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Severe Obesity and Bariatric Surgery Alter the Platelet mRNA Profile

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Abstract

Mechanisms explaining the relationship between obesity and cardiovascular disease (CVD) are needed. Despite growing recognition of the importance of the anucleate platelet transcriptome, low levels of RNA in platelets make assessment difficult. We sought to perform unbiased platelet RNA profiling in obesity by performing a prospective study of severe obesity and weight loss via bariatric surgery on platelet characteristics and mRNA profile in 26 pre-menopausal, non-diabetic women (31.6 ± 8.4 years; BMI 43.0 ± 6.5 kg/m^2) who underwent sleeve gastrectomy. Ten women of similar age with normal BMI served as controls. Platelet activation via flow cytometry was assessed before and after surgery. RNAseq was performed on platelet isolates from a subset of 13 subjects (8 obese women and 5 normal-BMI subjects). Platelet count, size and age did not differ between control and obese women. However, platelet surface P-selectin and CD40 were higher in obesity. RNAseq demonstrated 629 differentially abundant transcripts in obesity. Notably, *S100A9* and *AGER*, established markers of cardiovascular risk, were two of the most highly upregulated transcripts (each >2.5 fold). At 6 months post-operatively, subjects lost 26.1 ± 5.8% body weight and inducible platelet P-selectin expression was reduced. Expression of 170 transcripts was affected by surgery, but only a small fraction (46/629) were genes found altered in obesity. We demonstrate that obesity is associated with an altered platelet transcriptome and increased platelet activation, which is partly attenuated by bariatric surgery. These observations suggest that platelets may contribute to increased cardiovascular risk in obesity through a variety of mechanisms.

Keywords

platelet; RNA; obesity; bariatric surgery; cardiovascular diseases

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Declaration of Interests

All authors declare no conflicts of interest.

Introduction

Obesity contributes to global cardiovascular disease (CVD) burden and one potential mechanism for this heightened risk is platelet dysfunction in obesity (1–4). Beyond being crucial mediators of hemostasis, platelets are increasingly recognized as immune cells (5). Of particular relevance to atherothrombotic risk, products released during platelet activation, as well as platelet-derived RNAs and miRNAs (both directly and via platelet-derived microparticles) are known to affect function of both macrophages and endothelial cells (6,7).

Despite growing recognition of the importance of the platelet RNA profile, low levels of RNA in platelets make assessment difficult. Nonetheless, previous work targeted specifically at inflammatory genes demonstrated differential expression of a number of transcripts in obesity (8). One of these, *S100A9* (also known as *MRP14*), has been found to be predictive of adverse cardiovascular outcomes in coronary and peripheral artery disease (9,10). Recently, RNA-sequencing (RNAseq) technology has introduced a powerful tool for understanding roles of platelets in various disease states. Reports of whole transcriptomic profiling of platelets are few and largely limited to healthy individuals (11–15), although some reports in disease, specifically CVD, are available (16,17). Notably, unbiased platelet RNA profiling in obesity has not previously been performed. Herein, we report the first RNAseq analysis of human platelets in obesity and following substantial weight loss via bariatric surgery.

Methods

All premenopausal women aged 21–50 years undergoing sleeve gastrectomy (18) at Bellevue Hospital (NY, NY) over a 4 month period were approached for a prospective observational pilot study approved by the NYU School of Medicine IRB. A single gender and limited age range were chosen because of the known differences in platelet gene expression by age and gender (8,19). Patients were excluded if they were active smokers, taking lipid-lowering agents, diabetes medications, anticoagulants, or any medication known to affect platelet function.

Subjects providing informed consent presented to the NYU-HHC CTSI Clinical Research Center after an overnight fast, between 2–6 weeks prior to surgery (in a state of weight maintenance) and at 6 months following surgery, for anthropmetric measures and blood sampling. Blood draws were performed with 19 gauge needles without tourniquets into sodium citrate-containing tubes (BD Vaccutainer, Franklin Lakes, NJ). Assessments of platelet characteristics and platelet isolation for RNA collection began within 15 minutes of blood draw completion.

Premenopausal women, aged 21–50 years, recruited via flyers posted around the medical center, with normal BMI (18.5–24.9kg/m^2), but otherwise meeting all enrollment criteria and of similar race and ethnic background as obese subjects, were enrolled as controls. These subjects also were non-smokers and not taking lipid-lowering agents, diabetes medications, anticoagulants, or any medication known to affect platelet function. Control subjects attended a single study visit that was identical to those of obese subjects.

Measures of platelet characteristics and activation

Platelet count and mean platelet volume (MPV) were measured on a Coulter Ac T diff 2® (Beckman Coulter, Brea, CA). Assessment of surface markers of platelet activation (P-selectin with and without exposure to thrombin, and CD40), and reticulated platelets (thiazole orange) was performed on a C6 Plus flow cytometer (BD Accuri, San Jose, CA) as previously described (10). Statistical comparisons were performed with independent samples t-tests (obese versus normal BMI) and paired-samples t-tests (obese before versus obese 6 months after surgery) using SPSS (Armonk, NY).

Platelet RNA isolation and sequencing

RNA from washed platelets was isolated as previously described (17). In short, blood collected with sodium citrate as the anticoagulant was centrifuged (200g × 10min) to obtain platelet-rich plasma (PRP). PRP was centrifuged (1000g × 10min) and the platelet pellet resuspended in Tyrode's buffer. CD45+ and Glycophorin A magnetic beads (EasySep, STEMCELL, Vancouver, Canada) were used to to limit non-platelet contamination. Depletion effectiveness was assessed by flow cytometry and demonstrated a platelet-to-leukocyte ratio of $<1:1\times10^7$. Further, there was no detectable CD45 transcript in platelet RNA samples. TRIzol® Reagent (Invitrogen, Carlsbad, CA) was added to purified washed platelet pellets, the pellets disrupted and samples stored at −80C until isolation using Quick-RNA MiniPrep kits (Zymo Research, Irvine, CA) according to the manusfacturer's instructions.

Deep RNA sequencing of platelet RNA was performed in the NYU Genome Technology Core. Briefly, following demonstration of adequate RNA quality via RNA 6000 Pico kit (Agilent, Santa Clara, CA) analysis, rRNA was depleted using Ribo-Zero Gold (Illumina, San Diego, CA). RNASeq library preps were made using the Illumina TruSeq® Stranded mRNA LT kit on a Beckman Biomek FX instrument, using 500 ng of total RNA as input, amplified by 10 cycles of PCR, and run on an Illumina HiSeq 4000, as single read 50. RNAseq differential expression analysis was performed for three lanes of a single-read 50 Illumina HiSeq 4000 run. Per-read per-sample FASTQ files were generated using the bcl2fastq2 Conversion software (v2.17) to convert per-cycle BCL base call files outputted by the sequencing instrument into the FASTQ format. The alignment program, STAR (v2.4.5a), was used for mapping reads of samples to the human reference genome hg19 and the application FastQ Screen (v0.5.2) was utilized to check for contaminants. Statistical analysis was performed with the R DESeq2 package (Bioconductor v3.3.0).

Obesity- and bariatric surgery-affected genes were identified as those differentially expressed in obese versus control subjects or pre-operative versus post-operative samples, repectively, that met 1) p-value ≤0.05 and 2) abundance threshold cut-off of $>1/15000^{th}$ the expression of beta-actin (base mean counts ≥5). Pathway analyses were completed using IPA (QIAGEN Inc., Germantown, MD) and DAVID 6.8 (NIAID, NIH).

Transcript Validation

The QuantiTech Whole Transcriptome kit (QIAGEN Inc., Germantown, MD) was used to amplify cDNA from isolated platelet RNA according to the manufacturer's instructions. Fast

SYBR Green (Applied Biosystems, Foster City, CA) real time polymerase chain reaction (PCR) mix was then used according to manufacturer's instructions to quantify expression of several transcripts of interest in amplified cDNA on a QuantStudio 7 Flex (Applied Biosystems, Foster City, CA) quantitative PCR machine. Primer sequences are listed in Table 3.

Results

Surgical subjects were young (31.6 ± 8.4 years) and severely obese (43.0 ± 6.5kg/m^2). There was no difference in baseline characteristics between those 19 subjects who returned for six-month visits and the entire obese cohort (n=26). Normal BMI subjects were of similar age and ethnic background. At six months after surgery, subjects lost 26.1 ± 5.8% body weight (30.4 ± 9.1kg) and significantly reduced BMI and waist circumference (Table 1).

Platelet characteristics

Platelet count, size and age did not differ between obese and normal BMI subjects (Table 1). Further, these measures did not change at six months following sleeve gastrectomy. In contrast, platelet surface CD40 expression was significantly elevated in obese subjects relative to normal BMI women. Additionally, there was a trend towards elevated P-selectin expression on unstimulated platelets from obese subjects which became statistically significant with thrombin exposure (Figures 1a - 1c). Post-operatively, P-selectin expression to thrombin was reduced and similar to normal BMI subjects.

Platelet RNA profile

RNAseq in a cohort of 13 – 8 obese and 5 normal BMI subjects – identified 629 differentially expressed transcripts in the platelet RNA profile of obese versus normal BMI women (Figure 2a). Approximately 70% of these transcripts (443/629) exhibited greater expression in obesity, including 89 with more than 2-fold higher expression. 34 genes were expressed at <50% the level of normal BMI subjects. The top differentially expressed transcripts in obesity are presented in Table 2a. Obesity was associated with increased expression of genes involved in a number of pathways related to translation and metabolic regulation (Figure 2b). Upstream factors predicted to be involved in differential platelet RNA expression in obesity were transglutaminase 2, IFNγ, and CEBPα.

Among 5 women who had platelet RNA profiling performed before and 6 months after bariatrc surgery, 170 transcripts were differentially expressed (Figure 2c). Of note, 62% of these transcripts (105/170) were more highly expressed following surgery. The top differentially expressed transcripts following bariatric surgery are presented in Table 2b. 19 of the 629 transcripts differentially expressed in obesity were also found to be affected at 6 months after surgery (Figure 2e, Table 2c). Accordingly, pathway analyses modestly suggested that metabolic pathways different from those identified above (Figure 2b) were altered with surgical weight loss (Figure 2d). Upstream factors predicted to be involved in changes in expression at six months following bariatric surgery were E2F, TCF4, mir-30 and EGR1.

Real time quantitative PCR of several highly differentially expressed transcripts, as well as others in implicated pathways largely validated the RNAseq findings (Figure 3).

Discussion

We report the first unbiased assessment of the platelet RNA profile in severe obesity as well as the effects of >25% body weight loss following bariatric surgery on this profile. While we observed that common variables, including platelet count, age (reticulated platelets) and MPV did not differ, we found >600 transcripts to be differentially expressed between platelets of obese and matched, normal-BMI women. Additionally, several direct markers of platelet activation, surface CD40 and P-selectin, were greater in obese subjects.

As prior assessments of platelet RNA in obesity were limited to several dozen targeted genes (8,20), most transcripts and implicated pathways that we report are novel. Given increasing recognition of the importance of platelet RNA profile in mediating atherosclerotic cardiovascular disease (ASCVD) (5–7,9,10), we are excited by the wealth of insight potentially provided by our data. Notably, we identified *S100A9* as one of the most upregulated transcripts in obese versus normal-BMI subjects. This observation has precedence and profound relevance. Freedman et al. found *S100A9* to be one of the most highly upregulated transcripts in platelets in obese subjects in an analysis limited to inflammatory transcripts in the Framingham Offspring Study (8). Further, platelet *S100A9* mRNA is elevated in STEMI versus stable coronary artery disease, predictive of adverse ASCVD events, and implicated mechanistically in these adverse outcomes (9,10,21). Our study supports that increased expression of *S100A9* in platelets is a novel CVD risk factor associated with obesity.

S100A9 expression and BMI exhibited a strong correlation (r=0.64, p=0.02) in our subjects who were without significant obesity-related metabolic comorbidities. While this suggests that adiposity is the most important single factor driving overexpression of the transcript, *S100A9* was not one of the few transcripts affected at 6 months after bariatric surgery. In fact, expression of only 3.2% of obesity-altered transcripts was affected at six months following bariatric surgery. The plasticity of the platelet mRNA profile in obesity is indeed unclear. Recent work suggests that obesity-induced changes in hematopoetic stem cells persist even after normalization of body weight (22). Whether changes in megakaryocytes within the bone marrow (the source of platelet RNA) occurring with obesity are also durable remains unknown.

Beyond the known risk factor *S100A9*, our data support widespread alteration of the platelet RNA profile in obesity. Notable themes from our analyses include upregulation of several pathways, including: eIF2 and mTOR signaling, and translation broadly. Further, *AGER* was one of the most upregulated transcripts in severe obesity despite the absence of diabetes in our subjects. Although qPCR demonstrated detectable AGER transcript in >90% of platelet mRNA in this study, the protein product of *AGER* has yet to be identified in platelets, so the relevance of this observation remains uncertain.

Several additional transcripts influenced by obesity immediately strike us as potentially quite important: *ALOX5*, *CLEC2*(*B,D,L*), *CXCL3*, *MMP9*, *MYD88*, *SELP*, *S100A4*, *S100A6*, *TLR1* and *WAS*. Given platelets' ability to affect atherothrombosis via transfer of their cargo via microparticles to leukocytes or endothelial cells (6,7), an altered platelet RNA profile, as we demonstrate in obesity, may represent an additional measure of cardiometabolic risk that is separate and independent from more traditional measures of platelet activation and thrombotic potential. Of particular relevance in this regard, weight loss has previously been shown to reduce circulating platelet-derived microparticles in obesity (23).

Platelet expression of P-selectin and CD40 occurs with activation and both proteins mediate interactions of platelets with endothelial and immune cells (24,25). Platelet surface P-selectin and CD40 are felt to promote atherosclerosis through these cellular interactions (26). We specifically observed that P-selectin expression to thrombin was elevated in obese subjects, but that expression was normalized by 6 months after bariatric surgery. Our finding of elevated platelet surface P-selectin expression in severely obese subjects has precedence, and higher P-selectin is hypothesized to partly contribute to excess ASCVD in obesity (27). Elevated platelet P-selectin has been observed in obese adults both with the metabolic syndrome (28,29) and without other cardiovascular risk factors (27). Further, BMI has been independently associated with increased P-selectin expression to agonists among diabetics (30). Given that at 6 months after surgery, most of our subjects (11/19) remained obese (BMI 32.1 ± 6.0 kg/m^2), the observed normalization of P-selectin expression to thrombin at this time point suggests that obesity per se may not be a primary factor contributing to increased expression of P-selectin in our subjects pre-operatively, but a currently undetermined factor present in our metabolically healthy subjects that nonetheless improves following bariatric surgery.

While soluble CD40 ligand has been previously noted in obesity, and found to be modifiable with weight loss (31,32), to our knowledge, ours is the first study to directly measure platelet surface CD40 expression in obesity and with weight loss. In contrast to our findings with surface P-selectin, and prior reports of soluble CD40 ligand, platelet surface CD40 remained significantly greater in obese subjects following 6 months of surgical weight loss.

Our study has several limitations. Our cohort consisted of relatively young, metabolically healthy women, not taking antihyperglycemic, lipid-lowering, or platelet-affecting medications. This was purposefully done to reduce confounding from medications (and/or changes in medications following surgery) and the known differences in platelet gene expression with age and gender. Thus, it is not known if our findings can be extrapolated to men and older individuals, or how common medications may influence platelet mRNA profile in obesity. The absence of a comparator weight loss group(s) does not allow for assessment of any weight loss- versus surgical procedure-specific effects. Finally, findings from our 6 month post-sleeve gastrectomy time point may not reflect changes that occur with additional weight loss, non-surgical weight loss, or alternative surgical weight loss techniques.

Nonetheless, this is the first report of an unbiased analysis of the platelet transcriptome in obesity and after bariatric surgery. Our work supports prior reports that *S100A9* is increased

in obesity, and adds data that suggest modification of this risk factor may be difficult. We also contribute a novel list of differentially expressed transcripts which may lend mechanistic insight to the increased risk of ASCVD in obesity. Finally, in addition to improving established risk factors, we report that bariatric surgery reduces platelet activation and alters the platelet transcriptome. These observations suggest that platelets may contribute to increased cardiovascular risk in obesity through a variety of mechanisms. The relevance of the platelet mRNA changes and whether they are secondary to weight/adipose loss, myriad metabolic effects following bariatric surgery, or a combination, and the durability of these changes remain to be elucidated.

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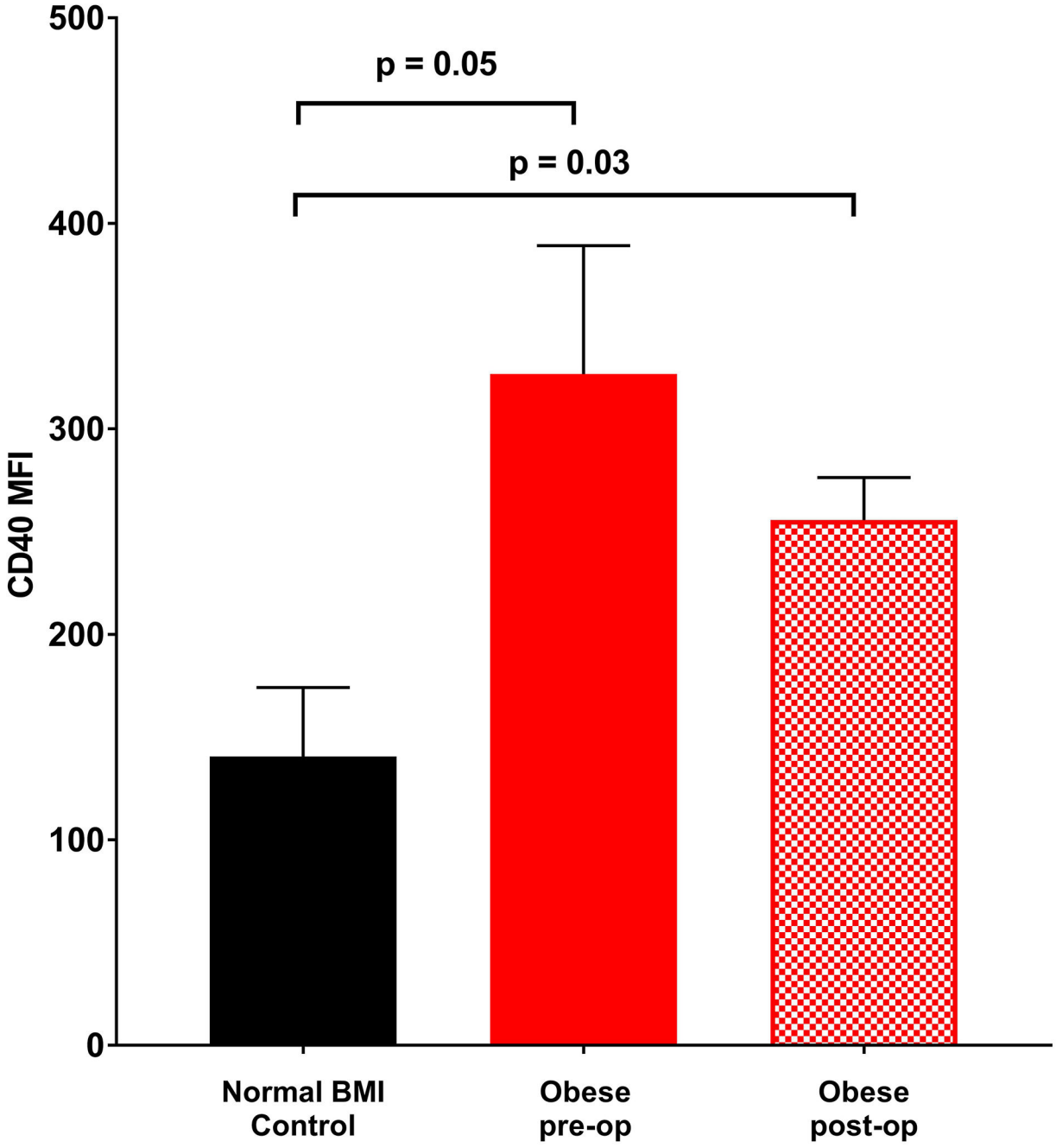
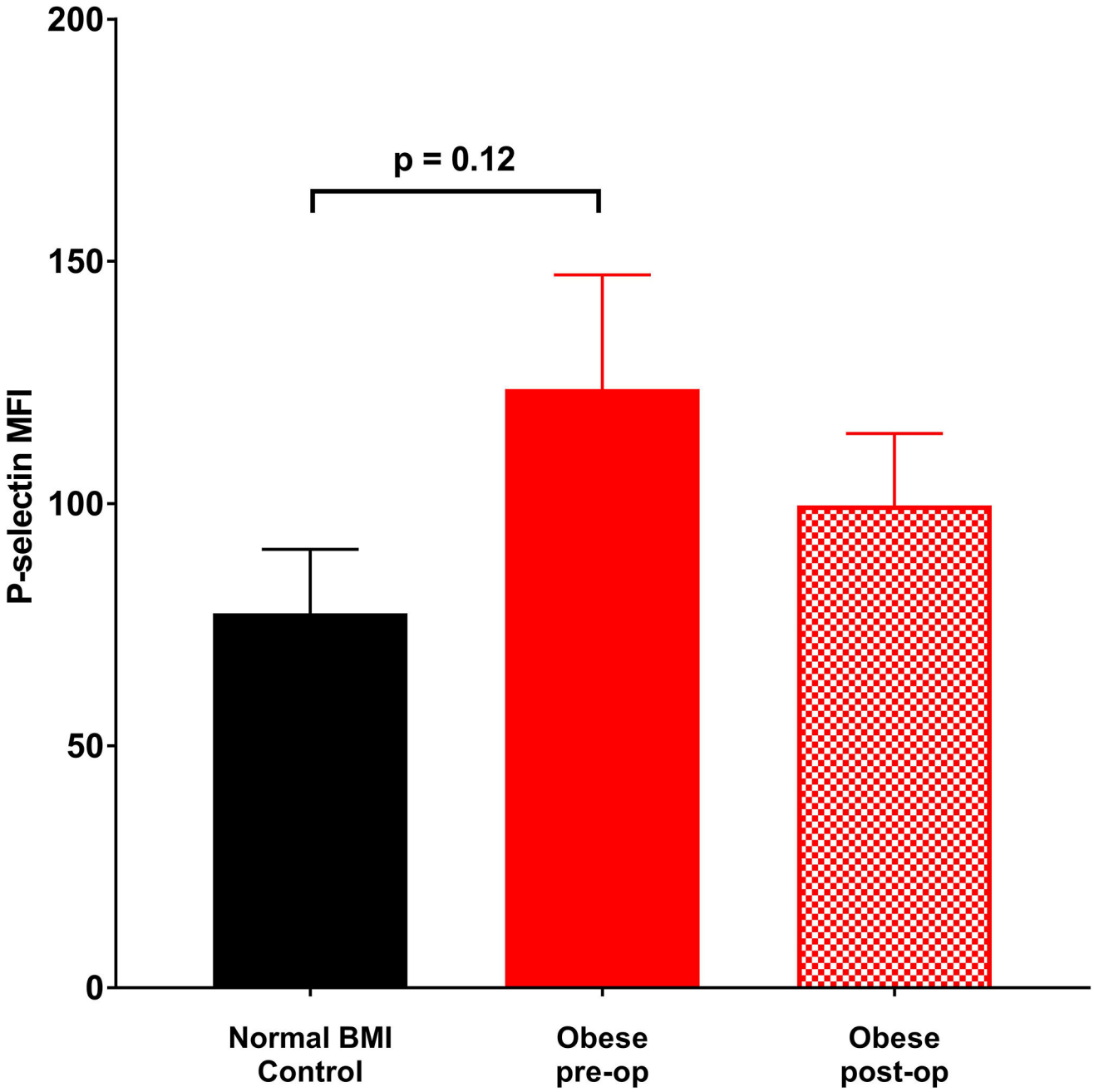




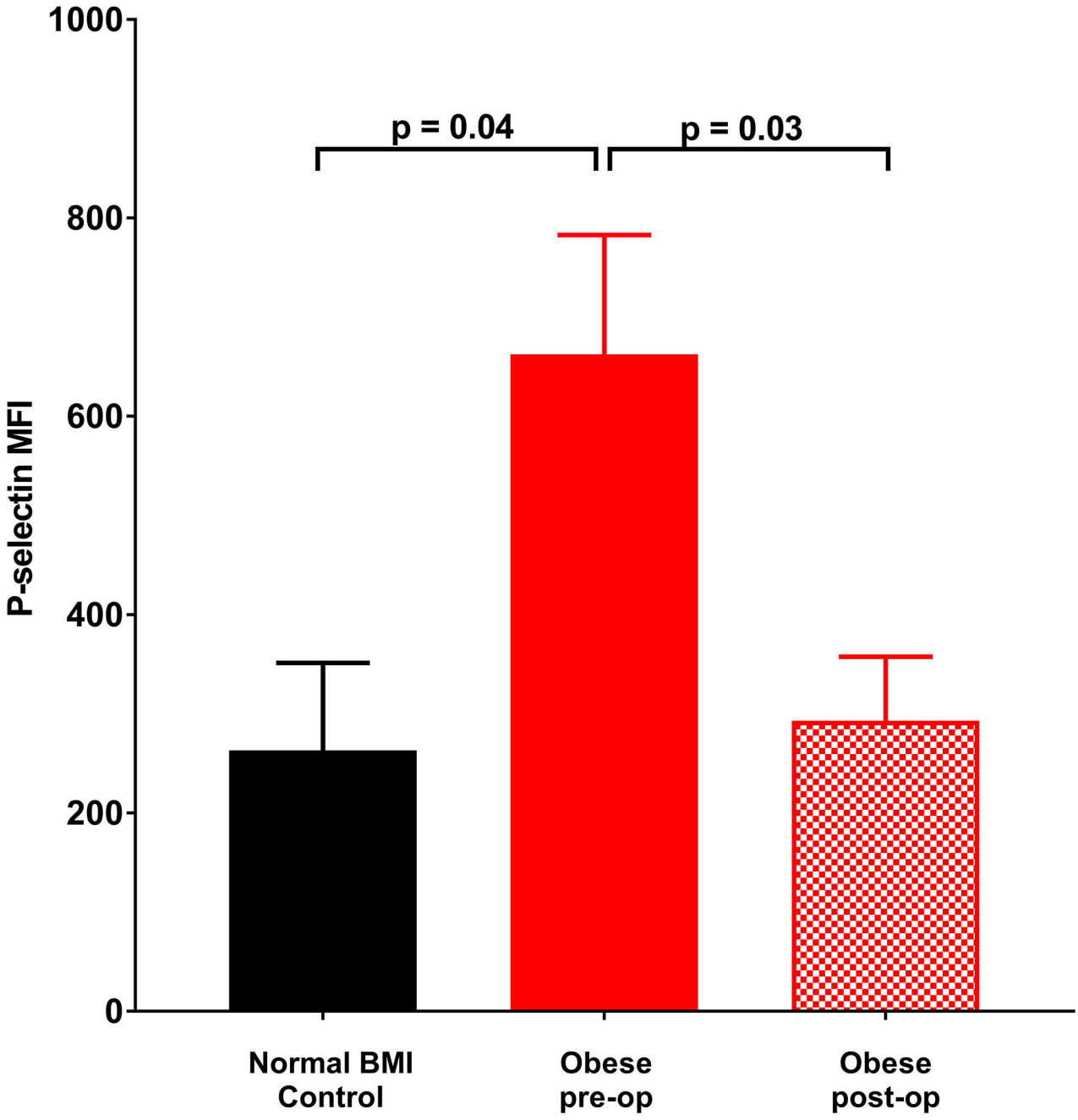
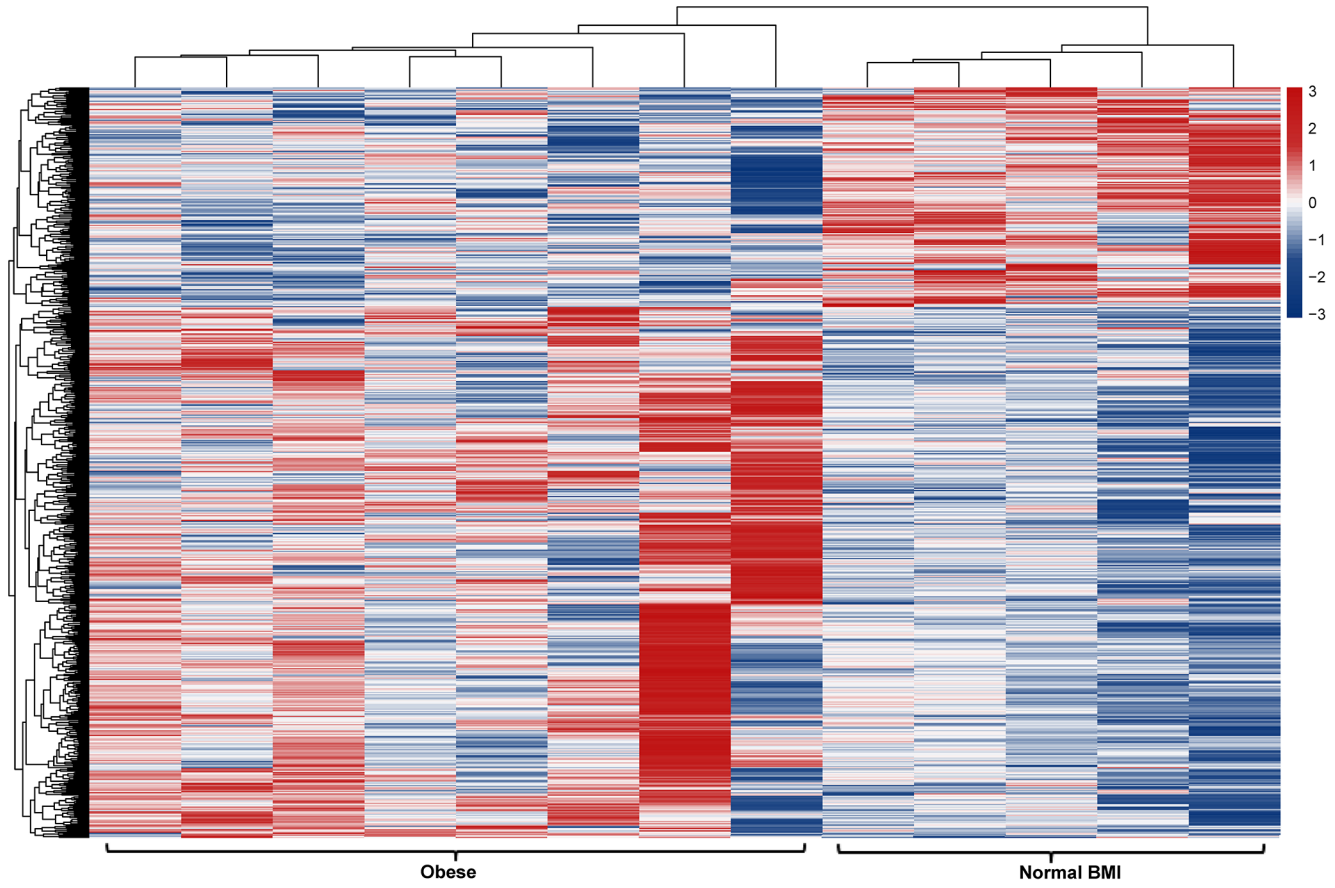
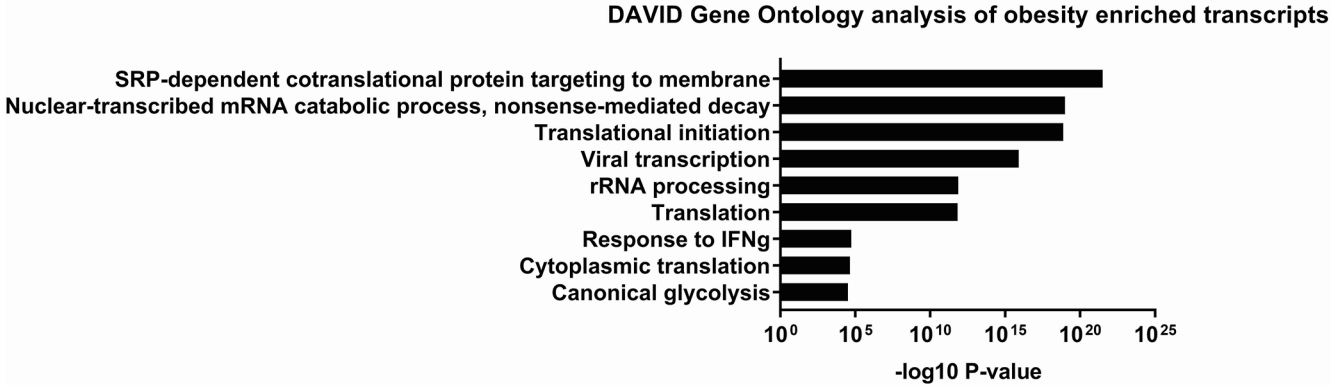
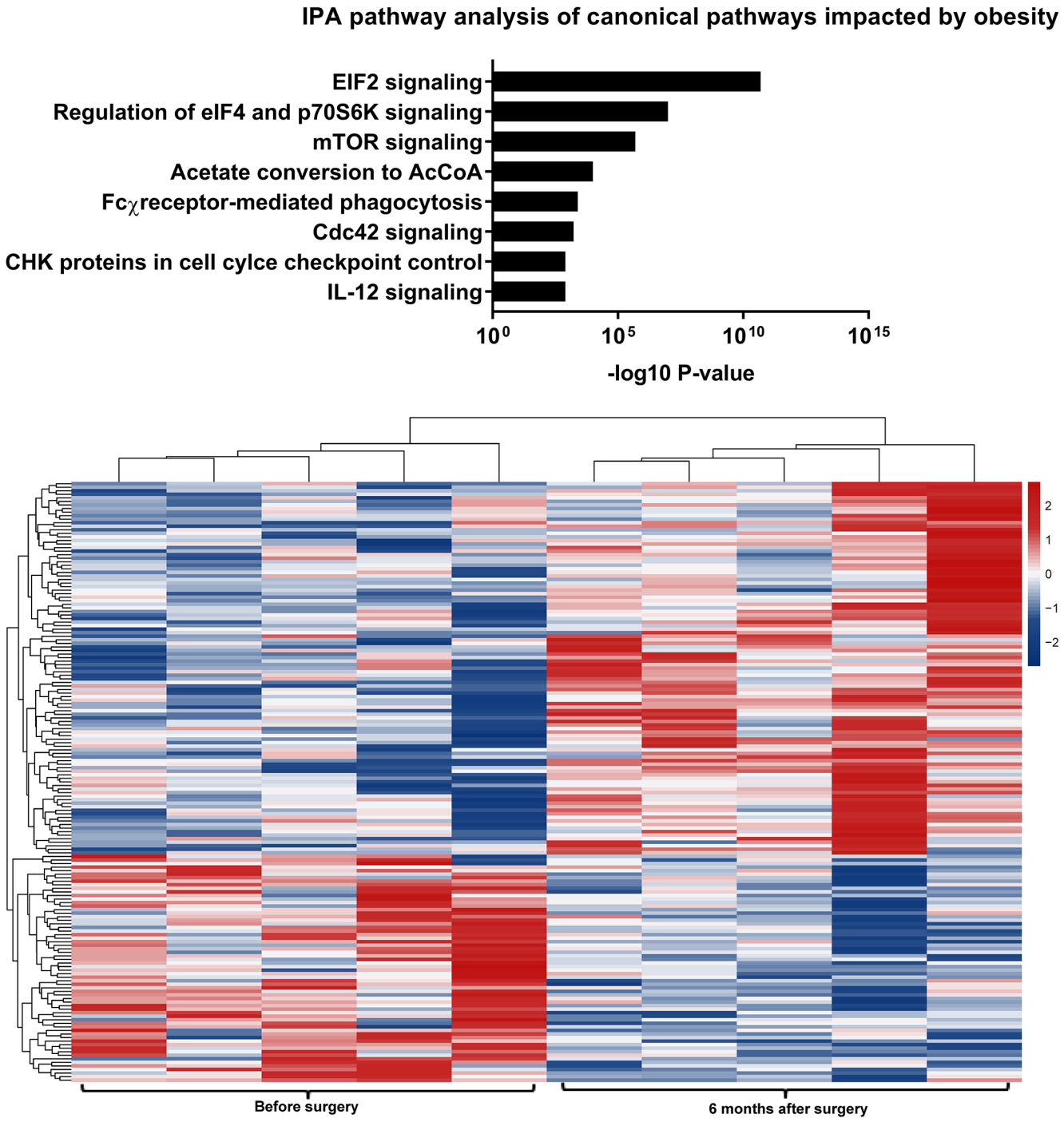


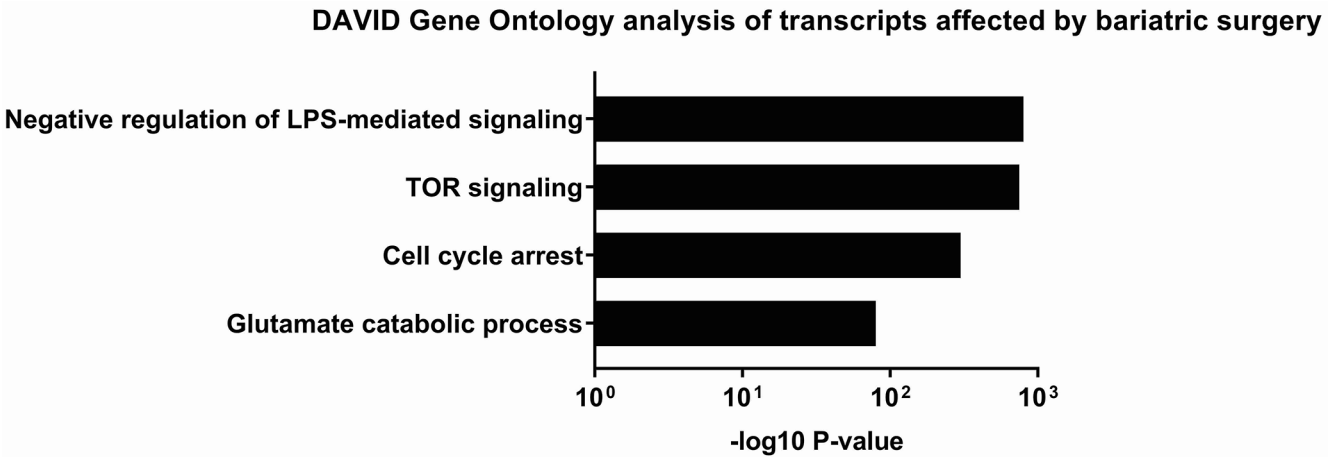
Figure 1.

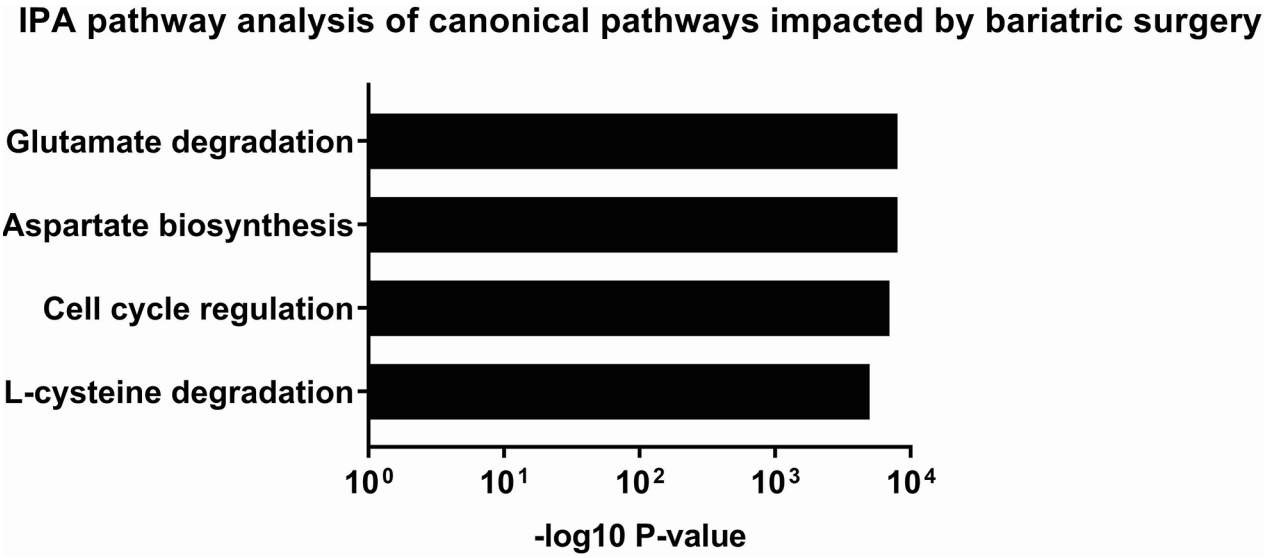
Mean fluorescence intensity (MFI) (corrected for CD42 MFI) indicative of surface staining of (a) CD40 in the absence of agonist, (b) P-selectin in the absence of agonist, (c) P-selectin on exposure to 0.025 U thrombin. Data represented as mean ± SEM.

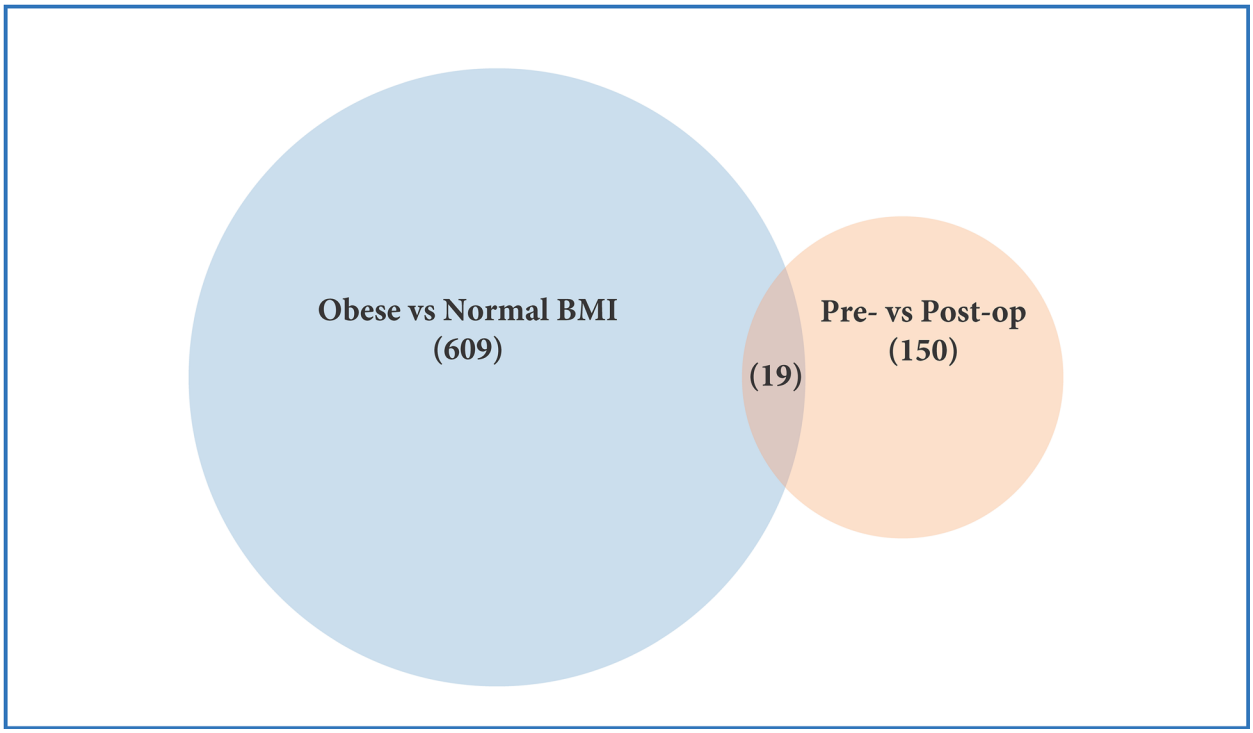


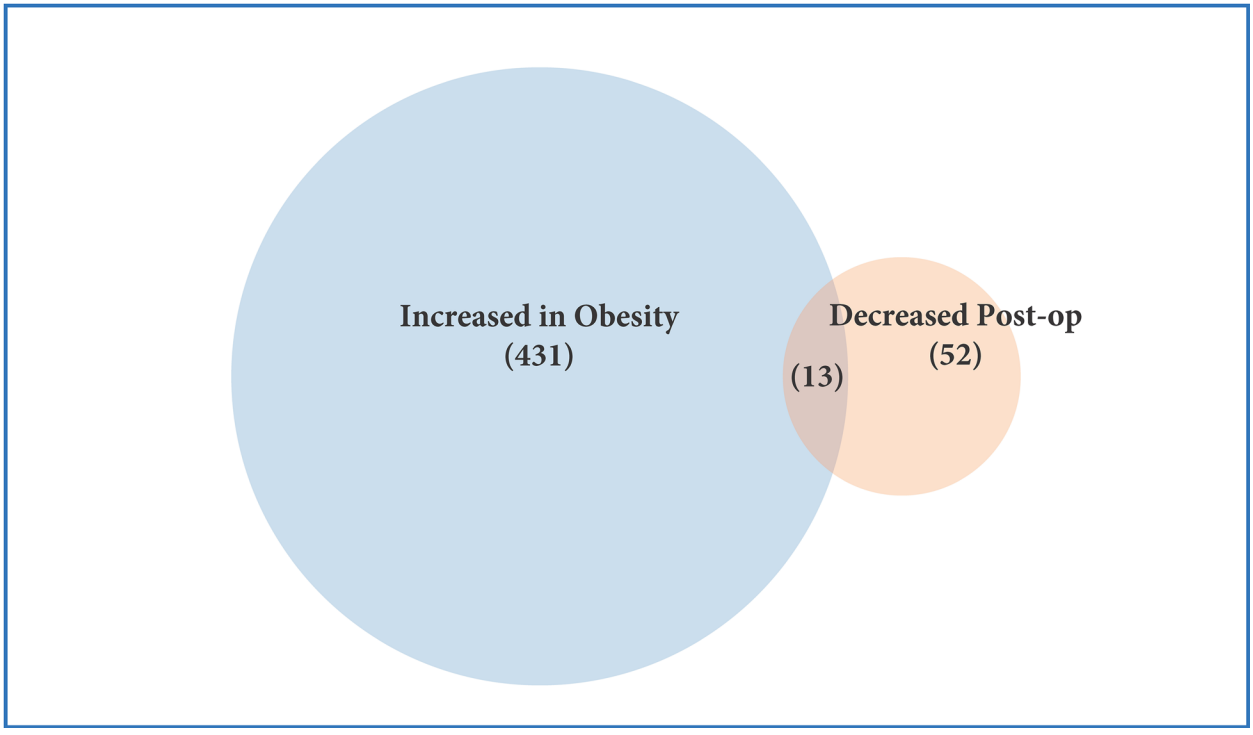












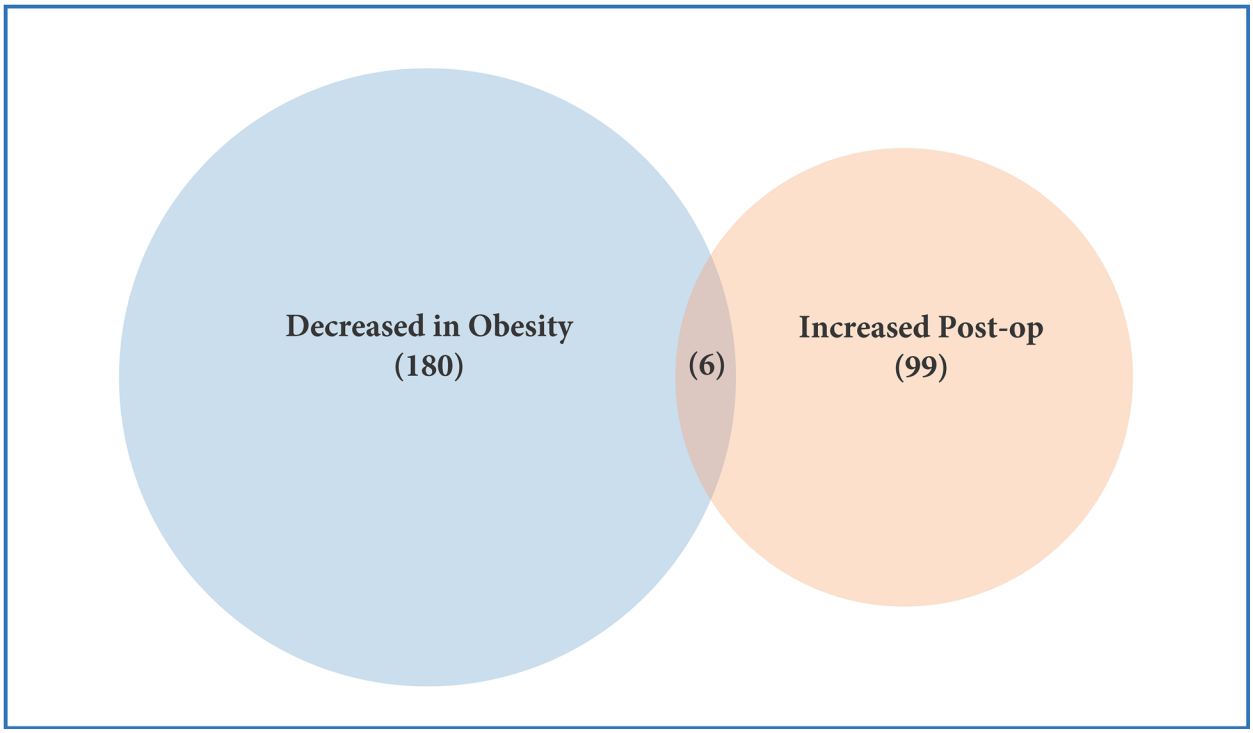


Figure 2.

Profiling of platelet mRNA expression in obese women. (a) Heatmap of platelet transcripts differentially expressed between obese women (n=8) and normal BMI women (n=5) with p-value <0.05 and base mean counts >5. (b) Top pathways of platelet transcripts differentially expressed in obese versus normal BMI women. (c) Heatmap of platelet transcripts differentially expressed between obese women (n=5) before and at six months following sleeve gastrectomy with p-value <0.05 and base mean counts >5. (d) Top pathways of platelet transcripts differentially expressed in obese women at six months after sleeve gastrectomy. (e) Venn diagrams representing platelet transcripts differentially expressed in obesity and bariatric surgery.

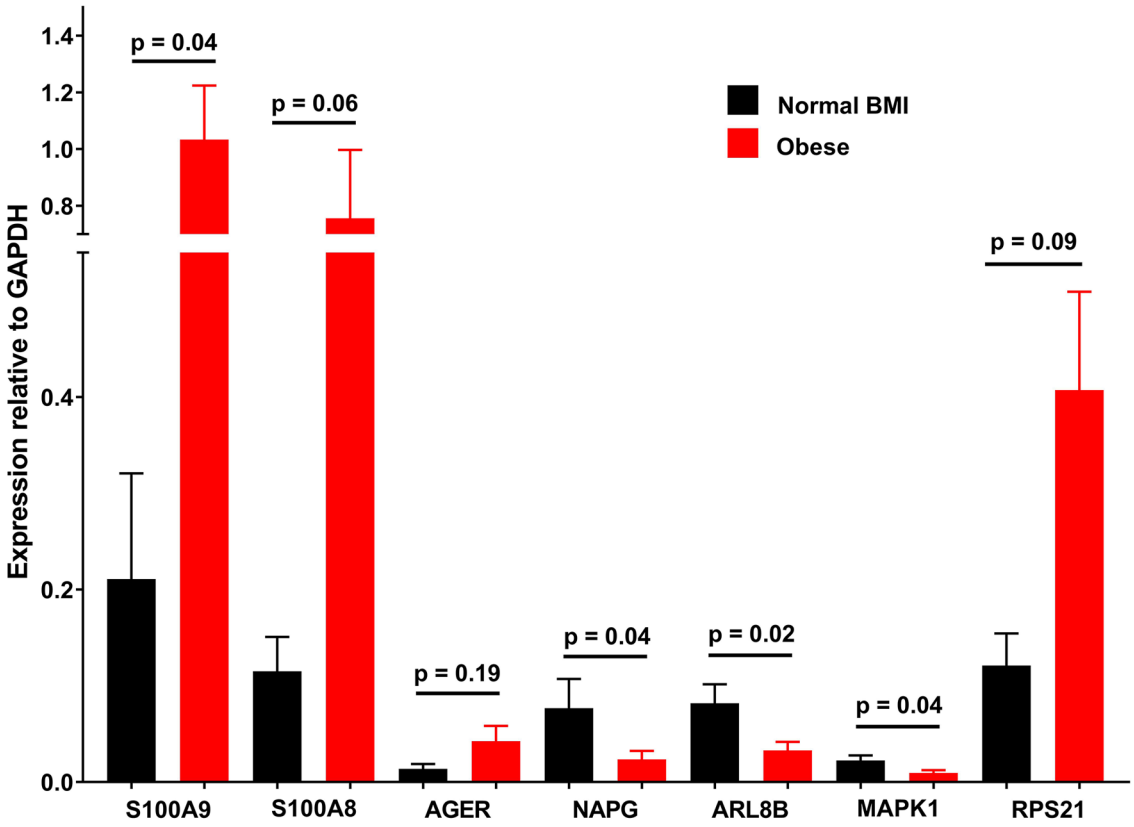


Figure 3.

Gene expression (relative to GAPDH) in platelet mRNA samples from obese and normal BMI control subjects measured with quantitative PCR. Data represented as mean ± SEM.

Table 1

Characteristics of obese and normal BMI subjects

	Obese - baseline	Obese - 6 months post-op	Control
	n = 26	**n=19**	**n = 10**
Female sex (percent)	100		100
Age (years)	31.6 ± 8.4		32.0 ± 3.7
Weight (kg)	116 ± 23	87 ± 22 **	58 ± 5 **
BMI (kg/m^2)	43.0 ± 6.5	32.1 ± 6.0 **	22.8 ± 2.3 **
Waist circumference (cm)	121 ± 14	96 ± 14 **	80 ± 5 **
Platelets (10^3/uL)	283 ± 60	297 ± 89	281 ± 80
Mean platelet volume	8.4 ± 0.8	8.3 ± 0.8	8.5 ± 1.0
Reticulated platelets (MFI)	81 ± 42	74 ± 15	73 ± 27

** p<0.01 for comparison with Obese – baseline

Platelets. Author manuscript; available in PMC 2020 January 01.

Table 2.

Top differentially expressed genes (a) in obesity and (b) 6 months following sleeve gastrectomy.

	Gene	Base mean expression	Fold different (obese vs normal BMI)	p-value
(a)	**LRRC8B**	1774.33	0.60	<0.00001
	RP11–757G1.6	36.64	2.17	0.00002
	PDE11A	5.49	0.30	0.00002
	QPCT	8.99	3.26	0.00003
	TRHDE	125.68	2.58	0.00003
	PCTP	646.76	0.40	0.00003
	S100A9	670.25	2.61	0.00007
	ANKRD36BP2	182.96	2.01	0.00008
	ARL8B	1022.41	0.58	0.00012
	AC093627.8	47.42	0.38	0.00013
	CLEC2D	23.42	2.67	0.00014
	BLVRB	33.01	2.32	0.00017
	TTF2	30.49	0.38	0.00032
	RP11–885N19.6	96.91	0.40	0.00038
	LCN2	2081.30	0.47	0.00038
	GABRR2	7.67	2.69	0.00039
	TES	18.13	2.68	0.00043
	AR	245.37	2.11	0.00045
	WRB	1591.17	2.11	0.00059
	FCGR3A	40.20	2.54	0.00064
	NAPG	655.18	0.49	0.00085
	AGER	7.09	2.55	0.00092
	PPP1R3E	33.15	1.88	0.00114
	MARCKS	28.13	2.42	0.00123
	CHRM3	34.49	2.29	0.00131

	Gene	Base mean expression	Fold different (pre vs post-op)	p-value
(b)	**AL161626.1**	3865.30	2.16	0.00005
	LTF	5.00	1.80	0.00078
	CBLB	19.20	0.53	0.00093
	CDH26	14.20	1.82	0.00179
	C14orf159	14.10	0.56	0.00215
	PLEKHM1P1	12.80	0.58	0.00272
	DXO	18.80	0.58	0.00373
	MTRNR2L12	82.40	0.57	0.00376
	AC015987.2	5.40	1.63	0.00469
	IER3IP1	7.90	1.64	0.00492
	SMIM6	13.50	0.59	0.00495
	PTPN9	8.40	0.60	0.00556
	RP11–672A2.3	15.60	0.59	0.00607
	EIF3J-AS1	6.30	0.61	0.00657
	YEATS4	20.00	1.68	0.00669
	PSMD8	44.50	0.63	0.00686
	RPIA	28.60	0.61	0.00728
	CCDC69	45.20	0.60	0.00731
	ATP6V1B2	296.50	0.61	0.00778
	TRIB1	7.90	0.63	0.00814
	L3MBTL4-AS1	28.80	0.61	0.00831
	GNG2	36.00	0.61	0.00880
	RP11–525A16.4	34.20	0.61	0.00952
	BTG1	96.60	0.62	0.00954
	GLOD5	20.7	0.64	0.00960

Table 2c.

Genes differentially expressed in obesity and affected by bariatric surgery

Gene	Fold different in obesity	Rank in obesity	Fold different after surgery	Rank in surgery
RP11–757G1.6	2.17	2	0.67	39
AC079949.1	2.15	37	0.65	40
PTPN23	2.33	38	0.43	164
CDK5RAP3	2.33	42	1.51	77
GFOD2	2.21	65	0.71	152
CDCA7L	2.21	73	0.61	29
CPNE4	0.50	100	1.60	37
ZNF711	0.52	127	1.53	64
MRPS30	2.07	129	0.68	128
MGAT3	0.49	148	1.51	89
NOP9	2.00	192	0.65	43
FAM26F	0.53	248	1.55	50
LTF	1.83	308	0.56	2
HIST1H3D	0.63	386	1.35	84
AL161626.1	1.69	418	0.46	1
SHQ1	1.60	419	0.69	104
PIGC	1.78	435	0.67	110
FAM212B	0.63	501	1.41	98
MDC1	1.76	544	0.65	59

Table 3

Primer sequences used in RT-qPCR validation

Transcript	Forward primer sequence	Reverse primer sequence
GAPDH	TGTGGGCATCAATGGATTTGG	ACACCATGTATTCCGGGTCAAT
S100A9	GGTCATAGAACACATCATGGAGG	GGCCTGGCTTATGGTGGTG
S100A8	ATGCCGTCTACAGGGATGAC	ACTGAGGACACTCGGTCTCTA
AGER	GTGTCCTTCCCAACGGCTC	ATTGCCTGGCACCGGAAA
NAPG	CTACCAGAGGCCGTTCAGCTA	CCTGTCGTAAGCGTTCTTCAT
ARL8	CATCGCGTCAGGTCAATTCAG	GTTGTCCTCCTATGTCCCAGA
MAPK1	TACACCAACCTCTCGTACATCG	CATGTCTGAAGCGCAGTAAGATT
RPS21	AGCAATCGCATCATCGGTG	CCCCGCAGATAGCATAAGTTTTA