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Peer Review File

Transcriptional dynamics in type 2 diabetes progression is linked with circadian, thermogenic, and cellular stress in human adipose tissue.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In “transcriptional dynamics in type 2 diabetes progression is linked with circadian, thermogenic, and cellular s tress in human adipose tissue”, authors study gene expression patterns in subcutaneous adipose tissue (SAT) of individuals of normal weight compared to individuals with obesity, obesity with type 2 diabetes, or alternatively type 2 diabetes. Cellular energetics were studied in beige adipocytes obtained from the patient groups. This study involved a total of 86 patients, most (60) being women. Additional markers, including BMI, HbA1c, cholesterol, and blood pressure were measured. The primary goal of this study was to identify metabolic changes in adipose tissue during metabolic disease progression. Generally, changes in gene expression in SAT were more pronounced between normal weight individuals compared to individuals with increasing disease progression. Though the work is largely descriptive, with little validation performed, the authors were successful in acquiring a relatively large number of individuals from which biopsies could be obtained.

1. When describing gene expression results, as written in the text it is not always clear whether a specific pathway is up- vs. down-regulated in the normal weight vs. metabolic disease groups.
2. Though it is interesting that epigenetic-regulatory enzymes genes are altered in some of these comparisons, it would be nice to actually provide a measure of said changes (i.e. by looking at histone acetylation, for example) in cultures from these individuals.
3. Since the authors mention alterations in circadian rhythms, they may want to discuss time of biopsy earlier in the article (even if it varies across individuals), as this is highly relevant considering the large amplitude of oscillation for some circadian genes. The reader does not come across this information until page 9.
4. CLOCK is generally not expressed with a very high amplitude. It is not clear why the authors did not base bmal1 and dbp (for example) or some other antiphase relationship when testing for associations with disease progression.
5. The authors should consider discussing the opportunity for performing serum shock on their beige cultures. Most cultured cells can by synchronized to study the robustness of clock expression across time in different cells types or in this case, across patients. Such an experiment could be very informative.

Reviewer #2

(Remarks to the Author)
The methods detail that overall, 60 females subjects were recruited compared with 26 males, but no further details are given regarding the sex distribution between the 4 subgroups of ~20 subjects, for which much fewer subjects are used for many of the subsequent analyses, that in some cases is as low as n=3. This raises the distinct possibility that some of the described differences may simply be due to an imbalance of sex distribution between groups. In addition, no account has been made of the multiple statistical comparisons made, whilst n=6 is NOT significant, especially with such low numbers of participants.

Reviewer #3

(Remarks to the Author)
In this manuscript, the anonymous authors demonstrate that metabolic dysfunction is associated with transcriptional

changes in adipose tissue including altered expression of clock- and mitochondrial- genes. The authors utilizes bioinformatic approaches to provide evidence suggesting that these processes are inter-regulated and involves the transcriptional regulator MAX as a key player. Transcriptional changes in adipose tissue from individuals with diabetes and obesity has been thoroughly studied however, the circadian mechanisms have often been overlooked and ignored. This study make an attempt to study the temporal aspect of metabolic dysfunction in human material and thus, provide valuable insight to the field. Yet, there are a few points that this reviewer would like to highlight:

1. One major limitation is of course that adipose tissue biopsies have been collected at one time point. The reviewer appreciate the challenge of taking biopsies at multiple circadian time points in humans however, this limitation should be clearly stated in the discussion.

2. In line with the previous point, the authors turn to an online database to extract information regarding the circadian rhythmicity of diferentially expressed genes in their dataset. The authors state that they overlapped the genes with circadian genes in different human and mouse tissues while their dataset is from human subcutaneous adipose tissue. Just because a gene is circadian in one tissue does not necessarily mean that it is circadian in another tissue. The same pertains to comparing circadian rhythmicity between species. Therefore, to gain insight on which diferentially expressed genes that are circadian, the authors should only overlap their data with the circadian dataset from human subcutaneous adipose tissue in the publicly available dataset.

3. A minor point: The time of biopsy collection should also be mentioned in the methods section.

4. The experimental design is very interesting and provides insight into what occurs in the adipose tissue at different stages of disease. The authors compare each disease state with the lean healthy group in each comparison. Would it be interesting to compare the diferentially expressed genes between each stage, i.e. NWvsOW, OWvsPT2D and PT2DvsT2D? If the differences are incremental and the statistical power is insuffcient to detect differences, this should be stated under the limitations of the study.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors went above and beyond to try to address my concerns. I am satisfied.

Reviewer #3

(Remarks to the Author)
The authors have addressed all my concerns, and I do not have any additional ones.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In “transcriptional dynamics in type 2 diabetes progression is linked with circadian, thermogenic, and cellular s tress in human adipose tissue”, authors study gene expression patterns in subcutaneous adipose tissue (SAT) of individuals of normal weight compared to individuals with obesity, obesity with type 2 diabetes, or alternatively type 2 diabetes. Cellular energetics were studied in beige adipocytes obtained from the patient groups. This study involved a total of 86 patients, most (60) being women. Additional markers, including BMI, HbA1c, cholesterol, and blood pressure were measured. The primary goal of this study was to identify metabolic changes in adipose tissue during metabolic disease progression. Generally, changes in gene expression in SAT were more pronounced between normal weight individuals compared to individuals with increasing disease progression. Though the work is largely descriptive, with little validation performed, the authors were successful in acquiring a relatively large number of individuals from which biopsies could be obtained.

1. When describing gene expression results, as written in the text it is not always clear whether a specific pathway is up- vs. down-regulated in the normal weight vs. metabolic disease groups.

Author's reply

Thank you for your comment. We have revised the manuscript and reorganized some paragraphs and figures as follows:

We included the up-regulated genes and their GO analysis in the new section, **“Subcutaneous adipose tissue undergoes progressive transcriptional alterations during T2D development.”** This is the first results section, which includes the exploratory results on the number of identified DEGs (Figure 1A-F), the new sexual dimorphism analysis (Supplemental Figure 1 and Supplemental Table 2), the GO analysis of the up-regulated genes (Supplemental Figure 2), and the new results about the histone modifiers (Supplemental Figure 3).

The results regarding the GO analysis of the downregulated DEGs and TH western blot are now part of the second section, **“Downregulation of catabolic and thermogenic genes during T2D development**,” which includes Figure 2A-E. Additionally, given that Figure 3 from the first version was complementary to these results, we have moved this figure to Supplemental Figure 4. We included a new quantification of circadian-expressed genes from adipose tissues (Supplemental Figure 4C, Supplemental Table 3).

In addition to stages S1-3, we identified the DEGs from additional comparisons OWvsPD (S4), OWvsT2D (S5), and PDvsT2D (S6) from the interactomes in

Supplemental Figure 14A, and Figures 6E and 7A in the new Supplemental figure 14. We also included the quantification of correlations of core-clock genes with clinical parameters (Supplemental Figures 9, 10). We eliminated Table 1, as this information is also included in Supplemental Table 1.

The results sections were reorganized accordingly to align with this new structure. Please refer to the blue text within the manuscript. We believe that this new organization is easier to follow.

2. Though it is interesting that epigenetic-regulatory enzymes genes are altered in some of these comparisons, it would be nice to actually provide a measure of said changes (i.e. by looking at histone acetylation, for example) in cultures from these individuals.

Author's reply

This is an intriguing question. At our current research stage, the analysis of epigenetic mechanisms fell outside our scope. However, we had planned to conduct chromatin immunoprecipitation experiments from the beige culture and have already completed a transcriptome analysis on those cells, the results of which will be part of another manuscript. Nevertheless, to honor this pertinent suggestion, we expanded our findings by constructing a protein-protein interactome network involving several histone modifiers, and the results were included in the new Supplemental Figure 3. We also added the following paragraphs in the results and discussion sections.

Text added in the section: **“The circadian clock is progressively altered during the progression of T2D”**

Lines 293-299

To gain additional insight into histone modification enzymes, we constructed a protein-protein interactome network using several histone-modifying enzymes, (Supplemental Figure 3A, B). We identified histone deacetylase 4 and 11 (HDAC4, 11), the histone methyltransferase complex regulatory subunit DPY30, and the histone H2A variant Y (H2AY) and the chromatin regulator ASF1A, whose expression was upregulated during the progression of T2D. (Supplemental Figure 3C). In this context, the upregulation of these chromatin remodelers had been linked to metabolic control, and cellular stress and senescence [44-51].

Text added in the section: “**Discussion.”**

Lines 545-562

Growing evidence highlights the importance of epigenetic modifications in the development of T2D [100,101]. For instance, ATAC-seq studies revealed differences in

chromatin accessibility in specific loci between diabetic and non-diabetic pancreatic islet donors [102]. However, research on adipose tissues has predominantly focused on DNA methylation [100,101]. In our study, we observed an elevated expression of chromatin remodelers, such as HDAC11 during the progression of T2D (Supplemental figure 3C). HDAC11 deficiency promotes thermogenesis and energy expenditure [44-47], suggesting a possible link between its upregulation and reduced thermogenic capacity in T2D.

Similarly, the histone H2A variant Y H2AY, (macroH2A1), is associated with regulating genes to lipid metabolism and adipocyte differentiation, in fact genetic ablation of this histone leads to increased leanness, improved glucose tolerance, and enhanced energy expenditure in mice fed with a high-fat diet [49]. Consistent with previous studies [51], we observed a progressive increase in H2A.Y expression from S1 to S3. Strikingly, H2AY acts as an epigenetic chromatin remodeler during the formation of senescence-associated heterochromatin foci-a complex process in which the chromatin regulator ASF1A plays a critical role [50]. Our data revealed an upregulation of ASF1A expression and other senescence-associated genes, including pro-inflammatory factors, interferon-regulated genes, (such as CXCL1 and NOX4). Notably, cellular senescence is closely linked to T2D [48]. This highlights the importance of chromatin remodeling mechanisms in the development of T2D.

3. Since the authors mention alterations in circadian rhythms, they may want to discuss time of biopsy earlier in the article (even if it varies across individuals), as this is highly relevant considering the large amplitude of oscillation for some circadian genes. The reader does not come across this information until page 9.

Author's reply

We appreciate this observation.

Text added in the section: “**Methods**.”

Line 101

“Subcutaneous adipose tissue (SAT) biopsies and blood samples were taken between 7:00 and 8:00 hours.”

4. CLOCK is generally not expressed with a very high amplitude. It is not clear why the authors did not base bmal1 and dbp (for example) or some other antiphase relationship when testing for associations with disease progression.

Author's reply

We appreciate this pertinent observation. While ARNTL and DBP are known to exhibit robust and antiphase oscillations, we observed a lower number of correlations with the metabolic parameters when using ARNTL:DBP ratio values compared to the ratio value

of the CLOCK:PER2 (Spearman correlation P<0.01 Figure 4F, G, Supplemental Figures 9, 10).

This discrepancy likely arises from differences in the Zeitgeber Time (ZT) at which each pair of core clock genes reaches its antiphase expression in our samples. For instance, according to the CIRCADB database, in mouse brown adipose tissue, CLOCK and PER2 are fully expressed in antiphase at ZT 1 or 13 (CLOCK acrophase = 1, PER2 acrophase = 13), whereas ARNTL and DBP peak at ZT 23 or 11 (ARNTL acrophase = 23, DBP acrophase = 11), approximately two hours earlier than CLOCK and PER2. However, due to the lack of acrophase information for these genes in our samples, we cannot determine the exact timing of their antiphase expression. Therefore, to improve our analysis, we selected a set of core-clock genes whose circadian expression patterns clearly display an antiphase profile in different tissues. We then calculated the Spearman correlation with blood glucose (glu) or HbA1c values, using the absolute (abs) expression values or the ratio between pairs of genes.

To analyze the effect on the number of correlations between the ratios of pairs of genes in phase or in antiphase, we classified the core-clock genes into 'Phase genes' (ARNTL, CLOCK, NFIL3, NPAS1, NPAS3) and 'Antiphase genes' (BHLHE40, BHLHE41, CRY1, CRY2, DBP, NR1D1, NR1D2, PER1, PER2) (Figure 4C). Additionally, due to our limited sample size, we also use a threshold of P<0.01 in the Spearman correlation analysis to obtain more robust correlations (Figure 4G, Supplemental Figure 10).

As previously noted in the first version of the manuscript, the number of correlations using absolute values (phase or antiphase genes), as well as the ratio values of pairs of genes with similar acrophases (ratios between phase:phase or antiphase:antiphase), were lower than those obtained from genes with 12-hour-separated acrophases (ratios between phase:antiphase) (Figure 4E). In this context, growing studies in both humans and rodent models support the idea that obesity-related metabolic changes are closely tied to alterations in the expression of the main core-clock genes [1-8]. Our results further suggest that disruptions in the circadian clock during disease progression—specifically, the expression patterns of antiphase core-clock genes—may reflect a progressive nature of clock alteration associated with disease development. However, given our limited sample size, larger studies are needed to enhance statistical power.

Text added in the section **"The circadian clock is progressively altered during the progression of T2D."**

Lines 393-412

Assuming that the conserved transcriptional-translational feedback loop mechanism controlling the circadian expression of core-clock genes (CoCGs) which is the first layer of transcriptional regulation for thousands of CCGs [66], we hypothesized that the expression level between CoCGs should preserve a specific expression relation at each time of the day [67]. Since the biopsies were collected in a narrow timeframe (7:00-8:00

hrs.), we calculated the ratio between the expression of different pairs of core-clock genes. We selected two sets of core-clock genes known to be expressed in antiphase: ARNTL, CLOCK, NFIL3, NPAS1, NPAS3 as “Phase” genes and BHLHE40, BHLHE41, CRY1, CRY2, DBP, NR1D1, NR1D2, PER1, PER2 as “Antiphase” genes [31] (Figure 4C, Supplemental Figure 8N-P). In line with the enrichment of circadian related genes (Figures 2A, D, Supplemental figure 4B), we observed a progressive effect across the different groups. This effect was inverse between antiphase genes as demonstrated by the *PER2* and *CLOCK* ratios (Figures 4D E, Supplemental Figure 8).

Indeed, the correlation analysis between the ratio values and the blood glucose or HA1Ab parameters, show that the absolute values (abs) or ratios values obtained from genes with similar acrophase (phase:phase or antiphase:antiphase) such as *CLOCK:ARNTL* or *CLOCK:NPAS3*, or *PER2:PER1* and *PER2:CRY2* gene pairs, showed significantly fewer correlations than those obtained from phase-antiphase gene ratios (Figure 4F, G, Supplemental Figure 9, 10). This finding highlights a progressive effect on the different groups, suggesting that disruptions in the circadian clock during disease progression—specifically, the expression patterns of antiphase core-clock genes—may reflect a progressive alteration of the circadian clock associated with disease development. This supports the notion that obesity-related metabolic changes are closely tied to alterations in the main core-clock genes[68-75].

5. The authors should consider discussing the opportunity for performing serum shock on their beige cultures. Most cultured cells can by synchronized to study the robustness of clock expression across time in different cells types or in this case, across patients. Such an experiment could be very informative.

Author's reply

This is a very pertinent observation. While we successfully synchronized adipose-derived stem cells (ADSCs) using fetal horse serum shock, we have not been able to synchronize differentiated adipocytes. These cells are more fragile than ADSCs or fibroblast cell lines, and our protocols have not been effective in preventing cell death during synchronization attempts. Indeed, to our knowledge, only ADSCs have been reported to be synchronized for circadian experiments [1]. Nevertheless, synchronized adipocyte cell culture will be crucial for deciphering in more detail how the circadian clock and clock-controlled genes are altered during the progression of T2D and other diseases.

Text added in the section: “**Discussion**.”

Lines 671-674

Another limitation of our study is that samples were collected at a single time point in the circadian cycle. This limits our ability to analyze how different genes with varying acrophases might be affected at other times of day. To gain a more comprehensive understanding of circadian system alterations in each stage, sampling at multiple time points or analyzing synchronized beige cultures would be necessary.

Reviewer #2 (Remarks to the Author):

The methods detail that overall, 60 females subjects were recruited compared with 26 males, but no further details are given regarding the sex distribution between the 4 subgroups of ~20 subjects, for which much fewer subjects are used for many of the subsequent analyses, that in some cases is as low as n=3. This raises the distinct possibility that some of the described differences may simply be due to an imbalance of sex distribution between groups. In addition, no account has been made of the multiple statistical comparisons made, whilst n=6 is NOT significant, especially with such low numbers of participants.

Author's reply

We appreciate the reviewer's comments and understand their concerns. We included a new table with the sex distribution of all participants and the samples used for the transcriptome (supplemental table 2).

Moreover, to determine if the identified DEGs would be influenced by sex dimorphism, we performed factor analysis of mixed data (FAMD) which combines the multiple correspondence analysis (MCA) and the principal component analyses allowing to integrate quantitative (gene expression) and qualitative data (sex or diagnosis). Individuals with similar transcriptomic profiles are closed to each other in the FAMD map. As noted in the new Supplemental Figure 1, when considering the diagnosis (NW, OW, PD, T2D), a clear separation is established between groups, segregating the NW into different quadrants. However, this separation is no longer evident when considering the variable of sex. Therefore, we consider that sex is not influencing our results. However, we acknowledge that due to the reduced sample size, additional experiments with larger sample sizes are necessary to increase statistical power. Nevertheless, we have included these important limitations in a new section.

In most of the results, we used n=7-8, and in the functional analysis in cell culture, we used n=17-20 per group. For the Western blot analysis (n=3), these experiments were conducted to gain insights into the protein expression of a few identified genes. At this point, we did not aim to analyze signaling pathways by examining post-translational modifications. It is worth noting that this sample size for Western blots has been used in other articles analyzing adipocytes [9-12]. However, to improve the statistical analysis,

we reanalyzed this data using more appropriate statistical methods. We first analyzed normality and homoscedasticity using the Shapiro-Wilk and Bartlett tests, respectively. We found that in all the Western blots, the data met these assumptions. Therefore, we also analyzed the data using one-way ANOVA, followed by Tukey's post-hoc test. We accordingly modified the results and included the methods in the figure footnotes.

We are currently investigating the molecular mechanisms in beige culture. We have already completed a transcriptome analysis of these cells and will complement it with additional experiments, including ChIP and western blots. However, these results will be part of another manuscript.

Text added in the section: "**Subcutaneous adipose tissue undergoes progressive transcriptional alterations during T2D development.**"

Lines 278-284

Given the established sexual dimorphism in various aspects of T2D, including diagnosis, prevention, and treatment [40,41], we aimed to investigate whether the identified DEGs would be influenced by sex (Supplemental Table 2). As anticipated, the FAMD revealed that at stages S1, S2, and S3, the sample distribution was grouped by diagnosis, with NW and OW individuals positioned in distinct quadrants (Supplemental Figure 1A-C). However, the sex distribution did not exhibit a significant separation between diagnoses (Supplemental figure 1D-F), suggesting that within the analyzed DEGs, sex may not have a substantial influence on the observed patterns in these samples.

Text added in the section "**Methods**."

Lines 247-257

Means ± standard error (SEM) were calculated, and different statistical tests were used. A t-test analysis was employed to compare two independent groups, a one-way ANOVA test followed by a post-hoc Tukey-Kramer test was applied to compare three or more independent groups, or Dunnet´s post-hoc when comparing a control group, provided that the variable data was normally distributed (Shapiro-Wilk $P>0.05$) and homoscedastic (Bartlett's test $P>0.05$). If the data did not meet the assumptions of ANOVA, the Kruskal-Wallis with Wilcoxon post-hoc test (non-normal and homoscedastic data) or Welch's ANOVA test with post-hoc Games-Howell test (for normal and heteroscedastic data) were used. The analyses were conducted using the 'moments', 'rstatix', and 'plotrix' packages for R [34-36]. Plots were obtained using GraphPad Prism 9.0. Factor analysis of mixed data (FAMD) was performed to analyze the association between quantitative (gene expression) and qualitative variables (diagnosis and sex). The FAMD was conducted using FactoMineR, and factoextra [37-39].

Text added in the section "**Discussion**."

Lines 664-682

Our study has certain limitations. While we conducted functional studies on cultured cells, most of our results were limited to transcriptional analysis with limited protein expressions and no post-translational modifications. Such modifications would more precisely reflect how these identified processes are being altered. In this context, proteomics and metabolomics experiments would be valuable for understanding how the identified transcriptional alterations affect cellular and biochemical processes during the progression of T2D. Additionally, chromatin immunoprecipitation experiments would be helpful for understanding the role of identified regulatory factors in the development of T2D.

Another limitation of our study is that samples were collected at a single time point in the circadian cycle. This limits our ability to analyze how different genes with varying acrophases might be affected at other times of day. To gain a more comprehensive understanding of circadian system alterations in each stage, sampling at multiple time points or analyzing synchronized beige cultures would be necessary. Additionally, the modest sample size, although representative, limits statistical power and subgroup analyses, such as exploring gender-specific effects. Larger cohorts would be necessary to confirm these findings and enhance the robustness of our conclusions. While obesity is a well-established risk factor for T2D, further exploration of the disease's progression in normal-weight individuals would provide valuable insights into mechanisms independent of adiposity.

Despite these limitations, to our knowledge, no previous study has examined the transcriptomic profile across the full spectrum of T2D in human adipose tissue. It highlights significant, stage-specific alterations in gene expression and offers a detailed view of the intricate changes that accompany T2D progression.

Reviewer #3 (Remarks to the Author):

In this manuscript, the anonymous authors demonstrate that metabolic dysfunction is associated with transcriptional changes in adipose tissue including altered expression of clock- and mitochondrial- genes. The authors utilize bioinformatic approaches to provide evidence suggesting that these processes are inter-regulated and involve the transcriptional regulator MAX as a key player. Transcriptional changes in adipose tissue from individuals with diabetes and obesity have been thoroughly studied however, the circadian mechanisms have often been overlooked and ignored. This study make an attempt to study the temporal aspect of metabolic dysfunction in human material and thus, provide valuable insight to the field. Yet, there are a few points that this reviewer would like to highlight:

Author's reply

We appreciate the comments and thoughtful observations of this reviewer.

1. One major limitation is of course that adipose tissue biopsies have been collected at one time point. The reviewer appreciate the challenge of taking biopsies at multiple circadian time points in humans however, this limitation should be clearly stated in the discussion.

Author's reply

Thank you for this pertinent observation.

Initially, we did not contemplate investigating circadian aspects. However, since thousands of genes fluctuate along the circadian spectrum, we considered obtaining the biopsies within as narrow a time period as possible. Nevertheless, our results, along with those of others, warrant future investigation in that direction.

Text added in the section "**Discussion**."

Lines 672-675

Another limitation of our study is that samples were collected at a single time point in the circadian cycle. This limits our ability to analyze how different genes with varying acrophases might be affected at other times of day. To gain a more comprehensive understanding of circadian system alterations in each stage, sampling at multiple time points or analyzing synchronized beige cultures would be necessary.

2. In line with the previous point, the authors turn to an online database to extract information regarding the circadian rhythmicity of differentially expressed genes in their dataset. The authors state that they overlapped the genes with circadian genes in different human and mouse tissues while their dataset is from human subcutaneous adipose tissue. Just because a gene is circadian in one tissue does not necessarily mean that it is circadian in another tissue. The same pertains to comparing circadian rhythmicity between species. Therefore, to gain insight on which diferentially expressed genes that are circadian, the authors should only overlap their data with the circadian dataset from human subcutaneous adipose tissue in the publicly available dataset.

Author's reply

This is a pertinent observation, and the reviewer is correct in stating that different tissues should display different clock-controlled genes. Originally, we included various tissues from both humans and mice because studies on human adipose tissue are very limited. Indeed, to our knowledge, there is only one public database with predicted circadian parameters from different tissues, including subcutaneous and visceral adipose tissues, using the CYCLOPS algorithm [13] from post-mortem biopsies (which itself represents an important limitation) with no information about the death time. Therefore, these data do not include the acrophase, only the predicted phase value in radians. Moreover, in this database, there are only seven Core Clock genes with

predicted circadian oscillation (Per3, Dbp, Nr1d2, Per2, Cry2, and Arntl). Therefore, it is likely that the number of core-clock genes and, consequently, core-controlled genes, would be underestimated in this database. Nevertheless, to provide more targeted information on adipose physiology, we have now included the list of genes with predicted oscillation from visceral and subcutaneous (SQ) adipose tissue, as well as from brown and white adipose tissue in mice (Supplemental Figure 4C, D; Supplemental Table 3).

Text added in the section: "**The circadian clock is progressively altered during the progression of T2D.**"

Lines 381-389

Given that the term "biological rhythm" emerged as the most represented process in the interactome from the down regulated DEGs in the S3 (Supplemental Figure 4B), we assessed the number of known circadian-expressed genes according to adipose tissue from human and mouse databases [31]. We found that 4.6% and 18.4% of the interacting genes to be expressed in a circadian manner in human and mouse adipose tissues, respectively (Supplemental Figure 4C, Supplemental Table 3). However, when considering various tissues from post-mortem human adipose tissue and mice adipose tissue we found that 95% of the interacting genes (Supplemental Figure 4D, Supplemental Table 3) were indeed expressed in a circadian manner ($P<0.05$ JTK-P) [65], this suggests that the circadian clock may play a significant role in the rhythmic expression of these genes.

3. A minor point: The time of biopsy collection should also be mentioned in the methods section.

Author's reply

We appreciate this observation.

Text added in the section: "**Methods**"

Line 101

"Subcutaneous adipose tissue (SAT) biopsies and blood samples were taken between 7:00 and 8:00 hours."

4. The experimental design is very interesting and provides insight into what occurs in the adipose tissue at different stages of disease. The authors compare each disease state with the lean healthy group in each comparison. Would it be interesting to

compare the diferentially expressed genes between each stage, i.e. NWvsOW, OWvsPT2D and PT2DvsT2D? If the differences are incremental and the statistical power is insuffcient to detect differences, this should be stated under the limitations of the study.

Author's reply

This is an important observation. At this time, given that our scope was to analyze the progression of T2D from the perspective of obesity as an inducing factor, we compared each overweight group with the NW group. Further analysis considering only the OW groups will be part of another manuscript. Nevertheless, as preliminary study, we compared the overweight groups as follows: S4 (OW vs PD), S5 (OW vs T2D), and S6 (PD vs T2D) from the interactomes and included the result in the new (Supplemental Figure 14A, B, C).

Text added in the section: "**Cellular integrity is compromised during the progression of T2D.**"

Lines 513-520

Finally, to gain insights into whether the identified processes are deregulated following obesity, we identified the DEGs by comparing the overweight groups as follows: S4 (OW vs PD), S5 (OW vs T2D), and S6 (PD vs T2D) from the interactomes (Supplemental figure 14A, B, C), which are involved in catabolism, inflammation, and cellular stress respectively. While we found a lower number of DEGs within the three comparison sets when comparing the stages S1-3, we observed a higher number of DEGs in the S6 from catabolism compared to the inflammation and cellular stress interactomes. This suggests that inflammation and cellular stress might precede metabolic regulatory processes during the development of T2D.

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