrong with that but it should be stated clearly.*

Author response: We thank the reviewer for this observation, and we have modified the title to make clear the type of cohort we are working with. The new title reads: Artificial intelligence-enabled deep phenotyping of human brown fat in a large clinical cohort.

2. *SUMMARY: ... “full clinical impact of BAT”... be careful with that type of statement. The section relevant to ARBs is premature (see general comments).*

Author response: We thank the reviewer for this comment. We have modified this statement to be more conservative given the observational nature of this study.

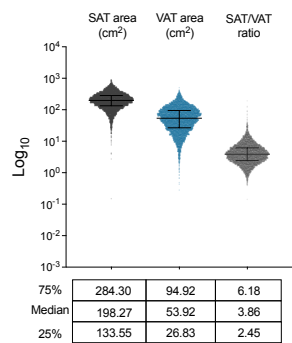
3. *INTRODUCTION: Obesity is heterogenous. You should refer to obesity phenotypes. Your second statement about adipose tissue dysfunction is more appropriate.*

Author response: We thank the reviewer for this comment. We have extended the introduction to discuss the heterogeneity in obesity and different obesity phenotypes.

4. *Because of its heterogeneity, would it be possible for this competent team to use AI to identify obesity phenotypes (i.e. subcutaneous vs visceral obesity) that could possibly be related to different BAT phenotypes? Would this be possible to generate that type of additional data from the already collected images?*

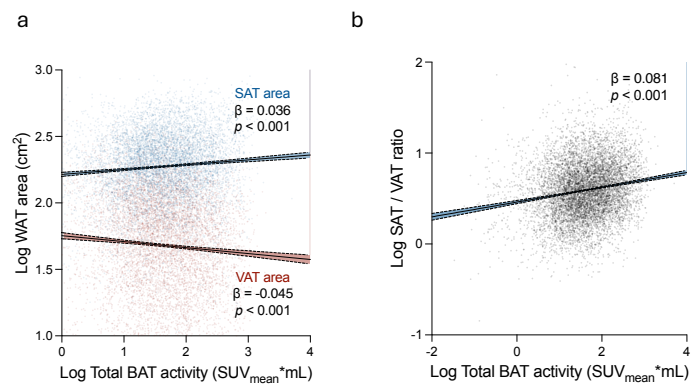
Author response: We thank the reviewer for this excellent suggestion. We want to respectfully make the reviewer aware of our prior publication (PMID: 34337558) where we reported differences in fat distribution between BAT+ and BAT- individuals. Indeed, it was found that BAT+ patients have a healthier fat distribution compared to BAT-

individuals (i.e. comparatively less visceral fat), and the metabolic benefits associated with BAT were still independent of the white fat phenotype. To complement these analyses and take advantage of the CT portion of the FDG-PET CT scans, we semi-automatically quantified subcutaneous (SAT) and visceral (VAT) adiposity in 8,040 BAT+ patients (92.3% of BAT+ patients from our PSM cohort). This allowed us to establish associations between total/depot-specific BAT metabolic activity and white fat distribution. Descriptive analyses of SAT, VAT, and SAT/VAT ratio are presented in Reviewer Figure 11. These data are included in Extended Data Figure 6.



Reviewer Figure 11. Quantification of SAT area, VAT area, and SAT/VAT ratio in a cohort of 8,040 BAT+ patients.

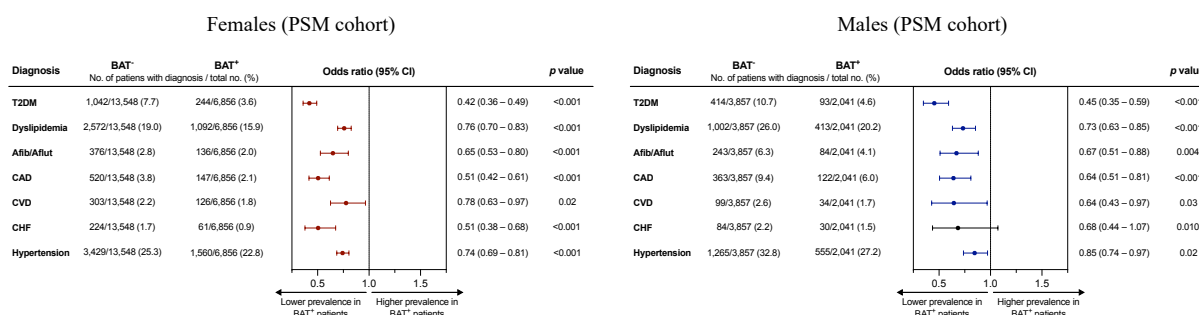
We also performed association analyses as suggested by the reviewer between WAT parameters and BAT metabolic activity. Similar to our previous publication (where manual measurements from ~850 patients were available), we found a positive correlation between total BAT metabolic activity and SAT area ($\beta = 0.036$, $p < 0.001$), whereas VAT area was inversely associated with BAT ($\beta = -0.045$, $p < 0.001$). As expected, the SAT/VAT ratio also exhibited a positive correlation with total BAT activity ($\beta = 0.081$, $p < 0.001$) (Reviewer Figure 12). These data are included in Figure 2.



Reviewer Figure 12. a) Association between total BAT metabolic activity, SAT area, and VAT area. b) Association between total BAT metabolic activity and SAT/VAT ratio

5. Since sex is an important determinant of differences in both white adipose tissue (WAT) distribution and BAT mass/activity, it would be important to conduct the analyses by sex rather than adjusting for sex. Indeed, pooling men and women is questionable for that type of analyses considering the sex dimorphism in WAT and BAT distribution, as well as in cardiometabolic risk.

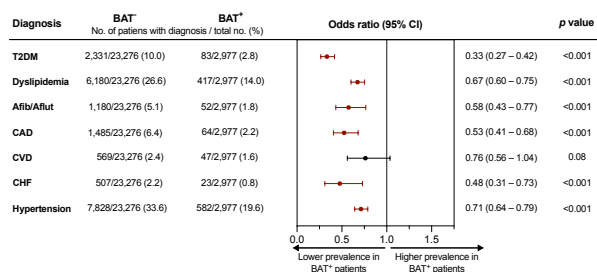
Author response: We thank the reviewer for this suggestion. To address this point, we conducted association analyses between BAT status and cardiometabolic diseases separately in males and females. In our PSM cohort, 20,404 patients were female and 5,898 were males. Upon conducting a multivariable logistic regression analysis controlling for age, BMI, ambient temperature, self-reported race and ethnicity, smoking history, family history of cardiovascular disease, cancer site, cancer stage, cancer treatment, and general groups of outpatient medications, we found that in both males and females BAT presence is associated with a decreased prevalence of cardiometabolic diseases (Reviewer Figure 13).



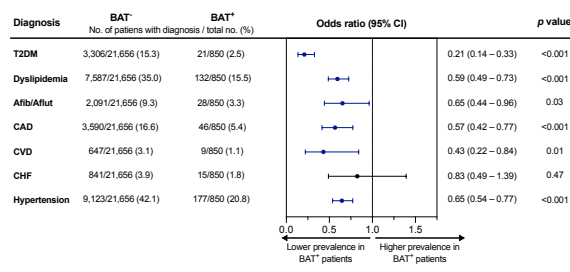
Reviewer Figure 13. Forest plots of odds ratios for the association between BAT status and cardiometabolic diseases in female patients only (left) (n=20,404) and male patients only (right) (n=5,898) from a propensity score matched (PSM) cohort. The odds ratio were obtained with the use of a logistic regression after adjustment age, BMI, ambient temperature, self-reported race and ethnicity, smoking history, family history of cardiovascular disease, cancer site, cancer stage, cancer treatment, and general groups of outpatient medications

We performed a similar analysis in patients from our BAT Extension Cohort (no propensity score matching) controlling for the same covariates. Our results again show that in both males and females being BAT+ is associated with reduced prevalence of cardiometabolic disease (Reviewer Figure 14).

Females (Extension BAT Cohort)



Males (Extension BAT Cohort)



Reviewer Figure 14. Forest plots of odds ratios for the association between BAT status and cardiometabolic diseases in female patients only (left) (n=26,253) and male patients only (right) (n=22,506) from the Extension BAT Cohort. The odds ratio were obtained with the use of a logistic regression after adjustment age, BMI, ambient temperature, self-reported race and ethnicity, smoking history, family history of cardiovascular disease, cancer site, cancer stage, cancer treatment, and general groups of outpatient medications

These analyses are included in Extended Data Figure 3.

6. L.119: replace “non-insulin dependent diabetes » by type 2 diabetes.

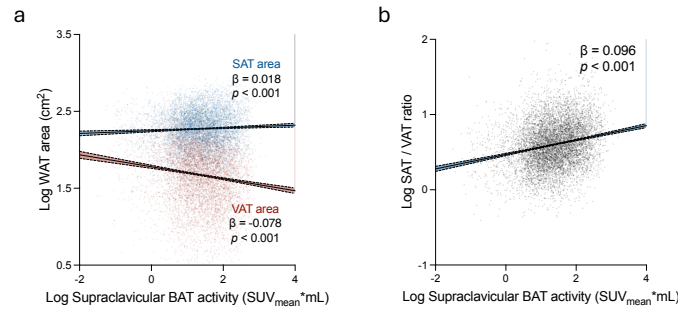
Author response: We have changed this as requested by the reviewer.

7. WAT distribution is related to depression and anxiety disorders, idem for sleep apnea. It is therefore of no surprise that individuals with type 2 diabetes with less BAT (and more abdominal visceral WAT) would show a greater prevalence of these conditions. The link between type 2 diabetes and these disorders is well established.

Author response: We thank the reviewer for this important point. We have included this in our discussion and describe how these diagnoses could be related.

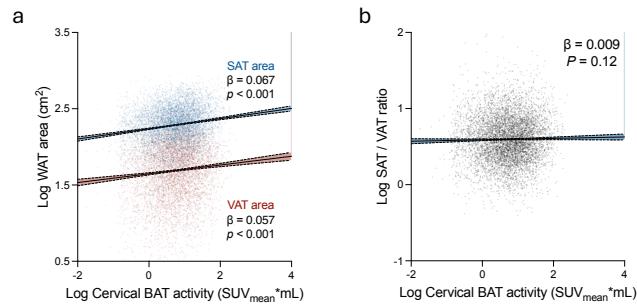
8. The findings on the distribution of the six BAT depots and clinical outcomes are quite novel and interesting. Considering the previous comments, it would be very interesting to probe the possible associations of these BAT depots with WAT distribution.

Author response: We thank the reviewer for this suggestion. As mentioned above, we were able to obtain SAT and VAT measurements in 8,040 patients, and we correlated them with each BAT depot individually. For the supraclavicular depot, we found similar associations as with total BAT activity, namely positive associations with SAT and SAT/VAT ratio, as well as a negative correlation with VAT (Reviewer Figure 15).



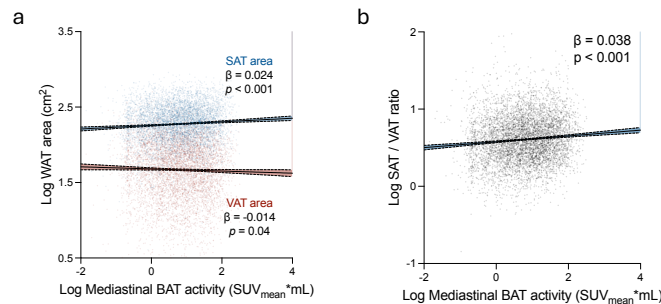
Reviewer Figure 15. a) Association between supraclavicular BAT metabolic activity, SAT area, and VAT area. b) Association between supraclavicular BAT metabolic activity and SAT/VAT ratio

Interestingly, for the cervical depot, we found positive associations with both SAT and VAT, which resulted in no correlation with SAT/VAT ratio (Reviewer Figure 16).



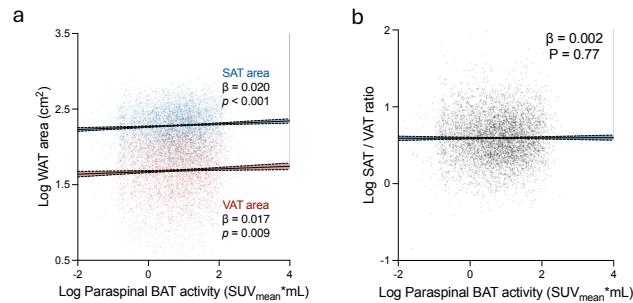
Reviewer Figure 16. a) Association between cervical BAT metabolic activity, SAT area, and VAT area. b) Association between cervical BAT metabolic activity and SAT/VAT ratio

The mediastinal depot also behaved similarly to total BAT metabolic activity (Reviewer Figure 17).



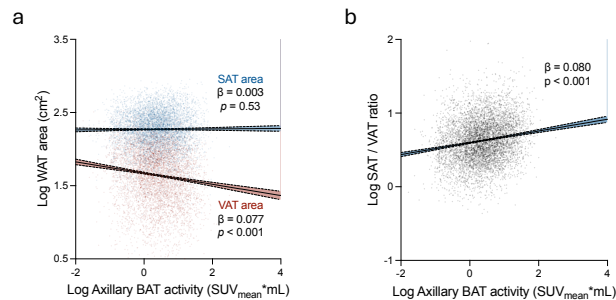
Reviewer Figure 17. a) Association between mediastinal BAT metabolic activity, SAT area, and VAT area. b) Association between mediastinal BAT metabolic activity and SAT/VAT ratio

On the other hand, the paraspinal depot behaved like the cervical depot, and it exhibited positive correlations with both SAT and VAT (Reviewer Figure 18).



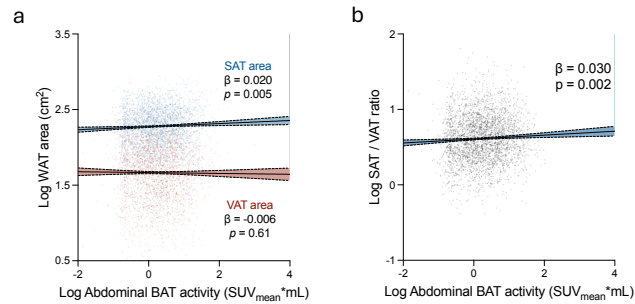
Reviewer Figure 18. a) Association between paraspinal BAT metabolic activity, SAT area, and VAT area. b) Association between paraspinal BAT metabolic activity and SAT/VAT ratio

Interestingly, the axillary depot exhibited the strongest negative association with VAT, resulting in a strong positive association in the SAT/VAT ratio (Reviewer Figure 19).



Reviewer Figure 19. a) Association between axillary BAT metabolic activity, SAT area, and VAT area. b) Association between axillary BAT metabolic activity and SAT/VAT ratio

Finally, the abdominal depot revealed a similar pattern of association as total BAT metabolic activity (Reviewer Figure 20)



Reviewer Figure 20. a) Association between abdominal BAT metabolic activity, SAT area, and VAT area. b) Association between abdominal BAT metabolic activity and SAT/VAT ratio

Of note, the axillary depot exhibited the most significant correlation with SAT/VAT ratio, and we demonstrate in the paper that increasing axillary BAT activity is most strongly associated with lower odds of all cardiovascular diagnoses in a stepwise fashion. This highlights the need to uncover differential biological effects between the different depots of thermogenic fat in humans. The compilation of these new data are included in Figure 2 and Extended Data Figure 6.

9. L.160: *..." specific benefits derived from this depot..." Please, rephrase. No evidence of a causal relationship.*

Author response: We rephrased as you have suggested.

10. *The section on AI-derived BAT activity vs. cardiometabolic risk variables is very interesting and novel and should be the main focus of the paper.*

Author response: We thank the reviewer for acknowledging the value of our AI-derived BAT activity platform and thanks to your suggestions we have expanded this work as detailed above. As mentioned in the general response, however, we also feel that there are other worthy findings in this paper, and we have included substantial additional data to make sure they provide the same depth of information as the AI observations.

11. *Pharmacological regulators of BAT: Please keep in mind that that, although large, this cohort is a convenience sample of patients followed at a cancer center. If you want to keep this section included the paper, you should remain very prudent in reporting these exploratory analyses. For instance, dexamethasone is positively associated with BAT presence!*

Author response: We thank the reviewer for this comment. We agree that a limitation of our work is that this is a convenience sample and a retrospective analysis. However, as

mentioned in the general comments, our analyses on medications like beta blockers, SSRIs, steroids, and mirabegron extensively confirm published findings from prospective human studies, which we feel supports the rationale of using our cohort as a tool for medication repurposing. We would like to draw the attention of the reviewer to the work by Potzch et al (PMID: 38081864) where they demonstrate that beta-adrenergic blockers decrease BAT activity in patients with lymphoma. Additionally, Suchacki et al (PMID 3753737) demonstrate that treatment with SSRIs in healthy volunteers decreases BAT activity, while dissecting the underlying mechanism. As for steroids, Ramage et al (PMID: 27411014) demonstrate these medications acutely increase BAT activity in humans depending on the duration of exposure. Moreover, extensive clinical trials by Aaron Cypess' group have demonstrated that mirabegron acutely and chronically increases BAT activity (PMID: 25565203, 31961829) in humans. We agree with the reviewer that we should remain prudent in discussing causality; however, our efforts in performing proof-of-concept experiments are meant to demonstrate that our findings are translatable to true biology.

12. DISCUSSION: The discussion is too speculative and sometimes falls in the trap of suggesting causality. I would respectfully suggest to these competent investigators to focus on the original, novel and interesting findings derived from their AI-generated analyses. If the heterogeneity in BAT activity (the 6 depots) could be related to the heterogeneity in WAT phenotypes and linked to health outcomes, this paper would make a very novel contribution to the already available literature on the topic.

Author response: We are deeply grateful to the reviewer for their suggestions on correlating WAT phenotypes with our novel, AI-derived BAT activity data. As presented above, we have done a large number of new analyses that confirm the association between total BAT activity and WAT distribution and also provide entirely new insights into depot-specific associations and SAT, VAT, and SAT/VAT ratio.

As for our studies on drug repurposing, as suggested by reviewer 1, we have significantly strengthened our proof-of-concept experiments demonstrating ARBs, specifically losartan, activate BAT. We have extended our studies and shown that losartan promotes Ucp1 expression via a PPAR γ -dependent mechanism. Additionally, we show in animal models that losartan increases BAT activity in response to the cold. Overall, these data demonstrate the validity of our utilizing this convenience cohort as a potential drug-repurposing platform.

13. This reviewer has tremendous respect for this established team of investigators, and the above comments are made in a constructive spirit with the hope of improving the quality of this paper.

Author response: We appreciate the constructive spirit of your comments. We feel that your suggestions have made this a much more compelling manuscript and hope that you will agree.

Authors' response to Referee comments:

We appreciate the time the referees took to carefully review our manuscript and to provide useful comments and suggestions. We have revised the manuscript accordingly and feel that this has greatly strengthened the paper.

We are pleased to see that Referees 2-4 feel that all of their concerns have been addressed.

Referee 1 provided some additional constructive feedback. To address these points, we have performed new experiments and have revised our text. Below is a point-by-point response to the concerns raised by Referee 1. For ease, the Referee's comments are displayed in italics, followed by our responses in standard font.

Referee #1 (Remarks to the Author):

In their revised version, the authors have improved the manuscript by the addition of new data. However, I am still concerned about the degree of conceptual advance as compared with their original publication in Nat. Med.

We thank the reviewer for acknowledging that the new data has improved our manuscript. Regarding the concern about the conceptual advance related to our 2021 Nature Medicine publication, we respectfully want to highlight the novelty of our findings, which address four large conceptual knowledge gaps in the field:

- 1) Does BAT affect other organ systems and clinical outcomes beyond the cardiometabolic realm? Our prior publication only looked at the association between BAT and cardiometabolic diseases. Here, for the first time, our manuscript examines the association of BAT with clinical traits in an unbiased manner. We describe several completely novel links between BAT and other diseases, including psychiatric diagnoses.
- 2) Does the degree of BAT metabolic activity matter in its association with medical outcomes? Our 2021 manuscript relied on a binary assessment of BAT activity. A consistent question that has been raised every time we have presented this work is whether there is a quantitative relationship between BAT metabolic activity and clinical outcomes. Until recently, it was not possible to answer this question. Here, we applied a newly developed AI tool to quantify BAT metabolic activity in the largest cohort of patients to date (~8,700 BAT+ patients) and established, for the first time, that higher BAT metabolic activity is associated with a stepwise decrease in the prevalence of cardiometabolic diseases.
- 3) Is all brown fat the same or do specific depots have unique roles? Thanks to our AI-based approach, we were able to dissect, for the first time, the association of specific BAT depots with clinical outcomes. Our analyses show that axillary BAT is

the depot associated with the greatest clinical benefit. These new findings highlight the heterogeneity of thermogenic adipose depots (as is now well accepted for white fat depots) and provide a strong rationale for future studies examining the mechanisms behind this association.

4) Are there safe and effective pharmacological approaches to activate brown fat?

The lack of drugs to safely activate BAT in humans has been a major barrier to clinical translation. Our unique dataset allowed us to establish associations between > 120 individual medications and BAT status with the goal of discovering approved drugs that could be repurposed to activate BAT in humans. To demonstrate the feasibility of this approach, we performed extensive proof-of-concept experiments illustrating that angiotensin receptor blockers (ARBs) activate BAT *in vitro* and *in vivo*. We further show that this action is not via the angiotensin receptor but instead through a PPAR γ -dependent mechanism.

We strongly believe that these four areas each represent important conceptual advances that have been consistently raised as key open questions by colleagues in the field. We also feel that the field needs a new, comprehensive resource on human brown fat, which our manuscript provides.

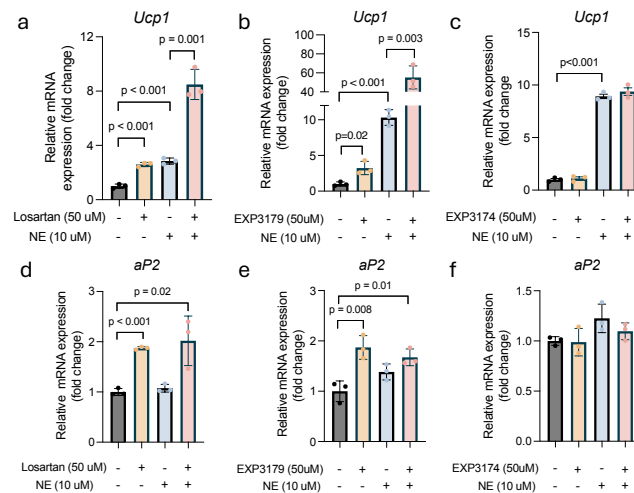
In addition, while they added new data on the mechanism of Losartan, there are still some inconsistencies in these experiments that require attention:

Thank you for raising this important point. Before addressing the specific comments, we want to respectfully point out that, in general, the inconsistencies noted by the Referee derive from experiments performed in two different systems: 1) *in vitro* differentiated brown adipocytes treated with losartan or its metabolites for 24 hours and 2) whole BAT obtained from animals treated with losartan versus control for at least 2 weeks. These two systems were employed to address distinct, but related, key questions. Differentiated brown adipocytes were used to assess the cell autonomous transcriptional response to acute losartan treatment. In contrast, whole BAT harvested after chronic losartan exposure represents an integrated tissue response which is heavily influenced by interactions of adipocytes with other cell types, the effects of losartan in other tissues, and compensatory adaptations. With these points in mind, however, we performed additional experiments, as detailed below, to provide further evidence for the effects of losartan on brown fat.

a) Reviewer Fig 1: There is inconsistent color coding of bars with respect to Losartan/NE treatments. Pls double check.

We thank the reviewer for pointing this out. The color coding for these figures has now been corrected. For this set of experiments, light yellow bars represent treatment with losartan/losartan metabolites, and light blue bars represent treatment with norepinephrine

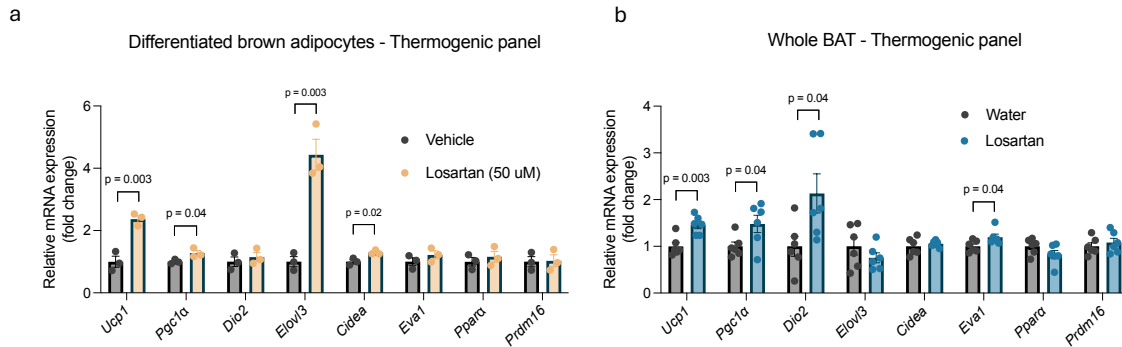
(Reviewer Figure 1). This has also been changed in Figure 4 and in Extended Data Figure 7.



Reviewer Figure 1. Mature immortalized brown adipocytes were pre-treated with losartan, EXP3179, or EXP3174 versus vehicle for 24 hours and then stimulated with norepinephrine (NE) vs. vehicle for 6 hours. a-c *Ucp1* mRNA levels quantified by qPCR. d-f *aP2* mRNA levels quantified by qPCR

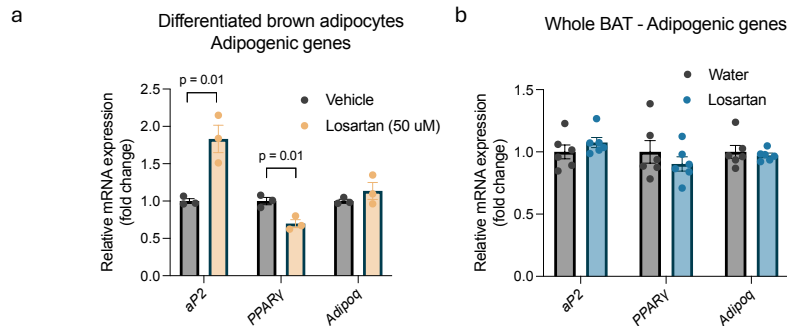
b) In contrast to Reviewer Fig 4, Losartan treatment in Reviewer Fig 1 triggers *aP2* expression as a single treatment while Losartan exposure *in vivo* leaves *aP2* levels unaffected. This also contrasts its effect on *UCP1* expression which is also induced by single Losartan exposure in isolated cells (Reviewer Fig 3). Thus, there is inconsistency between *in vitro* and *in vivo* data.

We thank the Referee for raising this point. As mentioned above, there are several biological and technical reasons why results from our *in vitro* and *in vivo* models do not completely match (cultured cells vs. whole tissue, acute vs. chronic treatment, adaptations in whole-body metabolism). However, we agree that additional information can help support our conclusion that losartan induces thermogenic gene expression. To that end, we performed new experiments expanding our gene expression panels in both systems to determine the degree of concordance between them. We now show data for a panel of eight thermogenic genes derived from other publications in the field^{1,2}. In terms of thermogenic genes, we observed an increase in the expression of *Ucp1* and *Pgc1a* in both systems, increased *Cidea* and *Elovl3* *in vitro*, and increased *Dio2* and *Eva1* *in vivo*. *PPARa* and *Prdm16* remained unchanged in both. We would like to note that while *PRDM16* is a key transcriptional regulator of thermogenic gene expression in brown and beige fat, *Prdm16* itself is mainly regulated post-translationally rather than transcriptionally³. Overall, losartan treatment in both our *in vitro* and *in vivo* systems leads to an enhanced thermogenic transcriptional program (Reviewer Figure 2). These data are included in Extended Data Figure 7 and 8



Reviewer Figure 2. a) Mature immortalized brown adipocytes were treated with losartan versus vehicle for 24 hours and expression of thermogenic genes was assessed via qPCR. b) BAT from WT mice treated with losartan in drinking water (100/mg/kg) for 2 weeks was harvested and the expression thermogenic genes was assessed via qPCR.

We also measured the effects of losartan on general adipogenic genes. We observed an increase in *aP2* expression, a decrease in *Pparγ* mRNA, and no change in adiponectin with losartan treatment *in vitro*. *In vivo*, there were no differences in the adipogenic transcriptional signature, likely due to the cellular heterogeneity of whole BAT, and the longer exposure to losartan, allowing for compensatory mechanisms (Reviewer Figure 3). These data were included in Extended Data Figure 7 and 8.

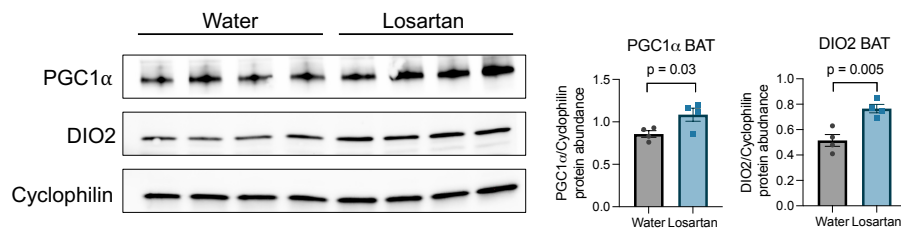


Reviewer Figure 3. a) Mature immortalized brown adipocytes were treated with losartan versus vehicle for 24 hours and expression of adipogenic genes was assessed via qPCR. b) BAT from WT mice treated with losartan in drinking water (100/mg/kg) for 2 weeks was harvested and the expression adipogenesis genes was assessed via qPCR.

c) Reviewer Fig 4: The impact of Losartan treatment on BAT gene programs is rather unimpressive at the RNA level. Please provide WB to assess Losartan effects on protein expression levels.

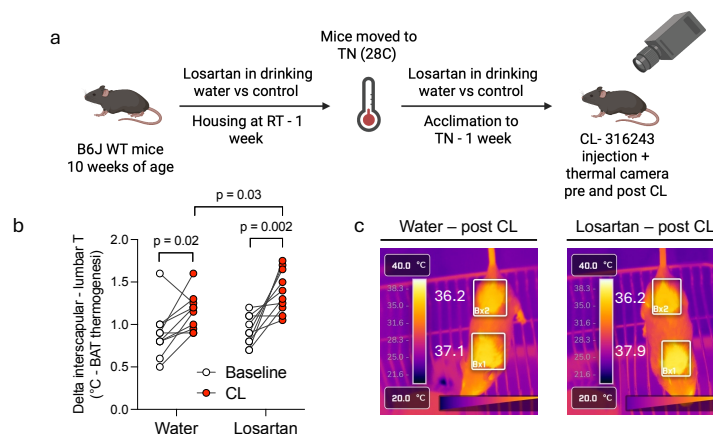
We thank the Referee for this observation. Given that the gene expression signature data comes from whole BAT, it is likely that the signal is diluted due to the cellular heterogeneity of this tissue. To address the concerns raised, we performed Western blots for two thermogenic proteins: PGC1α and DIO2 in BAT lysates from losartan-treated mice versus

controls. Losartan increased PGC1 α and DIO2 protein in BAT (Reviewer Figure 4). These data are included in Extended Data Figure 8.



Reviewer Figure 4. Representative Western Blot of PGC1 α and DIO2 of BAT protein lysates from losartan-treated mice versus controls. Quantification of PGC1 α and DIO2 protein relative to cyclophilin. P values were obtained with unpaired two tailed t-tests.

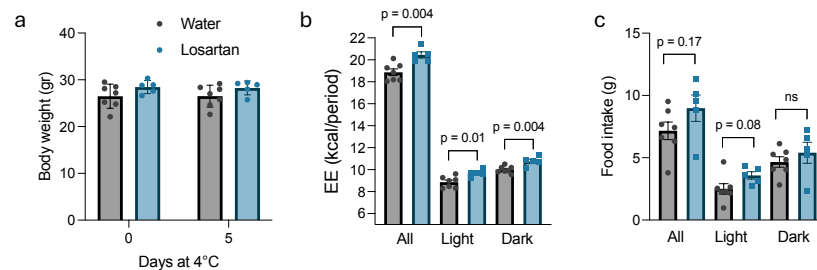
Moreover, we performed new experiments to functionally characterize the thermogenic potential of BAT upon losartan treatment *in vivo*. We feel this is a much more meaningful readout than analyses of RNA and protein alone, which serve as surrogates for thermogenic function. To that end, we treated mice with losartan or vehicle in their drinking water and acclimated them to thermoneutrality (to inactivate BAT). After 1 week, we injected mice with the selective beta3 adrenergic agonist CL-316,243 and measured interscapular (proxy for BAT heat production) and lumbar (proxy for systemic, non-specific heat production) temperature directly using a thermal camera. The absolute temperature difference between interscapular and lumbar temperature was used to quantify BAT thermogenesis⁴⁻⁷. As expected, losartan treated mice and controls exhibited an increase in BAT thermogenesis after CL injection. Importantly, this increase was significantly greater in the losartan-treated than the water treated control mice. These data confirm that losartan promotes increased BAT thermogenic capacity (Reviewer Figure 5) and are included in Main Figure 4.



Reviewer Figure 5. a) Schematic of experimental plan - B6J WT mice were treated with losartan in their drinking water (100 mg/kd/day) or regular water at room temperature (RT), followed by acclimation to thermoneutrality (TN) for 1 week, and then injected with CL-316243 at 1 mg/kg. Thermal camera images were obtained at baseline and 2 hours after CL injection. b) Quantification of BAT thermogenesis assessed by thermal camera. c) Representative thermal camera images after CL injection in losartan-treated and control mice.

d) Reviewer Fig 5: Please also show body weight development upon Losartan exposure. If EE is elevated with no effects on food intake and locomotor activity, it would be interesting to see the actual impact on body weight.

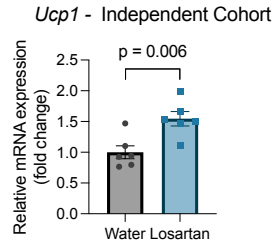
We appreciate the reviewer raising this important comment. The increased energy expenditure in losartan-treated mice was documented under cold conditions for 5 days, and during this time we did not detect changes in body weight. The overall increase in EE was ~8% in the losartan treated mice, with a concomitant, non-statistically significant trend towards higher food intake (Reviewer Figure 6). Given the limited duration of the experiment, it is likely that the compensatory increase in food intake in losartan-treated mice prevented us from detecting significant changes in body weight. We have added this finding to our manuscript and specifically discuss the trend towards increased food intake in losartan treated mice.



Reviewer Figure 6. a) Body weight at baseline and 5 days after cold exposure in losartan-treated mice versus controls. b) Energy expenditure (EE) (representative 24 hours) was measured in WT mice treated with losartan or water during a cold challenge (4°C), and average EE for 24 hours, dark and light phase are shown. c) Food intake (representative 24 hours) measured in WT mice treated with losartan or water during a cold challenge (4°C), and average food intake for 24 hours, dark and light phase are shown.

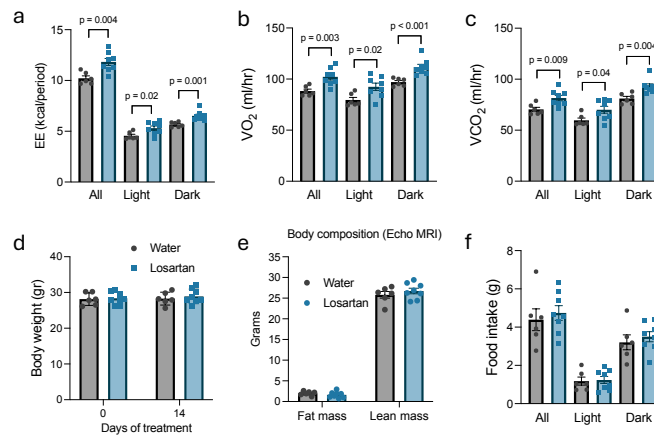
e) Reviewer Fig 6: Here Losartan only shows an effect upon elevated beta adrenergic signaling (= cold) which again is in contrast to Reviewer Fig. 1.

The reviewer is correct that in this figure whole BAT *Ucp1* mRNA does not change with losartan treatment in mice at room temperature (RT). We respectfully want to point out that these samples come from whole BAT (potentially diluting signals due to the cellular heterogeneity of the tissue as above), and we had 4 animals per treatment group, making this experiment underpowered to detect statistically significant differences. These samples were meant to illustrate the increase in *Ucp1* transcription with cold in losartan treated and control animals. We have previously shown with a higher number of samples that losartan treatment at RT increases *Ucp1* mRNA levels (see Reviewer Figure 2 above). To further address this point, we validated these observations in an independent cohort of mice treated with losartan at RT for 2 weeks (Reviewer Figure 7).



Reviewer Figure 7. a) BAT from an independent cohort of WT mice treated with losartan in drinking water (100/mg/kg) for 2 weeks was harvested and the expression of *Ucp1* was assessed via qPCR.

To further assess the effects of losartan on energy expenditure at RT, we assessed energy expenditure (EE) in WT mice pre-treated with losartan in drinking water for 10 days and recorded for 5 days at RT. Importantly, we observed an increase in EE, oxygen consumption, and carbon dioxide production with losartan treatment at these baseline conditions (Reviewer Figure 8a-c). The increase in EE was ~10% with no changes in body weight or body composition (Reviewer Figure 8d and 8e). However, we again detected a trend towards increased food intake (the outlier in the control group masks the overall effect) in the treatment group (Reviewer Figure 8f). Altogether, these data demonstrate that losartan promotes BAT activity and increased EE in baseline conditions and are included in Extended Data Figure 9. Please note this figure also includes temporal tracings and an ANCOVA analysis.



Reviewer Figure 8. a-c. WT mice treated with losartan or water for 10 days at RT were placed in metabolic chambers and indirect calorimetry was recorded for 5 days at RT. For a-c and f, representative 24 hours, dark, and light phase data are shown. a) Average EE. b) Average oxygen consumption (VO₂). c) Average carbon dioxide production (VCO₂). d) Body weight before and after losartan treatment. e) Body composition after losartan treatment as shown by Echo MRI. f) Average food intake.

Referee #2 (Remarks to the Author):

The authors have responded properly to the concerns raised by this reviewer. Additional work load by the group to improve the manuscript is greatly appreciated.

Referee #3 (Remarks to the Author):

Thank you for your revisions. I have no further comments.

Referee #4 (Remarks to the Author):

The authors have adequately addressed all my specific questions. The limitations of the study are better addressed and some of the added results have increased the level of originality of the paper.

We appreciate the comprehensive reviews from Referees 2-4 and are pleased to see that they feel all their concerns have been suitably addressed. Their constructive input has made this a much stronger manuscript for which we are grateful.

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- 7 Sakamoto, K. *et al.* Overnutrition causes insulin resistance and metabolic disorder through increased sympathetic nervous system activity. *Cell Metab* **37**, 121-137 e126 (2025). <https://doi.org/10.1016/j.cmet.2024.09.012>