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COL6A3 is regulated by leptin in human adipose tissue and reduced in obesity

--Manuscript Draft--

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Abstract:	Fibrosis of adipose tissue (AT) increases AT rigidity, reduces its expandability and contributes to metabolic dysfunction. Collagen type VI, alpha3 (COL6A3) encodes one subunit of a fibrotic extracellular matrix (ECM) protein highly expressed in rodent AT. Knock-out of collagen VI in rodent AT led to a significant improvement in metabolic health in obese, diabetic (ob/ob) mice however, it is unknown whether this collagen has the same metabolic significance in human AT. We therefore aimed to undertake a comprehensive assessment of COL6A3 in relation to human AT and obesity. Characterisation of COL6A3 in human AT showed 5fold higher expression in the stromalvascular fraction compared with adipocyte expression and significantly higher expression in subcutaneous than omental AT. In both depots COL6A3 expression appeared to be lowered in obesity, whilst diet and surgery-induced weight loss increased COL6A3 expression in subcutaneous AT and serum. Leptin treatment caused a dose dependent decrease in COL6A3 expression although no effect was seen with insulin or glucose treatment and no difference observed in subjects with diabetes. In addition, we found that the collagen expression profile in humans differs significantly from rodents as COL6A3 does not appear to be the predominant collagen

	in adipose, muscle or liver. Our findings oppose those initially seen in rodent studies and most importantly, demonstrate a direct regulation of COL6A3 by leptin. This highlights the importance of a paracrine leptin signalling pathway in human AT and suggests an additional mechanism by which leptin can regulate ECM composition and with it AT expandability.

Dear Professor Gore,

Re: McCulloch et al. “COL6A3 is regulated by leptin in human adipose tissue and reduced in obesity”

Thank you for giving us the opportunity to resubmit this manuscript. Additional data show that the increase of adipose tissue COL6A3 following bariatric surgery is due to its induction by stromalvascular cells and not adipocytes. As advised by the peer reviewer, we set up experiments to study the signal pathways by which leptin regulates COL6A3 in mouse cell lines. This showed that COL6A3 is not regulated by leptin in mouse adipocytes and is in keeping with the influence of SVF in COL6A3 regulation. We now feel that the manuscript has been considerably improved by this additional work, and we thank you and the peer reviewers for your suggestions for improving the manuscript. With your agreement this paper may be the first to show that higher COL6A3 in humans is not adversely linked to obesity or diabetes. This is unexpected and contradicts previous findings which sparked an interest in the potential role of collagens in human diabetes with the example of COL6A3.

We declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work which would have influenced its outcome. The manuscript has been read and approved by all named authors including the new collaborators.

We hope you find our manuscript suitable for publication and we look forward to hearing from you at your earliest convenience.

Yours sincerely,

Dr Katarina Kos

Senior Lecturer

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Reviewer #1

1. **We would like to thank the reviewers for their positive comments and recognising the novelty of our data.**
   The reviewer noted contradicting expression levels in the group of women with and without bariatric surgery: "as an example, in page 12, it is stated that COL6A3 expression was 1.2 ± 0.1 AU in subcutaneous adipose tissue in the cohort of healthy female subjects (whether lean or obese, it is not stated, but their BMI should be comprised between 22.5-28.4-33.7, according to that depicted in page 7), while in the cohort of subjects undergoing bariatric surgery (BMI: 44.4±7.3, Table 1; mostly women), the expression levels were 2.0±02 AU".

   An apparent contradiction in subcutaneous expression levels has been noted between healthy subjects and those undergoing bariatric surgery. However, we would like to draw the reviewer's attention to the fact that these two expression analyses were performed independently and therefore cannot be directly compared. We utilise the $2^{\wedge-\Delta\Delta CT}$ method of analysis, in which expression of individual genes are made relative to an arbitrary value within that probe set, i.e. Ct of the most highly expressed sample. This value will inevitably change between experiments due to the utilisation of unique samples, and the variance in this calibrator sample is the reason behind the varying expression levels (given in Arbitrary Units). On this basis we are only able to compare samples run during a single experiment, not between experiments.

2. **The reviewer points out that the analysis are mainly based on mRNA expression data suggesting that at least a group of samples could be employed for such an analysis. The method employed for RNA isolation in most of the studies (Tri reagent) enables the simultaneous isolation of proteins and, though not of the best quality, these could be employed for western blot analysis.**

   We are aware of the limitations regarding the use of mRNA in isolation without protein analysis. To overcome this we have previously attempted to isolate proteins from the organic phase of TRI reagent, however in our hands this has been of little success. As the reviewer points out, the protein obtained from TRI reagent is not of the best quality. Coomassie staining of proteins from multiple samples demonstrated non-uniform extraction from adipose tissue. As we want to compare directly between our samples we feel it would be inappropriate to use these TRI reagent extracted proteins as we are likely to identify false positives or negatives due to the variance in protein extraction. We included Immunohistology and serum protein analyses.

3. **The reviewer suggests protein analyses, e.g. by Western and Tri reagent and/or immunohistological staining and suggests that it could be that, in spite of the minor decrease in COL6A3 expression levels, COL6A3 protein content undergoes more important changes under the different conditions examined due to, for instance, increased translation efficiency, and/or decreased protein turnover.**

   We have included an additional figure (Figure 4 B-D) to show the immunostaining for COL6A3 in omental adipose tissue. Sufficient tissue was not available to perform a comparative analysis of lean vs obese tissue by Western. We have also documented these results on Page 15, Line 360.
   We have also analysed serum protein levels. We agree that the protein levels could be affected by other processes and discussed this in conjunction with protein levels.

Other comments:
The reviewer requests data on

4. **The insulin sensitivity/resistance of the cohort of subjects for the AT depot study:**

   Unfortunately we do not have blood samples from time of elective surgery for analysis of fasting glucose or insulin from subjects involved in the adipose tissue depot study who underwent elective surgery. We appreciate that data on insulin resistance and sensitivity would be beneficial, however in the depot study

analysis we are performing a within-person analysis (subcutaneous versus omental). Factoring in these variables is therefore unlikely to make a difference to the gene expression levels.

5. **The reviewer believes that the range of HbA1C levels of subjects with T2DM is too wide (from 34 to 62)**

   The HbA1c of subjects with T2DM depends on the effectiveness of their treatment on glycaemic control. In a clinical context the variance observed is not considered unusual and demonstrates good diabetes control which should not affect results. SEM has now been provided on Page 6, Line 132.

6. **The groups of patients for the in vitro explant analysis seem to be quite heterogeneous (at least those chosen for leptin treatment). It would be important to show the basic biochemical data from the patients included in the different groups (fasting glucose, insulin, HOMA-IR, HbA1c)**

   Unfortunately, as answered previously in question 4, bloods were not taken when adipose tissue samples were collected from the tissue bank and therefore we are not able to provide any further information on the biochemical data requested. However, we are performing a within-subject comparison and therefore the characteristics at the time of isolation are believed to have less effect on the outcome. We also performed the experiments on 9-10 subjects for each metabolic regulator to try to eliminate bias from any outliers. BMI mean±SEM for the insulin explants has now been provided on Page 8, Line 172.

7. **The summary provided in Table 2 could include the mean HbA1c levels of subjects in the very low calorie diet (VLCD).**

   Data regarding HbA1c has now been added to Table 2 as requested.

8. **The reviewers is concerned about speculative comments: 'Given that the increase in serum COL6A3 post-surgery did not reach statistical significance, the discussion on the relationship between this observation and the increased expression of this protein in AT and the reduced fat mass to secrete COL6A3 seems too speculative. '**

   We accept the comments made by the reviewer regarding the speculative nature of COL6A3 serum levels and have taken these sentences out of the discussion accordingly. It was replaced on Page 17, Line 401 with the following. "We observed an increase in COL6A3 in the serum of subjects post-surgery, however this did not reach statistical significance, nor did serum levels correlate with COL6A3 expression in SCAT (data not shown). Whilst this may require a larger sample size, collagen is also under continuous turnover by metalloproteinases and its inhibitors and other sources of COL6A3 levels other than AT. Due to scarcity of sample we were unable to directly assess COL6A3 protein levels in AT of these weight loss subjects.

   This will form an essential part of future analyses as it will be pertinent to assess whether an increase in mRNA expression following weight loss correlates with increased protein production, or whether there are post-transcriptional modifications such as reduced translational efficiency or increased protein turnover which may modulate COL6A3 protein levels.

9. **The reviewer is concerned that the relationship between increased leptin levels and decreased COL6A3 expression in adipose tissue might not be directly related if, as demonstrated in both rodents and human, increased fat mass is accompanied by decreased expression levels of the leptin receptors (Wang et al., 2005; Seron et al., 2006). The authors should consider this possibility, also with regards to the cartoon shown in Fig. 5.**

   We appreciate the reviewer's comments suggesting that although we have demonstrated leptin to have a role in regulation of COL6A3 in vitro, this may not translate in-vivo where obesogenic conditions may have rendered adipose tissue leptin resistant. This is a very pertinent point which may indeed affect leptin

action; however we feel that our choice of subjects for the leptin explant study circumvents this issue. SCAT biopsies were taken from four lean (<25kg/m^2) subjects and five obese (>30kg/m^2) subjects. If it were the case that obese subjects were very resistant to the effects of leptin then we would have expected to see very little change in COL6A3 in these five obese subjects. As this cohort of obese subjects comprises over 50% of our explant population we would be left with very little power to observe the effects of leptin on COL6A3 expression. However, when analysing the group of a whole we see striking results on COL6A3 expression across all subjects tested irrespective of obesity, suggesting that the leptin receptor remains sufficiently functional and active within the SCAT of obese subjects. Although we do appreciate that these experiments are taking place outside of the normal environment, we feel that our use of explants (containing multiple AT cell types) from multiple BMI donors makes this more physiologically relevant than simply using a cell line system. We have altered Figure 5 to include data relating to insulin resistance and mention in the discussion that: 'Adding to the complexity is the potential of peripheral leptin resistance in obesity which may selectively attenuate certain properties but not others'.

Minor questions:

How were the SCAT biopsies taken in the bariatric weight loss study at the last (second) time point tested?

SCAT biopsies were performed under local anaesthesia, small incisions (about 1 inch) were made in the abdominal region lateral and close to the umbilicus using a scalpel. This helped to ensure that we were extracting tissue in the same manner as within the original surgical procedure as we aware of the differences in gene expression seen when comparing needle and surgical biopsies. We have included words to this effect on Page6, Line 136.

Page 9, line 169: replace "stained with COL6A3" by "immunostained for COL6A3"

As requested – Now on Page 8, Line 181

Page 11, line 258: replace "Figure 1B" by "Figure 1D"

As requested – Now on Page 12, Line 282

Page 15, line 324, and Fig. 4: confirm whether it is COL1A3 or COL1A1

As requested, changed Page 15, Line 360 to confirm that we studied COL1A1 and not COL1A3

Reviewer #2

We thank the reviewer their constructive comments and we have addressed their concerns as listed below.

Major points

1. **What is the significance of COL6A3 higher expression in SC vs. OM? The two depots have different physiological role and this should be discussed, also with regard to possible implication in insulin resistance and type 2 diabetes.**

   We have now included a paragraph in our discussion commenting on the differential expression results. Page 16, Line 384: "*Whilst increased SCAT fat mass is metabolically more favourable, fibrosis in SCAT, as is also observed in subjects with lipodystrophy, is linked with insulin resistance/diabetes. With the finding of a higher expression in SCAT our results question whether COL6A3 takes part in obesity induced fibrosis given its reduced expression in obese SCAT".*

2. **Although the Authors show increased expression of COL6A3 under diet-induced weight loss, the data showing the same result in surgery-induced weight loss has already been reported, as also pointed out by the Authors (Henegar et al., Genome Biol 2008; Dankel et al., Plos One 2010). Therefore, the effect of diet is somehow expected and this findings appears not to be so novel.**

   Other studies of surgery induced weight loss have been performed on a genome-wide level, simply reporting up or down-regulation of genes. Although our study confirms the observation that COL6A3 is upregulated following surgery-induced weight loss, we are the first to correlate changes in COL6A3 gene expression following surgery induced weight loss with parameters such as circulating leptin which we feel makes our data novel. In addition, when comparing dietary with surgical induced weight loss, different processes play a role with surgery in which weight loss is typically greater there is also a change in appetite hormones and malabsorption which may affect adipose tissue differentially.

3. **What kind of cells were used to assess the effects of leptin? This is not clearly explained. Was it the stromal fraction? The Authors do not use a differentiation protocol, therefore these are not differentiated adipocytes. This should be performed in mature adipocytes, in order to clearly demonstrate the effects of leptin. Adipocytes from human tissue cannot simply be plated and cultured, they need to attach (as stromal fraction) and then differentiation protocols should be applied to induce maturation into adipocytes.**

   We accept the reviewer's comments that it was not clear which cells were used to assess the effects of leptin. We have now specifically altered wording in the methods to demonstrate that primary explant culture was used to determine the effects of physiological regulators of metabolism (Page 8, Line 186). In addition, we have also included an additional phrase in the results to clearly state that explants are fragments of whole adipose tissue (Page 15, Line 340).

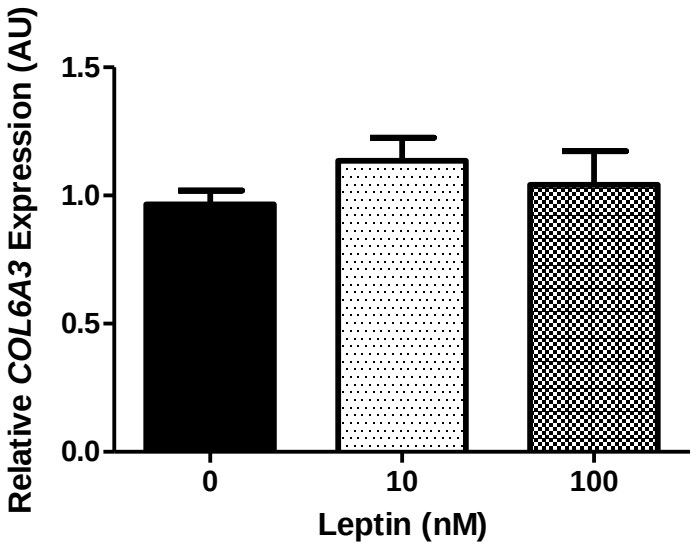
   As mentioned in our results we had previously determined that COL6A3 is expressed in both the mature adipocytes and SVF of adipose tissue. Therefore the advantage of studying explants as whole tissue in which adipocytes are embedded in a collagenous extracellular network containing other cells in the entirety of adipose tissue, and not ignoring the SVF contribution, which would have been the case had we chosen to study differentiated adipocytes.

4. **Figure 3: the error bar for leptin at 100 nM is quite high and is seems strange that the significance is **. In addition, the significance values are not reported in the Figure legend for Figure 3.**

   Please note that this Figure has now been altered to Figure 3A. As requested we have checked the error bar for leptin at 100nM and now report the significance values in the legend for Figure 3. Significance values were determined using a Friedmans One-way ANOVA with a post-hoc Dunns test, calculations have been re-checked and the data remains significant ($p<0.01$) for 100nM leptin.

5. **Experiments showing signaling pathways on the effects of leptin and COL6A3 could have been performed.the Authors state in the Discussion section that this is beyond the scope of their work. However, it is a fundamental point to be addressed, otherwise the paper is mostly descriptive. If not in primary adipocytes, which however may be obtained from the stromal fraction, signaling studies should be carried out, at least in adipocyte cell lines, such as mouse 3T3-L1 cells.**

In light of the reviewers comments we aimed to study the expression of leptin treatment on COL6A3 in mouse 3T3-L1 cells, more specifically to utilise blockers of the key leptin signalling pathways to determine via which cellular pathway leptin is able to mediate COL6A3 expression. However, in our differentiated mouse 3T3-L1 cells we were unable to identify any change in COL6A3 following leptin treatment (figure below from three independent experiments). These results are not necessarily surprising, as (a) the cells are mouse derived, and (b) they are a fully committed adipocyte cell line lacking any other cell type typically present within physiological adipose tissue and their interaction. Our data therefore support the notion that the effects of leptin on COL6A3 expression are likely to be mediated on other cell types present in adipose tissue. To strengthen this argument, and our manuscript, we have included additional data in our revised submission (Figure 2C&D) illustrating that the change in adipose tissue COL6A3 following bariatric surgery is due to alterations in SVF expression and not adipocytes. Therefore, it is not surprising that we are unable to see changes in a cell line system. Whilst the reviewer feels that the paper is mostly descriptive, we would like to highlight the human dietary interventions in this study as well as the study of leptin's effect on collagens in vitro which are functional experiments.



Reviewer #3

Major Points

We thank the reviewer for the encouraging comments and have addressed concerns as listed below.

1. **Regarding data on Table 2, statistical significance when comparing baseline week 0 with week 16 in the VLCD study should be indicated.**

   Statistical significance has now been indicated for all variables at week 16 as requested

2. **The reviewer is asking why BMI correlation was not examined in diet-induced weight change studies (VLCD and FF). A correlation analysis of COL6A3 with respect to serum adiponectin levels and to HOMA-IR should be performed in the VLCD study.**

   We chose to study the correlation between COL6A3 and BMI/serum leptin in the LBS study cohort. This population had a relatively wide spread of BMI (44.4±7.3 kg/m^2) in comparison to the FF group for example where the BMI was 21.4±2.4kg/m^2, or the VLCD group. Therefore we felt that studying the LBS cohort would provide greater power to observe significant correlations between the variables tested.

   We have now included further analysis within the VLCD cohort. COL6A3 mRNA expression levels have been tested for association with a number of other predictor variables including leptin, adiponectin and HOMA-IR as requested. Data are now included in results section, Page 14, Line 313 and in a new table (table 3).

3. **Data on the effects of leptin treatment of SCAT explants are very interesting and suggest a paracrine regulatory mechanism. The reviewer highlights that although not essential, assessment of whether leptin receptors are expressed in SCAT explants would be of interest.**

   Although of potential interest, there is already a substantial body of work in the literature which highlights the presence of the leptin receptor in human subcutaneous adipose tissue. Work performed in 2000 elegantly showed that leptin receptor mRNA is expressed in human white adipose tissue, and through immunohistochemistry demonstrated the presence of the receptor not only on mature adipocytes but also on cells of the stromalvascular fraction (Bornstein SR, Diabetes, 2000). In light of this previous data we have chosen not to assess receptor expression within our explants.

4. **It would be very interesting to analyze the effects of leptin on the expression of other collagen subunits to see whether leptin inhibitory effect is specific or not of COL6A3 expression, and/or whether expression of other collagen subunits is oppositely regulated, such as it has been reported by the authors to occur with the profibrotic protein SPARC (ref.8 in the manuscript).**

   We have performed expression analysis for COL1A1, COL3A1, COL4A1 and COL5A3 and included the data as supplementary information (Figure 3 B-E).

5. **Besides reported data on COL6A3 mRNA expression in AT, COL6A3 immunohistological staining reported as data not shown should be included in the manuscript.**

   We have included an additional figure (Figure 4B-D) to show the immunostaining for COL6A3 in omental adipose tissue. We have also documented these results on Page 15, Line 360.

6. **Data reported in Spencer et al, Am J Physiol Endocrinol Metab 299: E1016-E1027 (2010) should be recognized and discussed.**

Spencer has studied COL6A1 not COL6A3 expression and we have commented on the respective expression of COL6A1 in macrophages as identified by Spencer.

7. **In order to state that "diet and surgery-induced weight loss increased COL6A3 in serum" (line 52 abstract), data depicted on Fig. 2.C. should be statistically significant, and this is not the case. In order to reinforce this parameter, could it be possible to measure COL6A3 in serum in the diet-induced weight change studies (VLCD and FF)?**

Unfortunately there is insufficient sample remaining from the VLCD and FF studies to determine serum COL6A3 levels. We have amended the abstract, Page 3, Line 52 accordingly to take out any information relating to COL6A3 serum levels.

Minor points

Please indicate Sweden as the country of the Gothenburg and Linkoping Universities.

Updated on the title page as requested.

COL6A3 antibody used for immunostaining should be indicated.

Included product information on Page 8, Line 185.

Table 2. Please indicate whether data are expressed as mean +/- SD or SEM.

Updated in the footnote of the Table 2.

References. Please provide full reference of article number 6.

Updated in full Page 4, Line 87.

COL6A3 is regulated by leptin in human adipose tissue and reduced in obesity

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Abstract

Fibrosis of adipose tissue (AT) increases AT rigidity, reduces its expandability and contributes to metabolic dysfunction. Collagen type VI, alpha3 (*COL6A3*) encodes one subunit of a fibrotic extracellular matrix (ECM) protein highly expressed in rodent AT. Knock-out of collagen VI in rodent AT led to a significant improvement in metabolic health in obese, diabetic (ob/ob) mice however, it is unknown whether this collagen has the same metabolic significance in human AT. We therefore aimed to undertake a comprehensive assessment of *COL6A3* in relation to human AT and obesity. Characterisation of *COL6A3* in human AT showed 5 fold higher expression in the stromalvascular fraction compared with adipocyte expression and significantly higher expression in subcutaneous than omental AT. In both depots *COL6A3* expression appeared to be lowered in obesity, whilst diet and surgery-induced weight loss increased *COL6A3* expression in subcutaneous AT. Leptin treatment caused a dose dependent decrease in *COL6A3* expression although no effect was seen with insulin or glucose treatment and no difference observed in subjects with diabetes. In addition, we found that the collagen expression profile in humans differs significantly from rodents as *COL6A3* does not appear to be the predominant collagen in adipose, muscle or liver. Our findings oppose those initially seen in rodent studies and most importantly, demonstrate a direct regulation of *COL6A3* by leptin. This highlights the importance of a paracrine leptin signalling pathway in human AT and suggests an additional mechanism by which leptin can regulate ECM composition and with it AT expandability.

Introduction

Within adipose tissue (AT), mature adipocytes are responsible for the safe storage of dietary triglycerides during times of positive energy balance. Structural support for these lipid-laden cells is provided by the adipose tissue extracellular matrix (ECM), a dense network of fibrous proteins embedded in a proteoglycan-rich, gel like substance (1). The ECM provides structural integrity and transduces sheer stress away from the cells (2). Relative composition of the ECM and ratios of collagen subunits contribute to the mechanical properties of the tissue, such as tensile and compressive strength and elasticity (1). To maintain metabolic health, it is essential for the collagen network to retain flexibility (3). Dynamic remodelling of the ECM is key to allow tissue growth at times of positive energy balance by uncompromised cellular expansion, a process which occurs by adipocyte hypertrophy and adipogenesis (4).

Recently, studies relating to the composition of adipose ECM in obesity have generated substantial interest. Henegar *et al* demonstrated deregulated ECM composition in the AT of obese human subjects (5). Analysis of the AT transcriptomic signature demonstrated differential expression of multiple ECM genes between lean and obese subjects whilst histological analysis revealed increased interstitial fibrosis in obesity (5). Fibrosis is defined as an increased accumulation of insoluble fibers, described by increased synthesis, accumulation and reduced degradation of collagens (3). Increased AT fibrosis results in increased ECM rigidity and reduced expandability, and it is hypothesized that this drives ectopic lipid deposition, contributing to insulin resistance and impaired metabolic health. In our previous work we have shown that human omental (OM) tissue, which has a higher fiber content than abdominal subcutaneous (SC) tissue, is restricted with biomechanical tensile testing resulting in a higher elastic modulus, confirming that increased stiffness and decreased expandability are linked with increased collagen deposition in this depot (6).

In support of this initial work, microarray analysis of human AT following overfeeding and weight gain has demonstrated increased expression of multiple ECM genes (7), whilst more specifically levels of the ECM regulator and profibrotic protein SPARC have been shown to be upregulated in obese AT and associated with insulin resistance in humans (8). In rodent studies, Chun *et al* observed that ablation of an ECM remodelling enzyme, matrix metalloproteinase 14 (MMP14), prevented development of white AT leading to

a lipodystrophic phenotype in null mice (9) whilst the study of an obese, diabetic rodent model highlighted the ubiquitous up-regulation of multiple collagen subunits compared to control animals (2). Interestingly, amelioration of one collagen subtype, COL6, in obese rodents (ob/ob) led to increased AT expansion, improved insulin sensitivity and a reduced inflammatory profile, suggesting that targeting of this collagen subunit may be beneficial for the appropriate storage of triglycerides in AT (2). However, to date very little work has characterised the role of COL6A3 in human obesity and it is unclear to what degree COL6A3 contributes to the total ECM collagen fraction in human AT. As ECM remodelling genes are upregulated with overfeeding (7) we hypothesise that *COL6A3* will be increased with weight gain and obesity and downregulated with weight loss. Given the observation that *COL6A3* expression is higher in an insulin resistant population of Asian origin (2) we postulate that *COL6A3* expression will be higher in the AT of subjects with Type 2 diabetes and will be regulated by treatment with glucose or insulin.

The aims of our study were therefore to perform a comprehensive assessment of COL6A3 in human AT; to study its role in human obesity and diabetes, to assess the effect of weight change on expression levels and finally to identify potential regulatory mechanisms which drive *COL6A3* expression.

Materials & Methods

Subjects for AT depot study

Abdominal SC and OMAT biopsies were obtained from subjects undergoing elective gynaecological surgery at the Royal Devon and Exeter Hospital, Exeter, as part of an ongoing tissue bank initiative. All samples were collected from female subjects with full ethical consent. Subjects with diabetes or malignant diseases were excluded. Lean and obese subjects were matched on a pair-wise basis for age, gender, smoking status and activity levels where known and had the following characteristics: Lean vs Obese (n=15 pairs): Age (years) 44.1±11.6 vs 44.7±11.5 (mean ± SD), p=ns; BMI (kg/m^2) 22.5±1.6 vs 33.7±5.5, p<0.001. Individuals were selected from this cohort for within-subject depot specific analysis if both SC and OMAT was available (n=19, Age 45.8±13.6 years, BMI 28.4±7.7 kg/m^2).

Subjects for diabetes comparison study

All subjects used for the diabetes comparison analysis were female. SCAT biopsies were taken from Type 2 diabetes (T2DM) subjects undergoing bariatric surgery or from those enrolled onto a clinical trial intervention study at the Exeter Medical School. Samples from subjects without diabetes were collected via routine surgery as detailed above. All samples were collected with full ethical consent. Briefly diabetic and non-diabetic subjects were matched on a pair-wise basis for gender and BMI (36.1 kg/m^2 ± 7.6 vs 36.8 ± 6.4, p=ns, n=9 pairs). HbA1c (mean±SEM) of subjects with T2DM was 50.7±3.0 mmol/mol (range: 34-62).

Subjects for bariatric weight loss study

UK subjects undergoing weight-loss surgery (n=11) had SCAT biopsies taken during laparoscopic bariatric surgery and on average 9.5±1 months subsequently. Biopsies during the second visit were taken under local anaesthesia. A small incision was made in the abdominal adipose tissue lateral and close to the umbilicus using a scalpel to permit SCAT sampling. Subjects were included in the study if they were aged between 18-60 years and had no history of inflammatory disease, malignancy, uncontrolled thyroid disease, muscle conditions or chronic liver injury and had not recently been treated with steroids or hormone replacement therapy. All subjects had fasting bloods taken during both visits as well as a multitude of anthropometric

measurements including weight, waist circumference and body composition (measured by Air Displacement Plethysmography technology, Bodpod, Cranlea, UK). This study was approved by the National North West 3 Research Ethics committee-Liverpool East. Patient characteristics are reported in Table 1. Independently, severely obese subjects undergoing sleeve gastrectomy as previously described (10) were recruited and consented in Norway with approval by the Regional Committee for Medical Research Ethics (REK) in Western Norway.

Very low calorie diet study

To assess the effect of dietary induced weight loss, 24 obese subjects (BMI 37.6±4.9kg/m^2) undertook a 450kcal/day very low calorie diet (VLCD) for 16 weeks with a subsequent 2 week re-feeding period. Study assessments were performed at the beginning of the study (week 0), at two intervals throughout the study (week 8 and 16) and two weeks after the VLCD study (week 18) with SCAT biopsies obtained at each time point. The study was conducted in Gothenburg and approved by The Regional Ethics Review Board. Patient characteristics have been reported previously (8,11-13) and a summary is provided in Table 2. Analyses of association between clinical variables and COL6A3 expression were performed using Generalized Estimating Equations in which COL6A3 mRNA expression was set as the dependent variable, and values of each clinical variable, age, gender and week of follow-up were set as independent variables. Correction was done for repeated measurements in the same individual. All variables have been log transformed to perform the tests of association.

Hyperalimentation study

To study the effect of overfeeding, 6 lean subjects (BMI 21.4±2.5kg/m^2) were enrolled onto a fast food diet for 4 weeks in which caloric intake was doubled and exercise restricted to 5000 steps/day. The study protocol has previously been reported in detail (14) and was approved by The Regional Ethics Committee in Linköping. A summary of subject characteristics is provided in Table 2. Subjects had SCAT biopsies taken at the start (week 0) and end (week 4) of the study. Computed tomography (CT)-scans were also performed to assess the size of SCAT, visceral AT (VAT) and total AT (TAT) using a CT HiSpeed Advantage, RP 2.5 (GE Medical Systems, Milwaukee, Wisconsin) as previously described (15).

Subjects for in vitro explant analysis

Matched OM and SCAT biopsies were obtained from 28 female subjects undergoing elective gynaecological surgery as detailed above. The cohort of 28 was split into three groups and 9 matched biopsies processed and treated with recombinant human (rh) leptin, 10 with glucose and 9 with rh insulin. Briefly the characteristics of the individual treatment groups were: Leptin (mean±SD): Age 44.8±9.0 years, BMI 30.2±8.3kg/m^2; Insulin: Age 41.3±9.5years, BMI 25.5±5.5kg/m^2; Glucose: Age 45.1±13.3 years, BMI 27.1±3.3 kg/m^2.

Adipose tissue processing

AT biopsies for depot-specific analysis or for assessment of weight change related expression were snap frozen in liquid nitrogen at the time of biopsy before being transferred to -80°C for long-term storage. AT samples for *in vitro* explant analysis were placed into sterile containers containing collection media (1x Hank's balanced salt solution (HBSS), 0.01M Hepes, 2.5µg/ml Amphotericin B and 0.05mg/ml Gentamicin) for transport to the laboratory.

Immunohistochemistry

Immunohistochemistry was performed as previously described (16). In brief, formalin-fixed, paraffin-embedded AT sections were cut at a thickness of 4µm and collected on positive charged microscope slides. De-paraffinisation, rehydration and immunohistochemistry were carried out by the Bond™-MAX autostainer at room temperature. Slides were treated with or without COL6A3 antibody (1:100; Ab49273 [Abcam]). Staining was also performed in testes as a positive control.

Primary explant culture to assess physiological regulators of metabolism

Samples were processed for explant culture using a modification of Fried and Moustaid-Moussa (17). Briefly, samples were washed in 1xPBS (1% pen/strep) and minced into 5-10mg pieces. Tissue was rinsed through a 230µm nylon mesh using 1xPBS and M199 culture media. Samples were transferred to six well plates (~250mg/well) with 3ml M199 media, supplemented with 5% foetal calf serum and 1% penicillin/streptomycin. Explants were cultured for 24 hours at 37°C under an atmosphere of 5% carbon

dioxide/95% air in the presence of varying concentrations of glucose, rh leptin (Cambridge Biosciences, UK), or insulin (Sigma Aldrich, UK). All treatment conditions were performed in triplicate.

Adipocyte isolation protocol

AT biopsies were minced into small fragments using scalpels before sequentially washing four times in 1x Hank's buffered salt solution (HBSS) to remove contaminating blood. Tissue was enzymatically digested in 1mg/ml collagenase (Gibco, Life Technologies) in HBSS for 1 hour at 37°C with gentle agitation. Digested tissue was centrifuged at 360xg for 5 minutes (4°C) to separate out the lipid filled floating adipocytes from the stromalvascular fraction (SVF). Both fractions were washed in HBSS to remove residual collagenase solution before RNA extraction.

RNA extraction and processing

Total RNA from AT acquired in the VLCD study was extracted using the Qiagen RNeasy lipid tissue kit as per manufacturer's instructions. RNA from all other samples was extracted using the phenol-chloroform-guanidinium-thiocyanate method (18). Briefly ~100mg tissue was homogenised in Tri reagent (Life Technologies) using a Retsch Mixer Mill MM400 in accordance with manufacturer's instructions. RNA quantity was determined spectrophotometrically using Nanodrop technology. RNA was treated with DNase I (ThermoScientific) to remove residual genomic contamination before 500ng was reverse transcribed in a random primed single strand synthesis reaction using high capacity reverse transcriptase (Life Technologies).

Gene expression analysis

COL6A3 expression in subjects in the VLCD study was determined using the Human Genome U133A DNA microarray (Affymetrix) whilst in the hyperalimentation study, expression was determined using HG-U133Plus2.0 (Affymetrix) as previously described (19). *COL6A3* expression in all other samples was determined using qRT-PCR with Taqman technology. Briefly, cDNA was diluted in 0.01M Tris HCl before amplification with a *COL6A3* specific expression assay (Hs00915125_m1) or expression assays for the housekeeping genes *PPIA* (Hs99999904_m1) or *UBC* (Hs00824723_m1) using Universal PCR mastermix (Life Technologies). All samples were amplified in triplicate for each assay and were run alongside a

standard curve to allow assay efficiency determination. Expression analysis of COL1A1 (Hs00164004_m1), COL3A1 (Hs00943809_m1), COL4A1 (Hs00266237_m1) and COL5A3 (Hs00210526_m1) were also determined in AT explants following leptin treatment. *COL6A3* expression change following bariatric surgery was determined, alongside other collagen subunits, using a Taqman Low-Density Array, following normalising to the geometric mean of 4 housekeeping genes (*PPIA*, *UBC*, *GAPDH* and *PGK1*). Amplification was performed using an ABI7900 thermal cycler with the following cycling parameters: 50°C-2 minutes, 95°C-10 minutes, 40 cycles (95°C-15 seconds, 60°C- 1 minute). mRNA expression analysis in the Norwegian bariatric subjects was normalised to TBP. Gene expression levels were calculated using the $2^{\wedge-\Delta\Delta Ct}$ analysis method modified by Pfaffl (20) and are presented as Arbitrary Units (AU).

Serum analysis

Blood was taken from subjects during and 9 months post bariatric surgery for analysis of adipokines, glucose and insulin. The Homeostatic Model Assessment (HOMA) method was used to assess beta-cell function (HOMA-β) and insulin sensitivity (HOMA-IR) (21). Serum insulin levels were assessed using Immunoassay from Cobas-Roche (Sussex, UK) with sensitivity of 0.2μU/ml and intra-assay coefficients of variation of 2.5-4.9%. Serum leptin levels were assessed using ELISA from Millipore (Watford, UK) with sensitivity of 0.5ng/ml with intra-assay coefficients of variation of 2.6-4.6%. COL6A3 ELISA was purchased from USCN Life Science (E92148HU, Oxford Biosystems, UK) with sensitivity of 13.4pg/ml and intra-assay coefficient of variation of <10%. All ELISAs were performed in line with manufacturers' guidelines.

Statistical analysis

All data are presented as mean±SEM unless otherwise stated. Within-subject, lean versus obese and diabetic versus non-diabetic comparisons were made using the non-parametric Wilcoxon signed rank test. Comparison before and after weight change within subjects in the VLCD and hyperalimentation study were performed with a paired two-tailed t-test. For BMI and *COL6A3* expression analysis, data were log transformed to achieve normality before Pearson Correlation Coefficients were determined. For explant studies with varying treatment conditions, comparisons were made using Friedman's ANOVA with post-hoc

Dunn's test. Statistical analysis was performed using IBM SPSS v18 (SPSS, Chicago, US) and statistical significance achieved if $p<0.05$.

Results

COL6A3 depot specific expression

To begin, we determined in which AT cell type and depot *COL6A3* predominates. AT is comprised not only of adipocytes but also fibroblast derived preadipocytes, endothelial cells and immune cells which collectively comprise the stromalvascular fraction (SVF). To determine which cell types contribute to AT *COL6A3* expression, omental AT from six healthy subjects was fractionated using collagenase to separate mature adipocytes from the SVF. Expression analysis within these distinct cellular populations demonstrated that AT *COL6A3* mRNA levels are predominantly contributed to by cells of the SVF. Mean expression level (±SEM) within this fraction was 2.0±0.4 AU compared to 0.4±0.1 AU in mature adipocytes ($p<0.05$, Figure 1A).

Next we analysed the within-subject depot specific expression of *COL6A3* in two independent cohorts, on both occasions observing significantly higher *COL6A3* expression within abdominal SC compared to OMAT (Figure 1B&C). In the first population, a cross-sectional cohort of healthy female subjects, we observed that *COL6A3*expression was 1.2±0.1 AU (mean±SEM) in SC tissue compared to 0.9±0.1 AU within OM samples ($n=19$, $p<0.01$, Figure 1B). In a separate cohort of subjects undergoing bariatric surgery, consisting of both men and women, we again observed the same depot specific trend with SCAT showing higher expression than OMAT (SC v OM: 2.0±02 vs 1.2±0.1AU, $n=11$, $p<0.01$, Figure 1C).

COL6A3 expression and BMI

To determine whether *COL6A3* is differentially expressed with obesity, our Exeter depot cohort was subdivided into lean (BMI<25kg/m^2: 22.5±1.6 [mean±SEM] kg/m^2) and obese (≥25kg/m^2, 33.7.4±5.5kg/m^2) subjects. When comparing expression levels within OMAT we saw a trend towards reduced expression within obese subjects compared to lean (0.7±0.1 vs 1.0±0.1 AU, $n=13$, $p=0.22$, Figure 1D) although this did not reach statistical significance. A similar trend was also observed in SCAT although this once again failed to reach statistical significance (obese vs lean: 1.1±0.1 vs 1.3±0.1 AU, $n=11$, $p=0.47$, Figure 1D). SCAT is essential for buffering surplus free fatty acids in times of energy excess. Dysregulation of ECM within this depot is hypothesised to increase fibrosis, reducing the ability of AT to expand by suppressing adipogenesis

and limiting adipocyte hypertrophy, all of which contribute to ectopic fat deposition. Diversion of triglyceride storage from subcutaneous to ectopic tissues, as seen in lipodystrophy, increases insulin resistance and the risk of diabetes (22). With this in mind we focussed the majority of our further analysis on assessing the expression of *COL6A3* in the SCAT depot.

COL6A3 expression and diabetes

Kahn *et al* demonstrated significantly higher levels of *COL6A3* in a population of Asian Indians who show increased susceptibility to insulin resistance (2). To ascertain whether this correlation with impaired insulin sensitivity is true in other ethnic populations we determined *COL6A3* expression in the SCAT of Caucasian subjects with or without T2DM. All subjects were individually matched for gender and BMI. No significant difference in expression was determined between these two groups (0.8±0.1 vs 0.9±0.2 AU, n=9, p=ns, data not shown).

COL6A3 expression and weight change

Three independent cohorts were used to assess the effects of weight gain or weight loss on SCAT *COL6A3* expression levels. (1) Subjects gaining weight after four weeks of fast food dieting in the hyperalimentation study as described above, (2) subjects undergoing a 16 week VLCD diet followed by 2 weeks of refeeding, and (3) subjects before and after bariatric surgery. *COL6A3* expression was determined in SCAT biopsies before and after each of these dietary or surgical interventions.

(1) *COL6A3* and hyperalimentation

SCAT biopsies were taken from lean subjects at baseline and following four weeks of a high calorie diet (Table 2). As shown in Figure 2A, *COL6A3* expression was not significantly altered despite significant weight gain at the cessation of the study (week 0 vs week 4 [mean±SEM]: 2036±803 vs 2574±641 AU, n=6, p=ns).

(2) *COL6A3* and VLCD

As described in the method section, biopsies were taken from 24 subjects at baseline and following 16 weeks of a low calorie diet (Table 2). As shown in Figure 2A, *COL6A3* expression increased significantly with dietary induced weight loss (week 0 vs week 16 [mean±SEM]: 1725±76 vs 2142±98AU, n=24, $p < 0.001$).

Refeeding without weight gain (weeks 16-18) did not significantly affect *COL6A3*expression levels. We next determined the association between *COL6A3* and metabolic parameters during the course of the weight loss intervention (Table 3). *COL6A3* mRNA levels negatively correlated with BMI, HbA1c and HOMA-IR (if uncorrected for BMI). Of interest, *COL6A3* was found to be negatively associated with both leptin mRNA in SCAT ($p<0.0001$) and serum leptin levels ($p=0.0015$).

(3) *COL6A3* and bariatric surgery induced weight loss

SCAT biopsies were taken from 11 obese subjects during bariatric surgery and on average 9.5±1.1 months post-surgery. In this time period subjects lost on average 26.1kg, reducing their BMI by 8.5kg/m^2, and improving a number of other associated metabolic parameters as shown in Table 1. Following surgery, SCAT *COL6A3* expression was increased 1.6-fold (2.0±0.2 vs 3.2±0.6 AU, $n=11$, $p<0.05$, Figure 2B). There was a concomitant increase in serum COL6A3 levels post-surgery (133.3±12.2 vs 146.5±11.2ng/ml, n=8, p=ns, Fig 2E) whilst a significant reduction in circulating leptin levels was also observed at this time point (57.7±7.2 [mean±SEM] vs 18.2±5.1ng/ml, $n=11$, $p<0.01$, Figure 2F). Bivariate-Pearson correlation analysis highlighted a significant negative association between SCAT *COL6A3* expression and BMI ($r=-0.57$, $p<0.01$, Figure 2G), an association which was also observed when correlating SCAT *COL6A3* expression and circulating serum leptin levels ($r=-0.69$, $p<0.01$, Figure 2H).

To determine from which cell type the changes in COL6A3 mRNA were arising, we measured COL6A3 mRNA expression in adipocytes and SVF cells from an independent cohort of severely obese subjects before and one year after bariatric surgery (n=5, age 44.2±9.5 years, BMI 46.44± 4.8[mean±SEM] before and 33.34±6.3kg/m^2 after surgery). In isolated adipocytes there was no consistent change in *COL6A3* expression after profound surgical weight loss ($p=0.937$) (Figure 2C). On the other hand, in the SVF we found a significant 3.6-fold increase in *COL6A3* mRNA expression ($p=0.043$) (Wilcoxon signed rank test) (Figure 2D).

***COL6A3* expression and physiological regulators of metabolism**

The negative correlation between leptin and AT *COL6A3* in both the diet and surgery-induced weight loss cohorts prompted us to investigate whether *COL6A3* may be directly modulated by physiological regulators

of metabolism. In particular we wanted to study regulation by leptin and, due to the presumed association with insulin resistance as previously reported (2), we also wanted to assess modulation by glucose and insulin. SCAT explants (1mm^3 whole adipose tissue fragments) were treated with varying concentrations of rh leptin (0.01nM, 10nM and 100nM), insulin (0.01nM, 10nM and 100nM) and glucose (5mM, 10mM and 20mM) for 24 hours in culture and expression levels compared to control explants (explants cultured for 24 hours in the absence of the metabolic regulator). Treatment with insulin and glucose had no effect on *COL6A3* mRNA expression (Insulin: Control v 0.01nM vs 10nM vs 100nM – 1.5±0.3 vs 1.7±0.4 vs 1.5±0.3 vs 1.4±0.3 AU, n=9, p=ns. Glucose: Control vs 5mM vs 10mM vs 20mM – 1.8±0.3 vs 1.7±0.3 vs 1.6±0.2 vs 1.5±0.2 AU, n=10, p=ns). Treatment with leptin however caused a dose-dependent decrease of *COL6A3* (Leptin: Control vs 0.01nM vs 10nM vs 100nM – 1.6±0.2 vs 1.3±0.1 vs 1.1±0.1* vs1.1±0.3** AU, n=9, *p<0.05, **p<0.01, Figure 3A). Leptin treatment also caused a significant downregulation of *COL1A1* at 100nM vs Control (0.47±0.08 vs 0.68±0.07, n=9, p<0.05, Figure 3B). There was no change in expression of any other collagen subunit when cultured with leptin for 24 hours.

AT ECM collagen distribution

In order to establish whether, like rodents, *COL6A3* is the predominating collagen in human AT, we determined the expression profile of multiple collagen subunits across a range of metabolically relevant tissues. Using TLDA gene expression technology we simultaneously determined the collagen expression profile in human AT, liver and muscle biopsies, obtained from subjects undergoing bariatric surgery (described above). *COL5A3* was most highly expressed within liver, with levels four-fold higher than any other of the tested collagen subunits (Figure 4A). Interestingly, although *COL6A3* is one of the most predominantly expressed collagens within muscle, this same expression pattern does not translate to AT, with *COL6A3* expression within both OM and SCAT being strikingly lower than *COL1A1*, *COL3A1* and *COL4A1* (Figure 4A). Immunostaining showed that COL6A3 is distributed in the pericellular space of omental AT (Figure 4B).

Discussion

The ability of AT to expand in times of positive energy balance and retract during negative energy balance is an essential homeostatic mechanism, permitting buffering of daily lipid flux and mobilisation of an essential fuel source respectively. For AT to expand, remodelling of the ECM must occur, permitting both growth of existing adipocytes and differentiation of precursor cells. Increased deposition of fibrous proteins within the ECM has been shown to lead to increased rigidity and reduced AT expandability (9), and increased SCAT fibrosis is considered to be a driving force behind ectopic fat deposition. The negative health implications of ectopic fat deposition are clearly seen in patients with lipodystrophy, where storage of triglycerides in sites external to AT correlates with insulin resistance and diabetes (22). Khan *et al*. have previously highlighted the metabolic benefits of knocking out a fibrous ECM collagen subunit (COL6) in a genetically predisposed obese rodent model (2). Ob/ob mice, who are otherwise metabolically compromised, were shown to have increased insulin sensitivity, despite hypertrophic adipocytes, and a reduced AT inflammatory profile when COL6 was knocked out (2). Evidence from this study suggested that in rodents at least, accumulation of COL6 may be a significant factor contributing to reduced AT expandability with overfeeding (2).

Our study investigates whether the same applies to human physiology. Our data suggest that *COL6A3* expression is significantly higher in SCAT when directly compared to OMAT using within-subject paired analysis. This expression pattern was observed not only in a cross-sectional population of healthy female subjects, but also in subjects undergoing bariatric surgery, highlighting that the depot specific differences are not driven by BMI, but remain true across all weight ranges. Whilst increased SCAT fat mass is metabolically more favourable, fibrosis in SCAT, as is also observed in subjects with lipodystrophy, is linked with insulin resistance/diabetes (16, 22). With the finding of a higher expression in SCAT our results question whether COL6A3 takes part in obesity induced fibrosis given its reduced expression in obese SCAT given our observation of a consistent trend of lower expression with increasing BMI. Interestingly, our findings are in contradiction to those reported by Pasarica *et al* (23). In their depot specific analysis, significantly higher *COL6A3* expression levels in visceral compared to SCAT were found alongside a positive correlation between BMI and *COL6A3* SCAT expression (23). There are multiple explanations as to why our observed expression patterns vary including (1) differences in the site from which biopsies were

obtained, (2) variations in the methodology used to obtain biopsies, (3) design of probes to accurately quantify gene expression levels and/or, (4) variations in subject gender with lower *COL6A3* expression in women (23). To gain conclusive results regarding the expression levels of AT *COL6A3* with variable fat mass, we performed a more comprehensive assessment of *COL6A3* expression following diet and surgery-induced weight loss and dietary-induced weight gain, in subjects matched for gender where indicated.

In line with the findings in our initial cross-sectional study we found that weight loss, both diet and surgery-induced, led to an increase in SCAT *COL6A3* mRNA expression. Our findings are in line with other analyses in which *COL6A3* expression was found to be elevated following bariatric surgery (5,24), however as far as we are aware, we are the first to show that there is also a significant increase in expression following diet-induced weight loss, which was of a similar magnitude to surgery-induced weight loss. We observed an increase in COL6A3 in the serum of subjects post-surgery, however this did not reach statistical significance, nor did levels correlate with *COL6A3* expression in SCAT (data not shown). Whilst this may require a larger sample size, it is pertinent to remember that collagen is under continuous turnover by metalloproteinases and its inhibitors and other sources of COL6A3, other than AT, are likely to dilute any association. Due to scarcity of sample we were unable to directly assess COL6A3 protein levels in AT of these weight loss subjects. We extended our study to also assess *COL6A3* expression in relation to the hyperalimentation-induced weight gain but were unable to identify a significant change in expression. Though the number of AT biopsies obtained for this study was small (n=6) (as subjects who failed to adhere to the diet and/or failed to gain weight were excluded), the biopsies assessed were from subjects who achieved substantial weight gain as shown in Table 2 (14). Of note, the weight gain observed in our hyperalimentation study is of a similar magnitude to the weight gain reported in Pasarica's study, in which a significant increase in *COL6A3* was reported (23). Lack of correlation in our study may be explained by the small effect of weight gain on *COL6A3*, the relatively short duration of the study or higher baseline *COL6A3* levels in our subjects, which due to the low BMI of our subjects are less likely to increase, as suggested by Pasarica (23). Similarly, and consistent with this, we did not find a change in *COL6A3* expression after refeeding following dieting in the VLCD study.

We have not found elevated or different levels of *COL6A3* in subjects with T2DM when compared to BMI-matched non-diabetic subjects, which is similar to previous findings (23), nor did we observe changes in *COL6A3* expression in response to glucose or insulin treatment. However, one of the most interesting observations from our study is the strong correlation between *COL6A3* expression and leptin. Leptin is secreted from adipocytes in proportion to fat mass and is a regulator of satiety (25). In patients undergoing bariatric surgery, and those on a VLCD, we see a significant negative correlation between SCAT *COL6A3* expression and circulating serum leptin levels, suggesting that as leptin levels (and therefore obesity) increase, *COL6A3* mRNA levels decrease. To further investigate this novel association we treated primary human SCAT explants with rh leptin and observed a dose-dependent reduction in *COL6A3* expression with increasing leptin concentration. Our data therefore suggest a direct regulatory effect of leptin on the expression of *COL6A3*. Leptin receptors are present on the surface of adipocytes and autocrine/paracrine leptin signalling within adipocytes is known to occur to induce functions such as lipolysis (26,27). In rodents, AT-specific knockout of the leptin receptor was shown to result in increased adiposity and an impaired metabolic phenotype (28) whilst experimentally induced hyperleptinaemia was shown to deplete fat mass, which may in part be explained via increased lipolysis (29). This data clearly points to the presence of a paracrine feedback mechanism within AT by which leptin is controlling fat mass. There is also evidence of a paracrine feedback mechanism in human AT. In 2012, Singh *et al* demonstrated that leptin treatment of differentiating human adipocytes resulted in increased expression of the plasma membrane protein Caveolin 1, which in turn impaired leptin signalling preventing leptin-mediated reduction in lipid accumulation (30). This may suggest that in humans, feedback mechanisms exist by which leptin is able to mediate the storage of triglycerides in a safe manner in times of triglyceride excess and hyperleptinaemia.

Leptin binding to its receptor has been shown to activate a number of downstream signalling pathways and secondary messengers including ERK and JAK/STAT (30), all of which could be hypothesised to modulate expression levels of *COL6A3*. Investigation of the specific mechanisms of regulation were deemed to be beyond the scope of this manuscript but will warrant further investigation to determine how physiological regulators of metabolism are able to modulate ECM gene expression levels. Our observation that COL6A3

expression levels may change with weight only in the SVF, compared to adipocytes, needs further investigation. In particular, understanding the relevance of Col6 in endothelial maturation and its expression in newly formed vessels will be pertinent given the vascular rarefaction observed with obesity. This may go some way to explaining the higher levels observed with lower BMIs (31,32). Collagen VI is also expressed in AT macrophages as recently shown by Spencer at al (33) who identified an association between *COL6A1* expression and AT inflammation and reduced insulin sensitivity. However, we studied more specifically *COL6A3* expression and COL6A3 immunostaining and therefore our results are unlikely to be directly comparable with this study. Previous studies suggest non-coordinate regulation of the mRNA expression of COL6 alpha chains such as in wound healing (31) which may explain the different characteristics.

Our observation that AT *COL6A3* expression does not increase with obesity or metabolic parameters in human subjects is in stark and direct contradiction to previous studies performed in rodents (2). Although it is feasible that the disparity in findings may be due to species differences, we feel another possible explanation may pertain to the system in which COL6 was knocked down in rodents. Initial studies performed in db/db mice demonstrated elevated levels of AT *Col6a3* compared to wildtype animals whilst knockout of COL6 was performed in an ob/ob model (2). It is pertinent to consider that in both of these models of obesity and diabetes, the leptin signalling pathway has been genetically silenced at the level of the receptor or the adipokine respectively. Our data clearly demonstrate the importance of a paracrine leptin signalling pathway in regulation of human AT *COL6A3* expression, and it is therefore possible that loss of leptin signalling in the rodent models may be driving the increase in *Col6a3*, a phenomenon which may not necessarily be seen in physiological obesity, when leptin levels are high. Therefore in the future, it may be relevant to assess the effects of COL6 knockdown in an in-vivo situation in which leptin and its receptor remain functional.

Leptin is a pro-inflammatory cytokine and we have previously shown it is able to potently increase levels of the profibrotic matricellular protein SPARC in human AT (8). This would suggest leptin influences ECM

fibrosis. Data from our current study however suggests that leptin is also able to downregulate ECM components in an obesogenic environment. Although rodent data suggest *Col6a3* to be the most highly expressed collagen in AT (2), our human data suggest that the contribution of *COL6A3* to total collagen levels is minimal. Similarly immunohistochemical data from human subjects has shown that COL6A3 does not reside within fibrous bundles of AT, but was found by us and by others in the pericellular space surrounding adipocytes (23,34). Its position around the adipocytes may if in surplus limit the expandability of existing fat cells by increasing cell rigidity as demonstrated by Chun by which a rigid network limits adipocyte hypertrophy and adipogenesis (9). This would be a concern should we have found *COL6A3* to be increased in obesity yet our findings do not support its contribution to AT fibrosis if we assume to reserve this term to describe the obesity associated increase in AT collagens.

Leptin modulates ECM remodelling and as such its expansion by several mechanisms which we have outlined in a diagrammatic flowchart (Figure 5). It is expressed and secreted in relation to fat mass and upregulated by hypoxia, which amongst others develops as result of growing oxygen diffusion distance between increasingly distant blood vessel and hypertrophied cells in obese AT. Hypoxia increases leptin independently by amongst others an upregulation of *HIF1A* (35,36). As mentioned above, leptin may stimulate AT fibrosis by increasing expression of the fibrotic genes SPARC and TGF-beta, the latter as demonstrated in the peritoneal mesenthelial cells (37). In contrast to its profibrotic action, leptin has also been shown to increase expression of the membrane protein Caveolin which in turn impairs leptin signalling preventing leptin-mediated reduction in lipid accumulation (30). In line with this, we have shown that at high doses leptin decreases *COL6A3* which may allow free expansion and safe storage of surplus fatty acids. With its profibrotic and potentially proinflammatory actions, leptin promotes AT dysfunction whilst it also appears to be able to preserve AT expandability, a contradictory observation. Adding to the complexity is the potential of peripheral leptin resistance in obesity which may selectively attenuate certain properties but not others. Further research is required to disentangle in which tissue leptin takes on specific roles as it may have similar characteristics to SPARC which is multifunctional and its function dependent on cellular environment and tissue type (38).

In conclusion, we have demonstrated that in humans, unlike rodents, *COL6A3* expression does not correlate with impaired metabolic health, does not respond to variations in insulin but conversely decreased with obesity. The observation that *COL6A3* expression levels increase following weight loss, coupled with our data highlighting the direct repression of *COL6A3* following leptin treatment, suggest the presence of a potential paracrine regulatory mechanism in which leptin is able to modulate levels of this pericellular collagen to regulate ECM remodelling. This confirms previous observations of leptin as a potent modulator of the ECM in human AT.

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651 **Figure Legends**

652 **Figure 1. *COL6A3* expression and adipose tissue distribution. (A)** Omental AT (OMAT) biopsies from
653 six female subjects were digested and *COL6A3* expression determined in cells of the SVF (black bars) and
654 adipocytes (grey bars). *COL6A3* mRNA expression levels were determined in paired subcutaneous AT
655 (SCAT) and OMAT biopsies taken from female subjects undergoing gynaecological surgery (n=19) **(B)** and
656 from subjects undergoing bariatric surgery (n=11) **(C).** Expression was measured in the SCAT (black bars)
657 and OMAT samples (grey bars) and group means compared. **(D)** *COL6A3* mRNA expression levels were
658 determined in lean (black bars) and obese (grey bars) subjects matched for age, gender and ethnicity.
659 *COL6A3* mRNA expression levels were determined in SCAT (n=11 pairs, lean vs obese) and OMAT (n=13
660 pairs, lean vs obese). Expression is presented as mean±SEM (AU) and statistical significance determined
661 using Wilcoxon signed rank test, * $p<0.05$, ** $p<0.01$.

662 **Figure 2. *COL6A3* expression following weight gain and weight loss. (A)** *COL6A3* expression was
663 determined in SCAT of six subjects before and four weeks post (W4) exposure to hyperalimentation in the
664 fast food study (FFS, black squares), and dieting at baseline and week 16 (W16) of a VLCD (n=24). An
665 additional biopsy was taken at week 18 (W18) following two weeks of refeeding (black circles). Data are
666 presented in Signal Intensity Units. Black horizontal bars represent mean expression. **(B-H)**SCAT biopsies
667 and serum samples were obtained from subjects during and post bariatric surgery. **(B)** *COL6A3* expression
668 was determined in SCAT biopsies (n=11) before and after surgery. *COL6A3* expression in paired adipocytes
669 **(C)** and SVF **(D)** after bariatric surgery (n=5), different lines indicate the paired adipocyte and SVF samples.
670 COL6A3 levels were also determined in the serum of subjects pre and post surgery **(E).** *COL6A3* expression
671 and BMI correlation **(F)**. **(G)** Circulating leptin changes after surgery. Leptin (ng/ml) decreased post-
672 surgically as determined using ELISA's (n=9). **(H)** *COL6A3* expression was correlated to serum leptin. Data
673 are presented as mean±SEM, * $p<0.05$, ** $p<0.01$.

674 **Figure 3. AT *COL* expression following 24 hour culture with recombinant leptin**. SCAT biopsies from
675 nine female subjects were processed to explants and treated with recombinant leptin (0.1nM, 10nM and
676 100nM) for 24 hours. Expression levels of *COL6A3* **(A)** and other collagens **(B-E)** were determined using a

Friedman One-way ANOVA with a post-hoc Dunn test comparing to control samples. Expression levels are provided as mean±SEM in Arbitrary Units (AU). *p<0.05, **p<0.01.

Figure 4. ECM collagen distribution. (A) Comparative collagen expression in metabolically relevant tissues. Tissue biopsies were obtained from 11 subjects undergoing bariatric surgery. Expression levels of *COL1A1* (black), *COL3A1* (grey), *COL4A1* (black squares), *COL5A3* (white) and *COL6A3* (black diagonal stripes) were determined following normalisation to four endogenous controls (*PPIA*, *UBC*, *PGK1* and *GAPDH*). All expression levels are relative to *COL1A1* expression within the respective tissue. Immunostaining of COL6A3 in human omental adipose tissue**.** Immunohistochemical staining to detect COL6A3. Representative microphotographs showing collagen VI (brown) staining in extracellular spaces of omental adipose tissue **(B)** and testis (positive control, **(C).** Negative control showing omental adipose tissue processed without primary antibody **(D).** Nuclei were counterstained with DAPI (blue). Magnification (400x), scale bar = 2µM.

Figure 5. Leptin's regulation of fat mass in the development of obesity and relevant feedback loops. Leptin levels increase in relation to fat mass and is upregulated by hypoxia. It controls fat mass by several mechanisms including centrally by a reduction of appetite. In AT it acts in a paracrine/autocrine manner, it is decreasing fat mass by promoting lipolysis and may predispose to fibrosis by increasing SPARC and TGFbeta. This may in part attribute to leptin resistance as marked by the dotted line. Less understood are pathways by which it aids healthy fat expansion furthered by an increase in Caveolin1 and reduction in *COL6A3.*

Table 1
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Table 1. Clinical characteristics of subjects pre and post bariatric surgery

Parameters	Pre Surgery	Post Surgery
Population	11	11
Gender (M/F)	(3/8)	
Age at surgery (years)	46.3±8.1	
BMI (kg/m^2)	44.4±7.3	35.9±6.6**
Weight (kg)	129.3±27.8	103.2±22.5**
Systolic pressure (mmHg)	129±15.8	120±11.3
Diastolic pressure (mmHg)	86±10.7	81±7.1
Diabetes (%)	6(55%)	3(27%)
% Body fat	49±6.5	42.8±10.6*
% Lean mass	51.0±6.5	57.5±10.1*
Past smokers (%)	6(55%)	3(27%)
Current smokers (%)	1 (9%)	3 (27%)
Time since operation (months)	N/A	9.5±1.1
Fasting glucose (mmol/L)	5.3±0.8	4.7±0.5*
Fasting Insulin (mU/L)	19.9±8.0	10.9±4.3**
HbA1c (mmol/L) (range)	34-54	32-47*
HOMA-IR	4.7±1.9	2.3±1.0**
Triglycerides (mmol/L)	1.4±0.6	1.4±0.7
Total cholesterol (mmol/L)	4.3±1.0	4.8±1.0
Total cholesterol/HDL	3~~0~~.8±0.9	3.6±0.9
ALT (IU/L)	29.1±10.6	24.7±16.9

Data are means ± SD. *p<0.05, **p< 0.01.

Table 2
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Table 2. Clinical characteristic of subjects undergoing dietary intervention studies.

Parameters	VLCD Study				FF Study	
	Baseline	8 weeks	16 weeks	18 weeks	Baseline	Week 4
Weight (kg)[1]	119±20	101±17	91±16***	91±16***	64.2±9.3	71.5±13.0**
BMI (kg/m^2)[2]	37.6±4.9	31.8±4.1	28.6±4.1***	28.9±3.9***	21.4±2.5	23.7±3.3**
Waist (cm)[1]	123±12	110±12	101±13***	101±13***		
Fasting glucose (mmol/l)[2]	6.0±1.6	4.5±0.7	4.5±0.7***	5.0±1.0***	5.15±0.72	5.8±0.77
Fasting insulin (mU/l)[2]	16±7.4	7.0±4.1	4.3±2.2***	6.3±3.7***	4.2±2.7	8.8±2.9*
HOMA-IR[2]	4.4±2.7	1.4±0.9	0.9±0.5	1.5±1.3***	0.7±0.5	1.63±0.5*
HbA1c (%)	4.9±0.9	4.3±0.5	4.2±0.4	4.2±0.4		
SCAT area (cm^2)[3]	526±166		311±137**			
VAT area (cm^2)[3]	241±76		101±48**			
Systolic blood pressure (mmHg)[3]	138±17	121±12	117±14***	124±16***	115±5	125±14

Serum leptin[3] (ng/ml)	38.4±19	9.8±7.5	6.3±5.9	8.8±6.7***	6.4±10.9	14.8±17.7*
Serum adiponectin[3] (ng/ml)	9.0±5.3	11.5±7.3	13.6±6.4	15.2±7.9***		
CRP (mg/l)[4]	5.3±5.8	4.6±5.4	2.4±1.5*	2.4±2.2*		

The table shows subjects undergoing a very low calorie diet (VLCD) (n=24) or hyperalimentation by a fast food (FF) diet (n=6). Computed tomography was performed at weeks 0 and 16 for adipose tissue area calculations in the VLCD study. Data are presented as means ± SD, *p < 0.05, **p < 0.01,***p < 0.001 when comparing baseline week 0 with week 16, or week 18 in the VLCD study and with week 4 in the FF study. Data taken from [1] (12), [2] (11), [3](8), [4](10).

Table 3
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Table 3. Association analysis between COL6A3 and anthropometric variables and metabolic parameters during diet-induced weight loss

Predictor Variable	Estimate	P value
Leptin mRNA expression	-0.2075	<0.0001
Serum Leptin	-0.1098	0.0015
Adiponectin mRNA expression	0.0040	0.9717
Serum Adiponectin	0.0733	0.5339
HOMA-IR	-0.1347	0.0047
HbA1c	-0.7346	0.0167
BMI	-0.6087	0.0451

The model assumes that the relation (slope) between the given trait or parameter and the mRNA level is constant at all four study visits (weeks 0, 8, 16, 18), yet allows for week specific levels of mRNA. The table shows the estimated slopes together with the p-value from the tests for whether the slope of these four parallel lines is equal to zero (null hypothesis).

Figure 1

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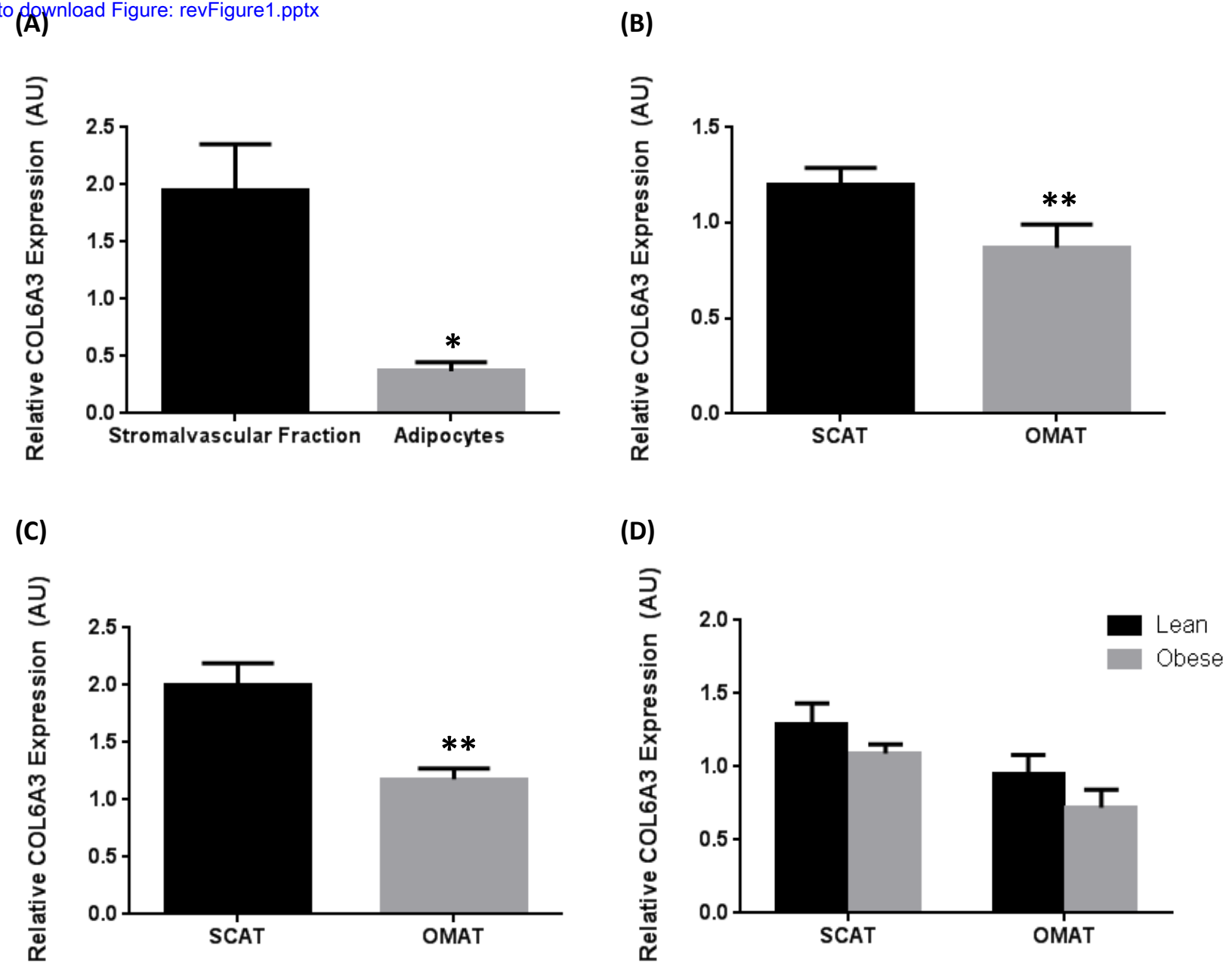

Figure 2

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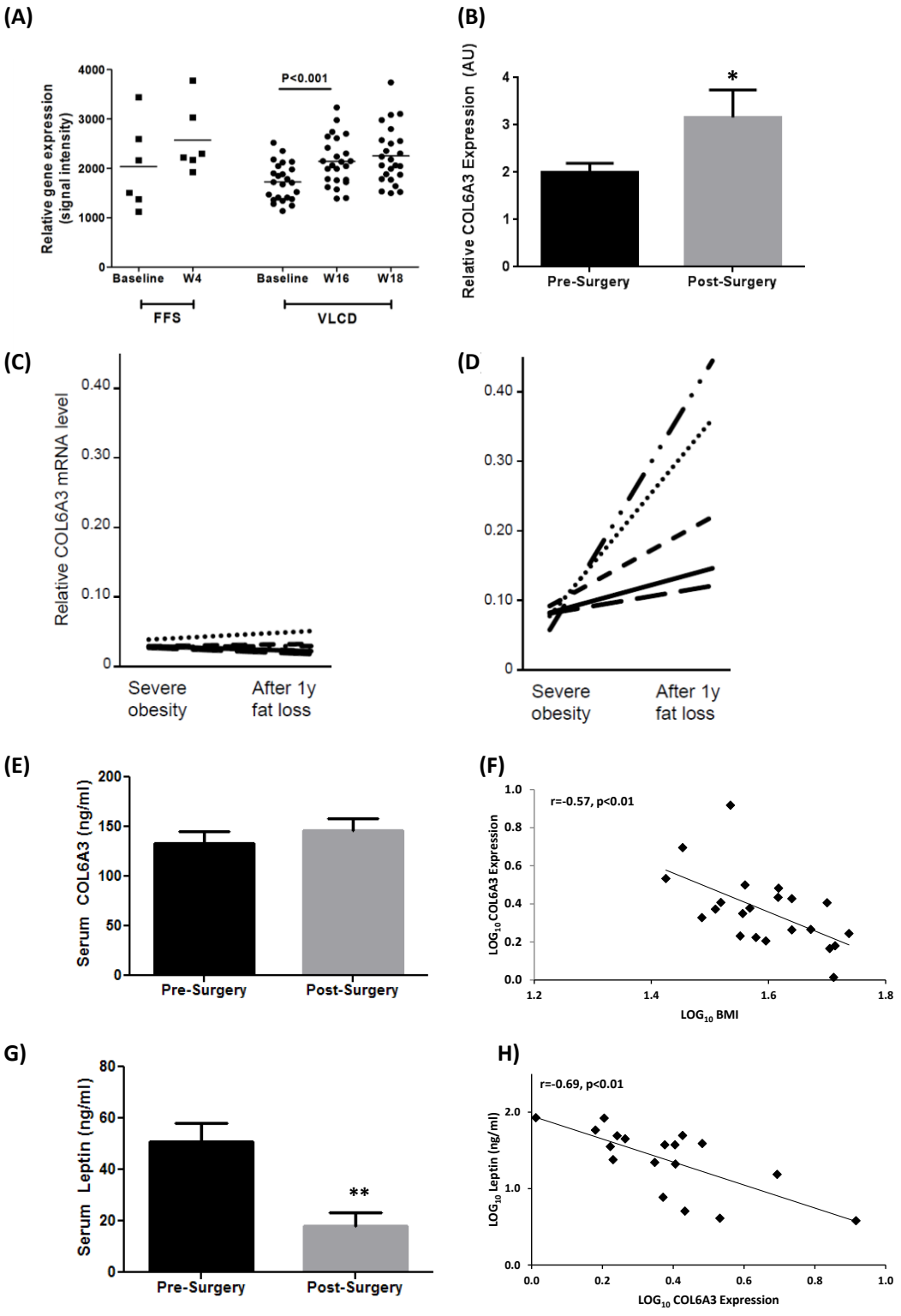

Figure 3

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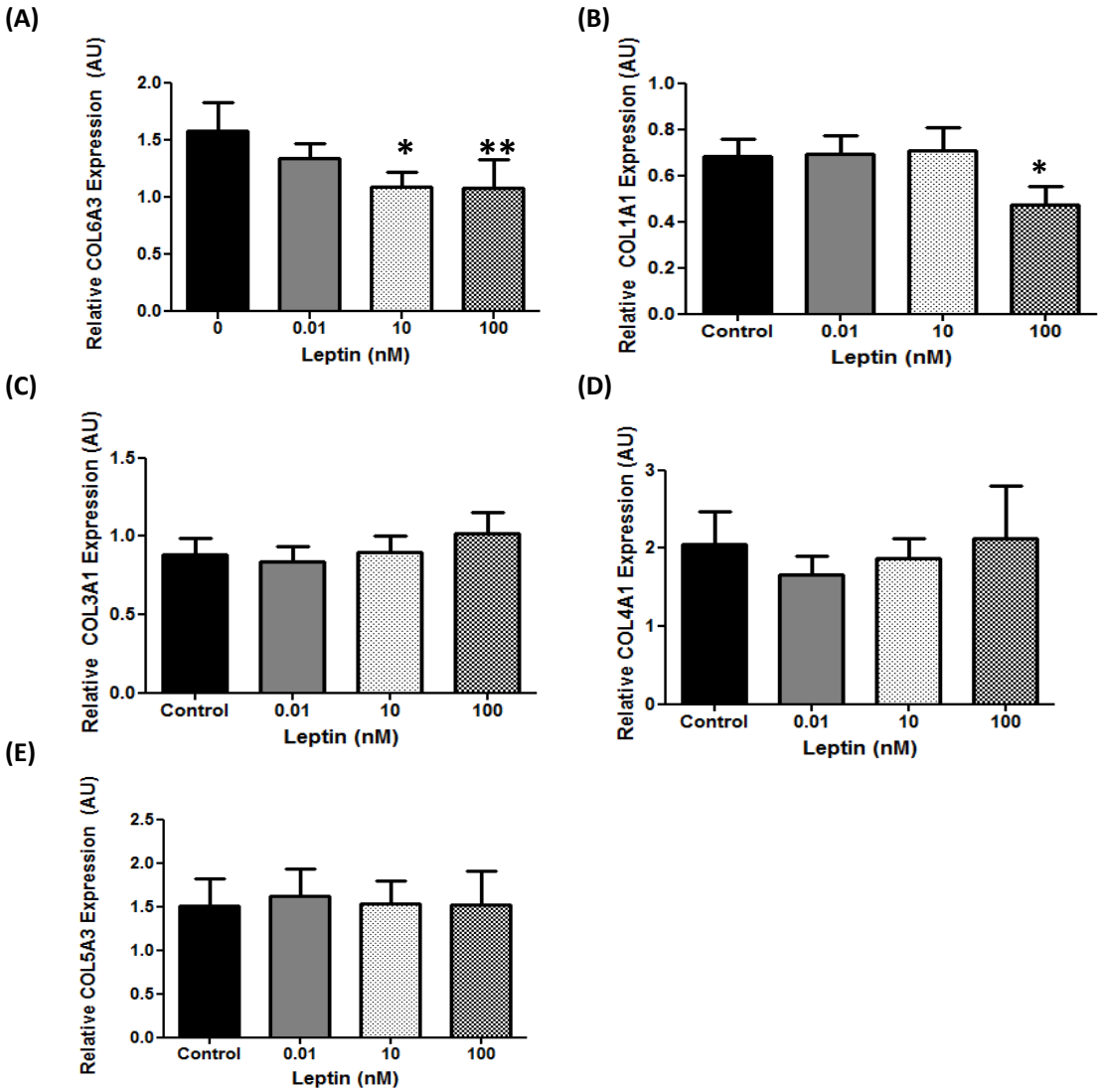

Figure 4

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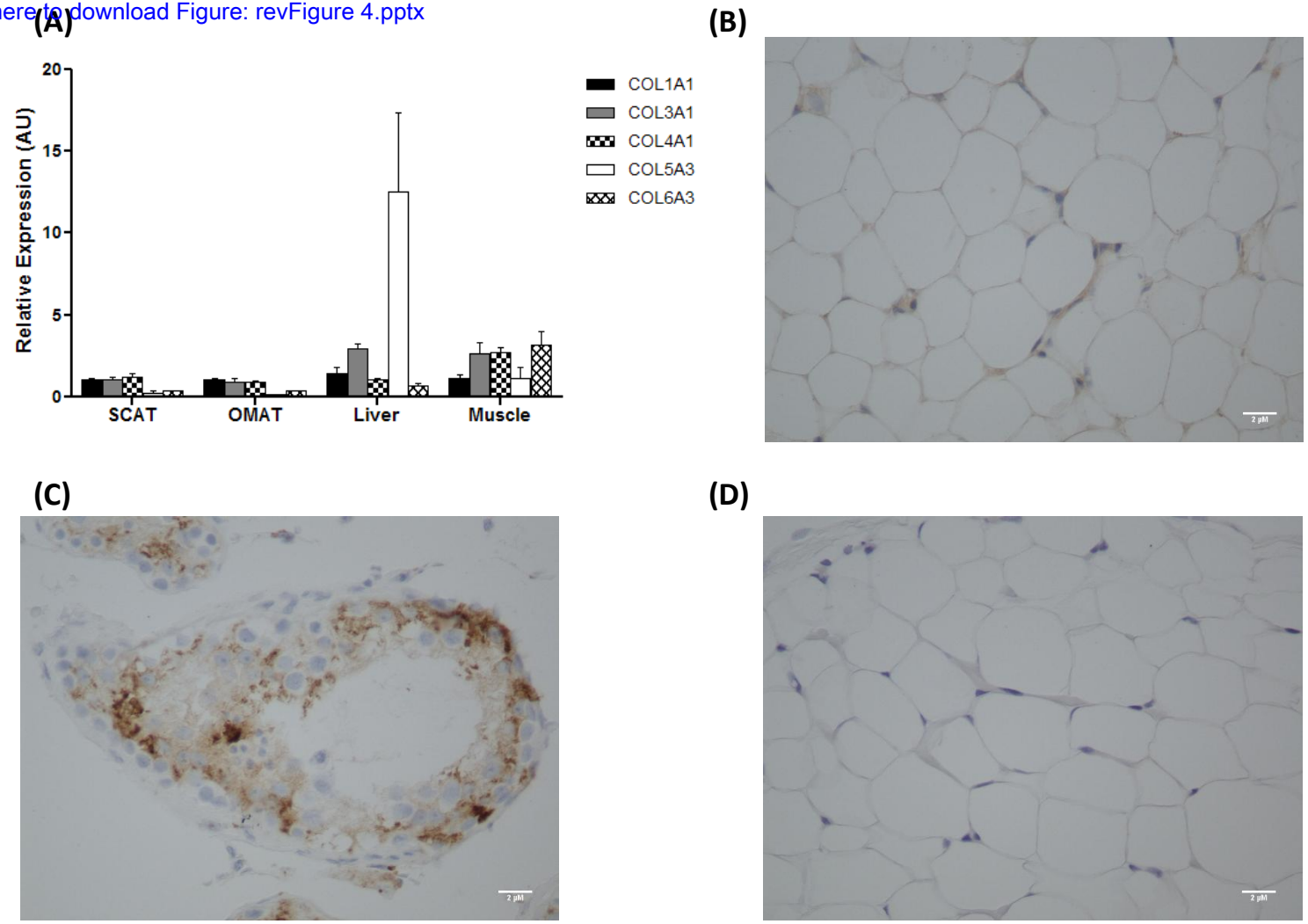

Figure 5

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