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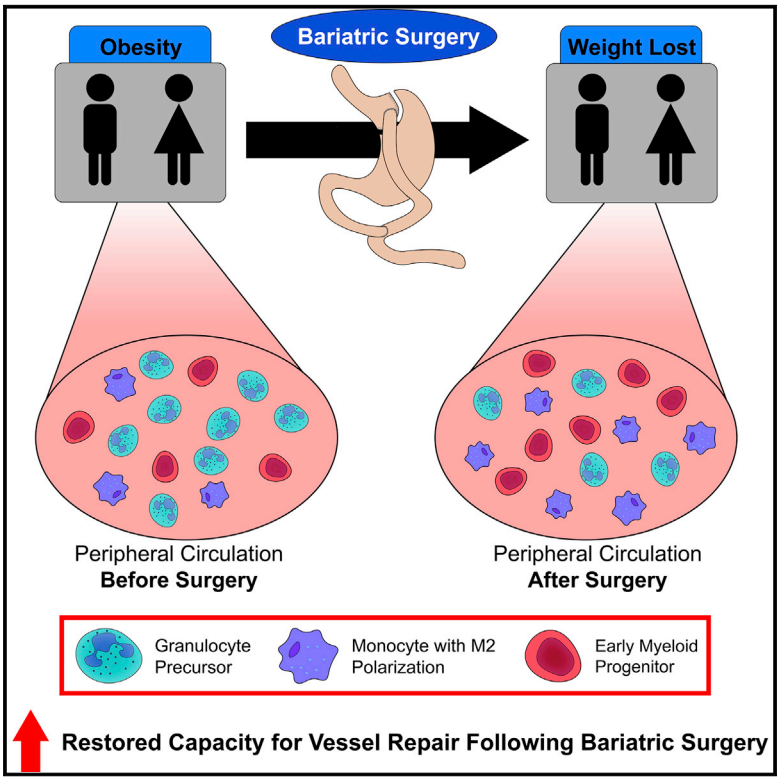
Vascular Risk Reduction in Obesity through Reduced Granulocyte Burden and Improved Angiogenic Monocyte Content following Bariatric Surgery

Graphical Abstract



Highlights

- Obesity features a low frequency of ALDH and CD133 co-expressing cells
- Bariatric surgery results in lower granulocyte precursors expressing ALDH
- Macrophage balance favors M2 polarization following bariatric surgery
- Cellular changes after bariatric surgery give insight into reducing CV risk

Authors

David A. Hess, Justin Z. Trac, Stephen A. Glazer, ..., C. David Mazer, Ori D. Rotstein, Subodh Verma

Correspondence

dhess@robarts.ca (D.A.H.), subodh.verma@unityhealth.to (S.V.)

In Brief

In Hess et al., the quantification of circulating progenitor cells in individuals with obesity, before and 3 months after bariatric surgery, demonstrates reduced granulocyte precursors and increased pro-angiogenic monocytes with M2 polarization. These alterations are consistent with improved vessel homeostasis and recovered cardiovascular health observed following bariatric surgery.

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Report

Vascular Risk Reduction in Obesity through Reduced Granulocyte Burden and Improved Angiogenic Monocyte Content following Bariatric Surgery

David A. Hess,[1,6,11,12,16,*] Justin Z. Trac,[2,6,16] Stephen A. Glazer,[13,14,16] Daniella C. Terenzi,[2,10] Adrian Quan,[2] Hwee Teoh,[2,3] Mohammed Al-Omran,[1,6,7,10] Deepak L. Bhatt,[15] C. David Mazer,[5,8,9] Ori D. Rotstein,[4,7,10] and Subodh Verma[2,6,7,10,17,*]

[1]Division of Vascular Surgery, Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, ON, Canada
[2]Division of Cardiac Surgery, Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, ON, Canada
[3]Division of Endocrinology and Metabolism, Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, ON, Canada
[4]Division of General Surgery, Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, ON, Canada
[5]Department of Anesthesia, Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, ON, Canada
[6]Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, Canada
[7]Department of Surgery, University of Toronto, Toronto, ON, Canada
[8]Department of Physiology, University of Toronto, Toronto, ON, Canada
[9]Department of Anesthesiology and Pain Medicine, University of Toronto, Toronto, ON, Canada
[10]Institute of Medical Science, University of Toronto, Toronto, ON, Canada
[11]Molecular Medicine Research Laboratories, Robarts Research Institute, London, ON, Canada
[12]Department of Physiology and Pharmacology, Western University, London, ON, Canada
[13]Department of Internal Medicine, Humber River Hospital, Toronto, ON, Canada
[14]Division of Endocrinology and Metabolism, Queen's University, Kingston, ON, Canada
[15]Brigham and Women's Hospital Heart and Vascular Center, Harvard Medical School, Boston, MA, USA
[16]These authors contributed equally
[17]Lead Contact
*Correspondence: dhess@robarts.ca (D.A.H.), subodh.verma@unityhealth.to (S.V.)
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SUMMARY

Bariatric surgery, in addition to the benefit of sustained weight loss, can also reduce cardiometabolic risk and mortality. Lifelong vessel maintenance is integral to the prevention of cardiovascular disease. Using aldehyde dehydrogenase activity, an intracellular detoxifying enzyme present at high levels within pro-vascular progenitor cells, we observed an association between chronic obesity and "regenerative cell exhaustion" (RCE), a pathology whereby chronic assault on circulating regenerative cell types can result in adverse inflammation and diminished vessel repair. We also describe that, at 3 months following bariatric surgery, systemic inflammatory burden was reduced and pro-angiogenic macrophage precursor content was improved in subjects with severe obesity, suggesting the restoration of a microenvironment to support vessel homeostasis. These data suggest that bariatric surgery may reverse deleterious events that predispose patients with morbid obesity to cardiovascular risk.

INTRODUCTION

Obesity is a global pandemic that affects >2 billion individuals[1] and dramatically elevates both cardiovascular risk[2] and all-cause mortality.[3] Obesity is also associated with heightened systemic inflammation, which is purported to interfere with blood vessel homeostasis and contribute to chronic ischemic deficits.

Bariatric surgery, a successful method of weight loss that is achieved through reducing stomach size and re-routing the gastrointestinal tract, has been reported to lower both adverse cardiovascular events and cardiovascular mortality in patients with obesity.[4] Therefore, we sought to determine whether chronic obesity is associated with "regenerative cell exhaustion" (RCE), a pathology whereby prolonged assault on circulating vascular regenerative cell types results in diminished vessel repair and eventually increases the rates of adverse cardiovascular events.[5] Although bariatric surgery has been shown to positively modulate metabolic factors such as glycemia, insulin

sensitivity, markers of inflammation, and oxidative stress,[6] whether bariatric surgery also improves blood vessel maintenance has not been previously studied.

Using a high-throughput, flow cytometry-based assay to quantify circulating pro-inflammatory and pro-vascular progenitor cell subsets in the peripheral blood of human subjects, we documented that the state of heightened oxidative stress in type 2 diabetes (T2D) was associated with a greater granulocyte burden, aberrant monocyte/macrophage (M1/M2) specification, and lower circulating pro-vascular myeloid progenitor cell content.[7] High aldehyde dehydrogenase ($ALDH^{hi}$) activity, a conserved protective function in progenitor cells from multiple lineages (hematopoietic, endothelial, mesenchymal), has been used to quantify circulating precursor cell subsets with enhanced resistance to oxidative stress.[8] Using the notion that as bone marrow-derived precursor cells differentiate toward more expendable effector cells in the periphery, self-protective ALDH activity is diminished,[5] we recently reported the recovery of circulating regenerative cell content in individuals with both T2D and documented coronary artery disease who had been treated with the antihyperglycemic agent empagliflozin.[9] Specifically, compared with placebo-assigned controls, participants randomized to empagliflozin had greater pro-angiogenic $ALDH^{hi}/CD133^{+}$ progenitor cell content and more monocytes with M2 polarization in the circulation. These changes may account, at least in part, for the lower rates of cardiovascular events observed with empagliflozin in a recent cardiovascular outcomes trial with individuals who had T2D.[10]

In the present study, peripheral blood was collected from 19 individuals meeting the selection criteria for bariatric surgery (BMI $\geq$ 35 kg/m^2 with obesity-related comorbidities or BMI $\geq$ 40 kg/m^2) before and at ~3 months after surgery. Comparative blood samples were drawn from 19 age- and sex-matched volunteers with BMI < 25 kg/m^2 (Figure S1). Circulating granulocytes, M1/M2 monocyte/macrophage polarization, and pro-angiogenic progenitor cell content were assessed by flow cytometry for elevated ALDH activity, with co-expression of selected cell surface markers. Serum was cryopreserved for examination of inflammatory cytokines or oxidative enzyme measurements before and at 3 months after surgery (Figure S1).

RESULTS

Individuals with Obesity Demonstrate Elevated Cardiovascular Risk

The mean baseline weight of those who underwent bariatric surgery was >2-fold greater than that of the control group (131.8 ± 6.3 kg versus 62.7 ± 1.9, $p < 0.001$); the mean BMI value for the former was double that of the latter (48.2 ± 2.1 kg/m^2 versus 22.1 ± 0.4, $p < 0.001$) (Table S1). Individuals who were scheduled for bariatric surgery had a higher prevalence of diagnosed comorbidities that included obstructive sleep apnea (68%), hypertension (58%), and T2D (26%), compared to the control group in which these diagnoses were predominantly absent. Additionally, compared to the control group, a larger proportion of individuals in the bariatric group prior to surgery were taking blood pressure lowering medications (Table S1). Levels of glycated hemoglobin (HbA1c) were generally higher among the bariatric surgery patients (mean 6.0% ± 0.2% versus 5.3% ± 0.1% for the control group, $p < 0.001$); 44% of the bariatric surgery patients had an HbA1c in the range of 6.0%–6.5%, which is considered to represent a state of pre-diabetes. The baseline lipid profiles of the two groups were similar. A history of smoking was more prevalent in the bariatric surgery group (47% versus 21%). Significant weight loss (−21.2 ± 2.3 kg, $p < 0.001$) and BMI reduction (−8.1 ± 1.8 kg/m^2, $p < 0.001$) was observed 3 months after bariatric surgery. There was also a concomitant reduction in HbA1c (−0.6% ± 0.2%, $p < 0.001$).

Reversal of M1/M2 Macrophage Polarization following Bariatric Surgery

Using cell surface marker analyses independent of ALDH activity, we previously reported that compared with healthy control subjects, individuals with T2D (duration >10 years) showed a higher frequency of circulating $CD80^{+}$ monocytes with M1 (pro-inflammatory) macrophage polarization.[7] In the present study, we sought to determine if comparable differences would be observed between healthy and obese subjects. Indeed, we noted significantly more $CD80^{+}$ monocytes with M1 polarization in the bariatric surgery group compared with the control group (3.1% ± 0.2% versus 1.9% ± 0.1%, $p < 0.001$; Table 1). After surgery, the frequency of cells expressing CD80 was reduced to levels that were equivalent to the control group (2.0% ± 0.2%, $p < 0.001$; Table 1). In addition, cells expressing the M2 (anti-inflammatory) polarization marker CD163 were concomitantly increased after surgery (7.0% ± 0.5% versus 9.2% ± 0.6%, $p < 0.01$; Table 1), similar to healthy controls, suggesting an adjustment of M1/M2 balance toward a less inflammatory phenotype.

ALDH Activity Identifies a Reversal in Granulocyte Burden following Bariatric Surgery

We incorporated functional ALDH activity analyses to quantify circulating progenitor cell subsets stratified for granulocyte, monocyte, or early myeloid and endothelial progenitor cell lineages.[7] Using the Aldeflour assay to identify precursor cells within subpopulations discerned by light side scatter (SSC) properties measuring cell granularity (complexity),[7] a reversible inhibitor of cytosolic ALDH1A activity, *N*,*N*-diethylaminobenzaldehyde (DEAB), was used to define mature versus primitive precursor cells with low versus high ALDH activity, respectively (Figure 1A). Figure 1B shows the accumulation of Aldefluor reagent within cells, inducing a right shift in fluorescence identifying (R1) $ALDH^{hi}SSC^{hi}$ cells, granulocytes that co-express neutrophil markers (CD15, CD16, CD66b)[7]; (R2) $ALDH^{hi}SSC^{mid}$ cells, circulating monocytes with intermediate cell complexity[7]; and (R3) $ALDH^{hi}SSC^{low}$ cells, rare circulating progenitor cells with pro-angiogenic function.[8,11] Although obesity was not associated with significant differences in the frequency of circulating $ALDH^{hi}SSC^{mid}$ or $ALDH^{hi}SSC^{low}$ subsets, bariatric surgery patients demonstrated a >2-fold higher frequency of circulating $ALDH^{hi}SSC^{hi}$ granulocyte precursors compared to the control group (9.7% ± 1.8% versus 4.7% ± 1.0%, $p < 0.05$; Table 1). This granulocyte burden was reversed at 3 months after surgery (5.0% ± 0.8%, $p < 0.01$; Figures 1C and 1D; Table 1), indicating a potential reduction in inflammation.

Table 1. Bariatric Surgery Was Associated with a Decreased Granulocyte Cell Burden and Increased Circulating Pro-angiogenic Monocyte Content

Marker Expression	Normal-Weight BMI <25 kg/m^2 (n = 19)	P-value Normal Weight versus Before Surgery	Bariatric Patients Before Surgery (n = 19)	P-value Before Surgery versus After Surgery	Bariatric Patients 3 Months After Surgery (n = 19)
CD45$^+$	97.8 ± 0.3	0.047*	95.4 ± 1.0	0.88	95.3 ± 0.6
CD33$^+$	43.2 ± 3.9	0.12	34.5 ± 3.8	0.01*	45.0 ± 3.0
CXCR4	76.2 ± 3.0	0.60	78.2 ± 2.4	0.93	78.0 ± 1.7
CD34$^+$	0.38 ± 0.05	0.046*	0.27 ± 0.03	0.32	0.31 ± 0.04
CD133$^+$	0.38 ± 0.03	0.98	0.37 ± 0.06	0.75	0.35 ± 0.03
CD14$^+$	8.5 ± 0.7	0.13	6.7 ± 0.8	0.002†	9.5 ± 0.6
CD80$^+$ (M1)	1.9 ± 0.1	0.0001‡	3.1 ± 0.2	0.001†	2.0 ± 0.2
CD163$^+$ (M2)	8.2 ± 0.8	0.18	7.0 ± 0.5	0.01*	9.2 ± 0.6
ALDH^{hi}SSC^{hi}	4.7 ± 1.0	0.02*	9.7 ± 1.8	0.01*	5.0 ± 0.8
ALDH^{hi}SSC^{mid}	5.6 ± 1.0	0.13	3.8 ± 0.6	0.002†	6.3 ± 0.8
CD14$^+$	91.0 ± 1.2	0.001†	81.9 ± 2.2	0.0001‡	93.1 ± 0.8
CD14$^+$/CD80$^+$	1.8 ± 0.2	0.001†	3.3 ± 0.4	0.03*	2.2 ± 0.2
CD14$^+$/CD163$^+$	74.8 ± 2.8	0.06	67.0 ± 3.0	0.02*	77.1 ± 1.9
ALDH^{hi}SSC^{low}	0.042 ± 0.004	0.08	0.054 ± 0.005	0.002†	0.036 ± 0.004
CD34$^+$	79.0 ± 3.8	0.47	74.7 ± 4.3	0.40	78.6 ± 3.2
CD133$^+$	40.1 ± 2.4	0.002†	30.2 ± 1.7	0.0004‡	45.4 ± 3.4
CD34$^+$/CD133$^+$	39.1 ± 2.4	0.004†	29.8 ± 1.8	0.001†	44.7 ± 3.4

Values are presented as a frequency. Data are presented as means ± SEMs. ALDH, aldehyde dehydrogenase; BMI, body mass index; SSC, side scatter.

*$p < 0.05$, †$p < 0.01$, and ‡$p < 0.001$ per the Student's t test.

ALDH^{hi}SSC^{mid} Cells Demonstrate Restoration of M1/M2 Precursor Polarization following Bariatric Surgery

The ALDH^{hi}SSC^{mid} compartment primarily comprises circulating monocytes (>80% CD14$^+$) that differentiate into macrophages when exposed to local signals. The overall frequency of circulating ALDH^{hi}SSC^{mid} monocytes was increased following bariatric surgery (3.8% ± 0.6% versus 6.3% ± 0.8%, $p < 0.01$) to levels similar to those in the control group (5.6% ± 1.0%; Figure 1E; Table 1). This is consistent with findings that examined a lowered frequency of classical (CD14$^+$/CD16$^-$) monocytes in obese individuals compared to normal-weight controls and subjects with diet intervention.[12] Macrophage polarization is a classification spectrum based on phenotype and secretory function that permits a better understanding of the multiple roles of macrophages in innate immunity and tissue repair. In general, M1 polarization is associated with classical inflammatory processes, whereas M2 polarization describes regulatory macrophages with anti-inflammatory and tissue repair properties. Within the ALDH^{hi}SSC^{mid} population, the frequency of monocytes with pro-inflammatory M1 polarization (CD14$^+$/CD80$^+$ co-expression) was decreased after bariatric surgery (3.3% ± 0.4% versus 2.2% ± 0.2%, $p < 0.05$; Figures 2A and 2B; Table 1). Conversely, the frequency of monocytes with M2 polarization (CD14$^+$/CD163$^+$ co-expression) was increased after bariatric surgery (67.0% ± 3.0% versus 77.1% ± 1.9%, $p < 0.05$; Figures 2C and 2D; Table 1) to levels equivalent to the control group (74.8% ± 2.8%; Table 1). Thus, bariatric surgery induced a transition from M1 to M2 features in circulating ALDH^{hi}SSC^{mid} cells, a phenotype previously associated with improved pro-angiogenic capacity.[13]

ALDH^{hi}SSC^{low} Cells with CD133 Co-expression Increased following Bariatric Surgery

The detection of circulating ALDH^{hi}SSC^{low} cells with progenitor surface marker expression is central to assessing endogenous pro-vascular regenerative capacity. ALDH^{hi}SSC^{low} cells from human bone marrow have been shown to support angiogenic vessel formation in preclinical models of limb ischemia.[8] These cells are rare in the circulation (<0.1%), but they possess a pro-angiogenic secretome and can stimulate endothelial cell colony and tubule formation in Matrigel.[11] Bariatric surgery patients at baseline showed a similar frequency of circulating ALDH^{hi}SSC^{low} cells compared to the control group (Figure 1F; Table 1), and we observed a consistent decrease in the frequency of ALDH^{hi}SSC^{low} cells after surgery (0.036% ± 0.005% versus 0.054% ± 0.004%, $p < 0.01$; Figure 1F; Table 1). Although these cells represent extremely rare but potent subsets, and analyses would benefit from absolute quantitation using flow cytometric counting beads, we further analyzed the ALDH^{hi}SSC^{low} sub-population for co-expression of well-established primitive cell surface markers (CD34, CD133) associated with hematopoietic and endothelial progenitor cells with revascularization functions in pre-clinical and clinical studies.[14] The frequency of circulating ALDH^{hi}SSC^{low} CD34$^+$ cells before and after bariatric surgery was equal to the frequency in the control group (Figures

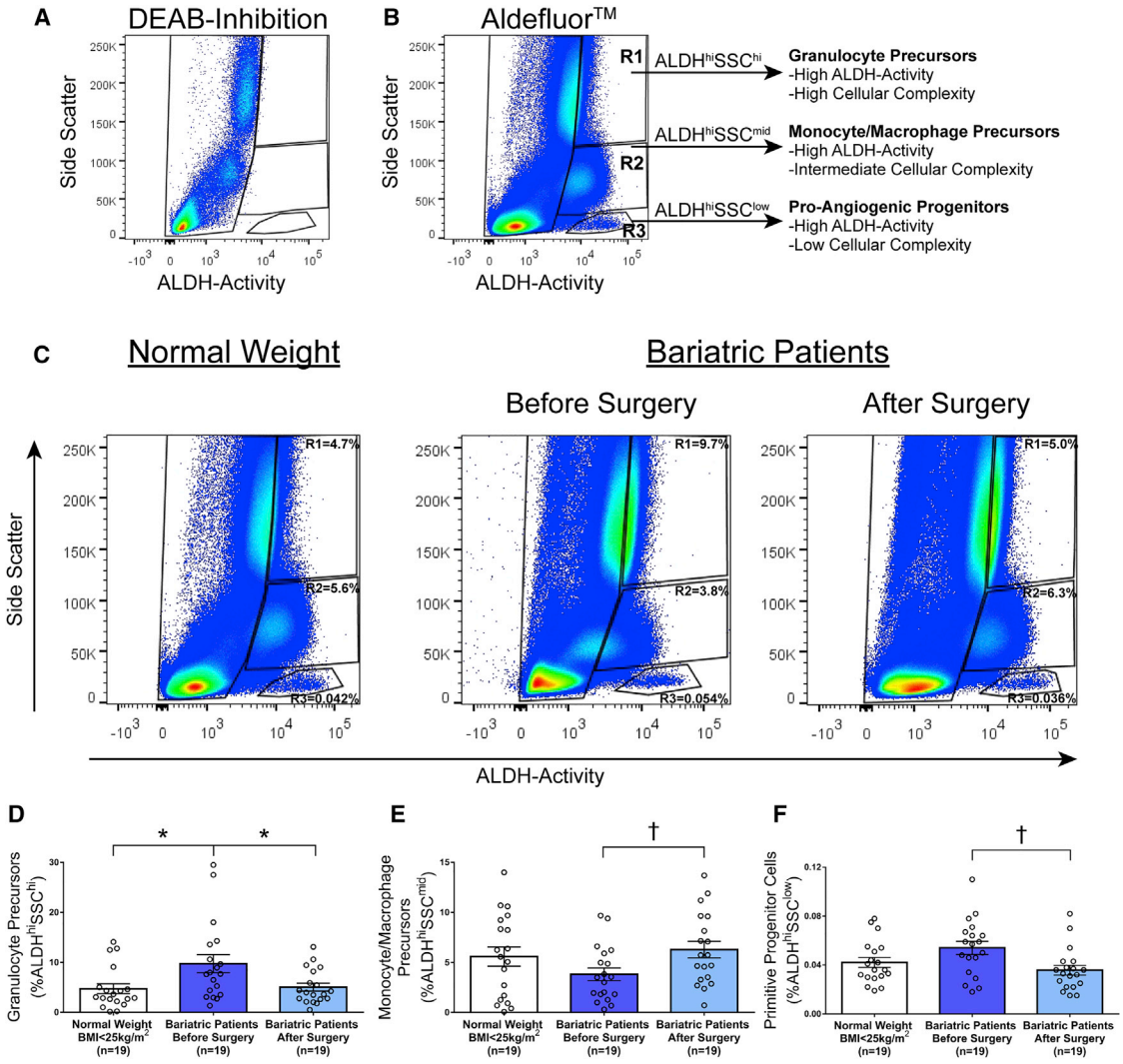

Figure 1. Bariatric Surgery Was Associated with a Decreased Circulating Pro-inflammatory Granulocyte Burden and Increased Pro-angiogenic Monocyte Content

(A) Representative flow cytometry plot showing inhibition of ALDH activity to establish gates marking cells with low versus high ALDH activity.

(B) Cells with high ALDH activity (right shift) were separated into subgroups based on side scatter properties (R1 = $ALDH^{hi}SSC^{hi}$ granulocyte precursors, R2 = $ALDH^{hi}SSC^{mid}$ monocyte/macrophage precursors, R3 = $ALDH^{hi}SSC^{low}$ primitive progenitor cells).

(C) Representative flow cytometry plots showing the frequency of $ALDH^{hi}$ cell subsets in normal-weight individuals and in patients before and 3 months after bariatric surgery.

(D) The frequency of circulating $ALDH^{hi}SSC^{hi}$ granulocyte precursors was higher in patients before bariatric surgery compared to normal-weight individuals and was normalized after bariatric surgery.

(E) The frequency of circulating $ALDH^{hi}SSC^{mid}$ monocyte/macrophage precursors was increased after bariatric surgery.

(F) The frequency of circulating $ALDH^{hi}SSC^{low}$ progenitor cells was decreased after bariatric surgery.

Data are presented as means ± SEMs. *$p < 0.05$ and †$p < 0.01$ per the Student's t test.

3A and 3B; Table 1). In contrast, $CD133^+$ co-expression within $ALDH^{hi}SSC^{low}$ cells was significantly increased after bariatric surgery (30.2% ± 1.7% versus 45.4% ± 3.4%, $p < 0.001$) to levels equivalent to the control group (40.1% ± 2.4%; Figures 3C and 3D; Table 1). Pro-angiogenic $ALDH^{hi}SSC^{low}$ cells with $CD34^+$/$CD133^+$ co-expression were also increased ($p < 0.001$; Figures 3E and 3F; Table 1), suggesting that bariatric surgery increased $CD133^+$ cells previously associated with vascular regenerative functions.[8]

The Circulating Antioxidant Enzyme Catalase Was Increased following Bariatric Surgery

We have previously shown that empagliflozin treatment correlated with increased pro-vascular cell content, stabilization in serum Nox1 expression, and an increase in reactive oxygen species (ROS)-reducing catalase levels compared to placebo treatment.[9] Although Nox1 acts to increase systemic levels of oxidative stress, we did not observe any difference in serum Nox1 levels in patients after bariatric surgery (745.2 ± 33.5 versus

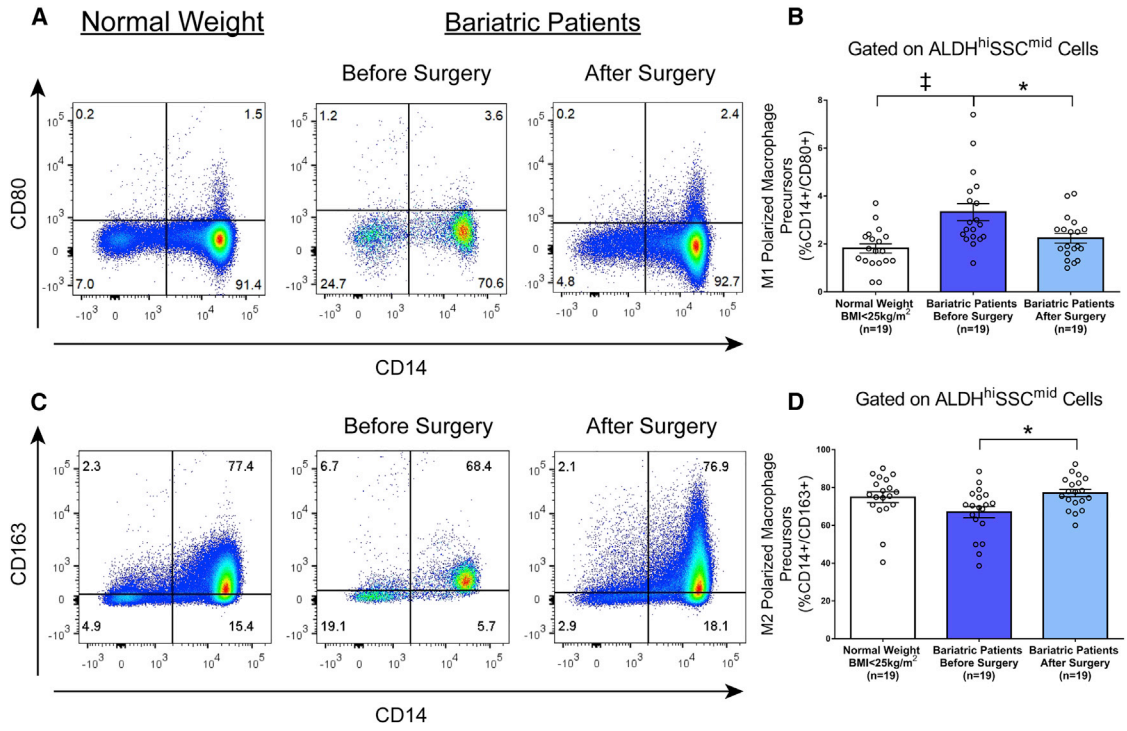

Figure 2. Bariatric Surgery Normalized the Balance of Circulating ALDH^{hi}SSC^{mid} Monocyte/Macrophage Precursors with M1 versus M2 Polarization

(A) Representative flow cytometry plots showing the frequency of ALDH^{hi}SSC^{mid} cells with CD14 and CD80 (M1) co-expression in normal-weight individuals and in patients before and 3 months after bariatric surgery.

(B) The frequency of circulating ALDH^{hi}SSC^{mid} cells with CD14 and CD80 co-expression was higher in patients before bariatric surgery compared to normal-weight individuals and was normalized after bariatric surgery.

(C) Representative flow cytometry plots showing the frequency of ALDH^{hi}SSC^{mid} cells with CD14 and CD163 (M2) co-expression in normal-weight controls and in patients before and 3 months after bariatric surgery.

(D) The frequency of circulating ALDH^{hi}SSC^{mid} cells with CD14 and CD163 co-expression was lower in patients before bariatric surgery compared to normal-weight individuals and was normalized after bariatric surgery.

Data are presented as means ± SEMs. *$p < 0.05$ and ‡$p < 0.001$ per the Student's t test.

739.3 ± 43.8 ng/mL) or when compared to the control group (686.2 ± 33.0 ng/mL). In contrast, catalase accelerates the decomposition of ROS and protects cells from oxidative stress. Serum catalase concentration was significantly higher in bariatric surgery patients at baseline compared to the control group (41.9 ± 7.2 ng/mL versus 22.1 ± 3.4, $p < 0.05$; Table S2) and was further increased (55.6 ± 6.5 ng/mL) 3 months after surgery. Thus, reduced granulocyte burden and improved M1/M2 balance after bariatric surgery correlated with increased ROS-reducing serum catalase levels.

Circulating Interleukin-10 (IL-10) Levels Suggest Reduced Inflammation following Bariatric Surgery

Because bariatric surgery efficiently reduced systemic inflammation via a shift in the balance of monocyte phenotype from M1 to M2 polarization, we also examined whether bariatric surgery was associated with altered circulating anti-inflammatory (IL-10) cytokine levels. IL-10, also known as cytokine synthesis inhibitory factor, is produced primarily by monocytes and mediates pleiotropic effects in immunoregulation. IL-10 downregulates inflammatory cytokine secretion, major histocompatibility complex (MHC) class II antigens, and co-stimulatory molecule expression on myeloid and lymphoid cells. Serum IL-10 levels were equivalent when comparing bariatric surgery patients at baseline with the control group (5.9 ± 1.0 versus 6.2 ± 0.9 pg/mL). At 3 months after bariatric surgery, serum IL-10 concentration was increased compared with baseline (7.1 ± 1.0 pg/mL, $p < 0.05$; Table S2). Cytokines associated with inflammation have been previously shown to decrease following bariatric surgery,[15] and we observed an increase in IL-10 concentration at 3 months, consistent with enhanced M2 macrophage polarization. As indicated by cell phenotype analyses, bariatric surgery efficiently reduced several measures of systemic inflammation and oxidative stress.

DISCUSSION

Bariatric surgery can confer immediate benefits that affect metabolism, including improved insulin sensitivity in a weight loss-independent manner.[4] In our 3-month study, we observed significant weight loss and improved HbA1c alongside reduced granulocyte burden and a shift toward monocytes with M2

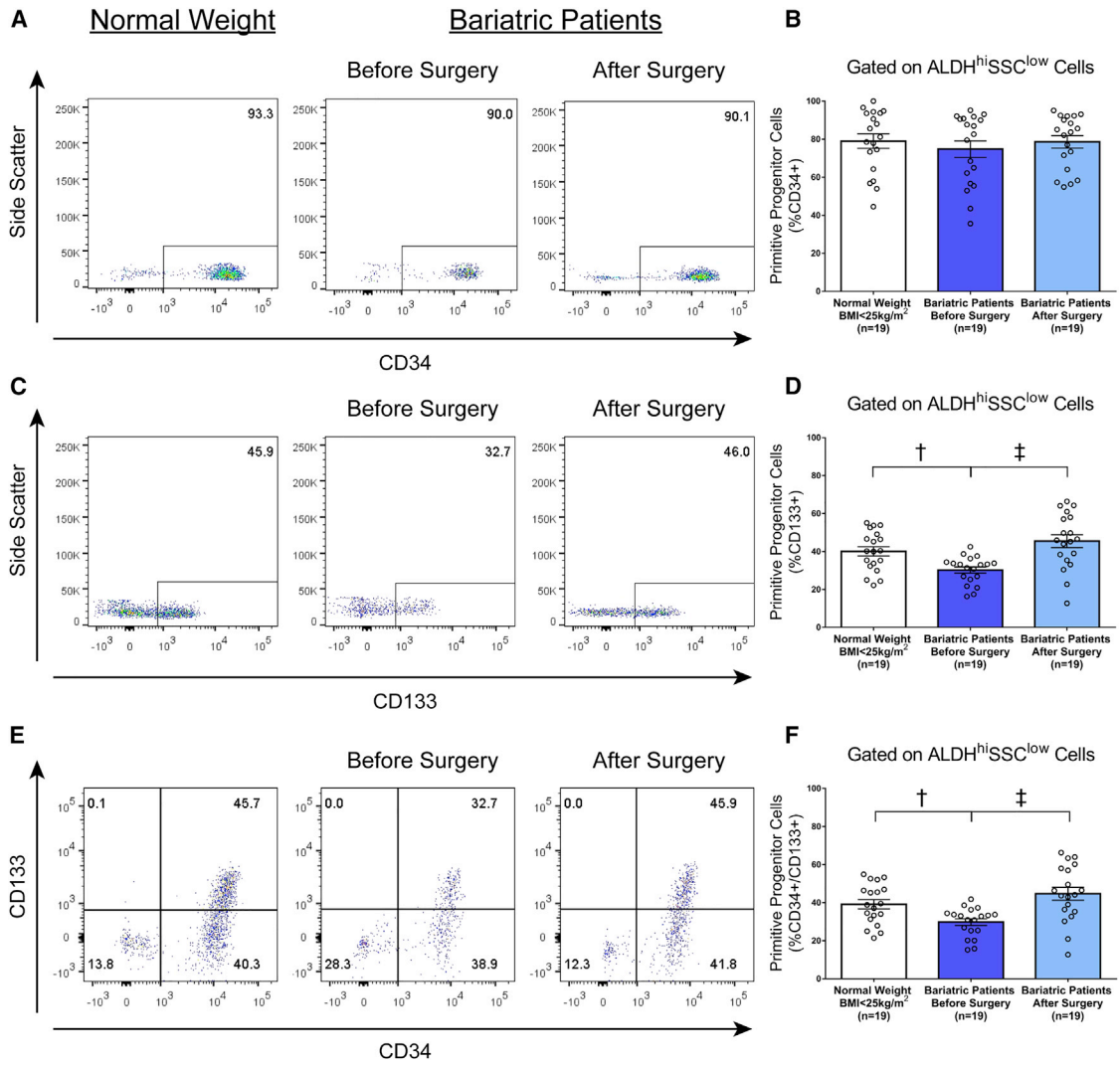

Figure 3. Bariatric Surgery Was Associated with an Increased Frequency of Circulating ALDH^{hi}SSC^{low} Progenitor Cells with CD133 and CD34 Co-expression

(A) Representative flow cytometry plots showing the frequency of ALDH^{hi}SSC^{low} progenitor cells with CD34 co-expression in normal-weight individuals and in patients before and 3 months after bariatric surgery.

(B) The frequency of circulating ALDH^{hi}SSC^{low} progenitor cells with CD34 co-expression was equal in patients before bariatric surgery compared to normal-weight individuals and was not changed after bariatric surgery.

(C) Representative flow cytometry plots showing the frequency of ALDH^{hi}SSC^{low} progenitor cells with CD133 co-expression in normal-weight individuals and in patients before and 3 months after bariatric surgery.

(D) The frequency of circulating ALDH^{hi}SSC^{low} progenitor cells with CD133 co-expression was lower in patients before bariatric surgery compared to normal-weight individuals and was normalized after bariatric surgery.

(E) Representative flow cytometry plots showing the frequency of ALDH^{hi}SSC^{low} progenitor cells with CD34 and CD133 co-expression in normal-weight individuals and in patients before and 3 months after bariatric surgery.

(F) The frequency of circulating ALDH^{hi}SSC^{low} progenitor cells with CD34 and CD133 co-expression was lower in patients before bariatric surgery compared to normal-weight individuals and was normalized after bariatric surgery.

Data are presented as means ± SEMs. $^{\dagger}p < 0.01$ and $^{\ddagger}p < 0.001$ per the Student's t test.

phenotype. Our study represents additional confirmation that chronic metabolic disturbances, such as T2D and severe obesity (BMI > 40 kg/m^2), can induce circulating pro-vascular progenitor cell depletion defined by high ALDH activity.[7] Functional analyses of ALDH^{hi} progenitor cell subsets in patients with T2D and obesity are pending.

Bariatric surgery is commonly referred to as a metabolic surgery because improvements in glucose homeostasis, insulin sensitivity, and decreased use of T2D medications can result before significant post-operative weight loss has occurred.[6] Consistently observed after bariatric surgery is a sharp postprandial rise in several gut-secreted peptides, including

cholecystokinin, glucose-associated ionotropic peptide, glucagon-like peptide-1 (GLP-1), and protein YY. Laferrère et al.[16] have shown that bariatric surgery resulted in a 5- to 6-fold increase in GLP-1 levels and insulin secretion after glucose load, when compared with equal weight loss through low caloric intake. Although incretin hormones are likely a central effector in a mosaic of metabolic changes that accompany gastric bypass surgery, GLP-1 has also been implicated in the reduction of inflammation and oxidative stress. Thus, studies addressing the effects of GLP-1 on circulating pro-vascular progenitor cells in the context of obesity are now warranted. Because GLP-1 is rapidly degraded in the circulation by dipeptidyl peptidase-4 (DPP-4), GLP-1 agonists and DPP-4-inhibitors, two newer classes of glucose-lowering therapies, may both affect the reversal of pro-vascular RCE. In addition, transcriptomic and proteomic approaches using highly purified progenitor cell subsets are warranted to address gene and protein expression alongside phenotypic characterization of circulating ALDH^{hi} cell subsets.

A limitation of this study is that the pro-vascular cell phenotype does not necessarily correlate to functional capacity. As a result, cell sorting to isolate ALDH^{hi} cell subsets for the subsequent analysis of tube formation, migration, matrix remodeling, and paracrine cytokine secretion are under way. Furthermore, additional measures of inflammatory function and intracellular oxidative stress are proposed for future work. Despite demonstrating increased circulating IL-10 levels after bariatric surgery, a more comprehensive profile including other inflammatory biomarkers such as tumor necrosis factor-alpha, interleukin-6, C-reactive protein, and the measurement of other antioxidants such as glutathione would provide further evidence for the reversal of regenerative cell exhaustion following bariatric surgery.

Although our study was sufficiently powered for flow cytometric studies based on previous literature, with many comparisons reaching statistical significance, interpretation of these data would benefit from a larger cohort. For example, most of our patients who received bariatric surgery were female. Having more male subjects would permit comparisons between the response of female versus male patients after bariatric surgery. Finally, determining whether other weight loss regimens can similarly produce improvements in circulating progenitor cell content remains to be addressed in future studies.

In summary, the resolution of cardiovascular events and reduced cardiovascular mortality after bariatric surgery are likely influenced by multiple factors. We provide herein a translational link between bariatric surgery and the improvement of several circulating pro-vascular ALDH-expressing progenitor cell subsets in subjects with obesity. At only 3 months following bariatric surgery and using ALDH activity, circulating granulocyte precursor content was reduced and pro-vascular monocytes and CD133-expressing myeloid cells were increased. The reversal of obesity-induced inflammation and the influence of these cells may confer significant cardiovascular benefit following bariatric surgery in patients with obesity.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xcrm.2020.100018.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD133 (Clone AC133) - PE	Miltenyi Biotech	Cat# 130-113-108; RRID: AB_2725937
Anti-human CD34 (Clone 561) – PE, BV421	BioLegend	Cat# 343606 and 343610; RRID: AB_1732008 and RRID: AB_2561358
Anti-human CD38 (Clone HIT2) – APC	BioLegend	Cat# 303510; RRID: AB_314362
Anti-human CD33 (Clone P67.6) – PE/Cy7	BioLegend	Cat# 366618; RRID: AB_2566420
Anti-human CXCR4 (Clone 12G5) – BV421	BioLegend	Cat# 306518; RRID: AB_11146018
Anti-human CD45 (Clone 2D1) – APC	BioLegend	Cat# 368512; RRID: AB_2566372
Anti-human CD14 (Clone M5E2) – APC	BioLegend	Cat# 301808; RRID: AB_314190
Anti-human CD80 (M1) (Clone 2D10) – PE	BioLegend	Cat# 305208; RRID: AB_314504
Anti-human CD163 (M2) (Clone GHI/61) – PE/Cy7	BioLegend	Cat# 333614; RRID: AB_2562641
Anti-human CD15 (Clone HI98) – PE	BD Biosciences	Cat# 555402; RRID: AB_395802
Anti-human CD66b (Clone G10F5) – PerCP-Cy5.5	BD Biosciences	Cat# 562254; RRID: AB_11154419
Anti-human CD16b (Clone CLB-gran11.5) – BV421	BD Biosciences	Cat# 744821; RRID: AB_2742507
Critical Commercial Assays		
Ammonium chloride lysis buffer	STEMCELL Technologies	Cat# 07850
ALDEFLUOR™ kit (Progenitor Cell Isolation)	STEMCELL Technologies	Cat# 01700
Human Nox1 (NADPH oxidase 1) ELISA kit	MyBioSource	Cat# MBS2540334
Human Cat (Catalase) ELISA kit	MyBioSource	Cat# MBS2600178
Human IL-10 Quantikine ELISA kit	R&D Systems	Cat# D1000B
Deposited Data		
Raw and analyzed data	Flow Repository	ID: FR-FCM-Z2KZ or https://flowrepository.org/id/FR-FCM-Z2KZ
Software and Algorithms		
FlowJo V10	Becton, Dickinson & Company	https://www.flowjo.com/solutions/flowjo/downloads; RRID: SCR_008520
GraphPad Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/; RRID: SCR_002798
Other		
SepMate™ PBMC isolation tubes (15mL)	STEMCELL Technologies	Cat# 5420
EDTA Vacutainer (10mL) – Lavender Top	VWR	Cat# CABD366643L
SST Serum Separation Vacutainer (5mL) – Gold Top	VWR	Cat# CABD367988L
Blood Collection Set with Tubing (21 Gauge)	VWR	Cat# CABD367296

RESOURCE AVAILABILITY

Lead Contact

Additional information and inquiries for reagents and resources should be directed and will be fulfilled by the Lead Contact, Subodh Verma (Subodh.Verma@unityhealth.to)

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The datasets generated during this study are available at Flow Repository: FR-FCM-Z2KZ. https://flowrepository.org/id/FR-FCM-Z2KZ.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study design, participants, and setting

This was a single site study that enrolled individuals who were undergoing bariatric surgery and individuals classified as normal weight using the National Institute of Health criteria. The study was reviewed and approved for initiation on May 1, 2018 by ADVARRA, a central ethics review board. Permission was given to screen, contact and recruit individuals so long as participants consented and were fully informed of study details. Written informed consent was obtained from all study participants. Eligible normal weight participants were between 18 and 55 years of age, with a BMI < 25kg/m^2, and who presented with no major health issues. They attended one study visit. The inclusion criteria for the bariatric surgery group were a documented BMI of 35-40 kg/m^2 with an additional comorbidity, or a BMI greater than 40 kg/m^2. The participants in this group were scheduled for bariatric surgery and attended two study visits, the first just before surgery and the second 3 months post-surgery. Recruitment was equally split between the normal weight control (n = 20) and bariatric (n = 20) group. Medical information and peripheral blood were collected at all study visits. Detailed group information can be found in Table S1.

Sample size estimation and allocation

The sample size was estimated based on comparing two means for a continuous variable:[17]

$$n = \frac{2\left(Z_{(a/2)} + Z_b\right)^2 \times \sigma^2}{d^2}$$

$Z_{(a/2)}$, represents a confidence interval of 95% for a two tailed distribution, an alpha set at 0.05 and the critical value being 1.96. Z_b represents a power of 80%, beta set at 0.2 and the critical value being 0.84. σ^2 represents the population variance for which we determined using previous literature examining progenitor cells in a similar condition. d represents the difference that we plan to detect based on previous literature published in this area.[7,9] The population variance and expected difference were based specifically on the $ALDH^{hi}SSC^{hi}$ populations.[7,9] Following all sample size estimations for both; normal weight controls versus individuals with obesity and before versus following bariatric surgery comparisons, the largest value estimated for a group was 15. Therefore, the aim was to recruit up to 20 individuals per group (normal weight control and bariatric surgery) to allow for a drop out rate of 25%.

METHOD DETAILS

Blood Samples

A total of 50 mL of peripheral blood was collected into both EDTA-lined and spray-coated silica and polymer gel vacutainers (BD Biosciences) at each study visit.

Some samples were processed for multi-parametric progenitor cell analyses by flow cytometry. Peripheral blood mononuclear cells were isolated from whole blood by Ficoll density gradient centrifugation. Next, cell lysis with ammonium chloride buffer followed by PBS wash removed remaining red blood cells and cellular debris. N,N-diethylaminobenzaldehyde (DEAB), a cellular inhibitor of ALDH, was added to a separate tube to establish gates for low versus high ALDH activity. Tubes containing ALDEFLUOR™ substrate were incubated for 30 min at 37°C in ALDEFLUOR™ buffer. Fluorochrome-conjugated anti-human antibodies were added, and the cells incubated for an additional 30 min at 4-8°C.

Samples collected in spray coated silica and polymer gel vacutainers were allowed to clot for at least 30 min at room temperature then centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was collected, divided into aliquots of 0.5 mL and stored at −80°C.

Flow Cytometry Analysis

Progenitor cell phenotype was first assessed for ALDH activity. Precursor cells with high ALDH activity were sub-divided into three groups based on light side scatter (SSC) properties to discern cells with high (granulocytes), intermediate (monocytes) or low (progenitor cells) cellular complexity. Next, identification of cell surface marker co-expression within each aforementioned region was examined using primitive progenitor cell (CD34, CD133, CD33) and M1/M2 macrophage (CD80, CD163) markers. Circulating inflammatory and progenitor cell content was acquired using a 6-color LSR Fortessa X-20 flow cytometer (BD Biosciences) at the Li Ka Shing Knowledge Institute's flow cytometry core facility. Data were analyzed using FlowJo software.

Enzyme-linked immunosorbent assay (ELISA)

Oxidative Stress

Levels of Nox1 and catalase were analyzed using kits from MyBioSource (MBS2540334 and MBS2600178). Serum samples were diluted 1:100 and analyzed in triplicate. Following a 90 min incubation at 37°C, 100 μL of the biotinylated detection antibody was added. After a second incubation of 60 min at 37°C, the horseradish peroxidase-conjugate was added (100 μL) and the samples allowed to sit for 30 min at 37°C before being exposed to the substrate reagent (90 μL) for 15 min at 37°C. The plate was read at 450 nm with a SpectraMax M5 Multi-Mode Microplate reader (Molecular Devices) after the Stop (50 μL) solution was added.

Systemic Inflammation

Serum interleukin 10 (IL-10) levels were measured in duplicate with a kit from R&D Systems (D1000B). Samples (200 μL) were mixed with the assay diluent (50 μL) and allowed to incubate for 2 hr at room temperature before human IL-10 conjugate (200 uL) was added for 2 hr at room temperature. This was followed by the addition of the Substrate solution (200 μL) and incubation for 30 min at room temperature before the Stop solution (50 μL) was added. The plate was read at 450 nm as described above.

QUANTIFICATION AND STATISTICAL ANALYSIS

Comparison between continuous variables of normal weight controls versus bariatric patients prior to surgery was conducted using the Student's t test on GraphPad Prism software and presented as mean ± SEM. Categorical variable analysis between the former groups was conducted using the Fisher's exact test on GraphPad Prism software and presented as n (%). Quantification of cellular frequencies was performed using FlowJo software and negative controls to determine positive marker signals. Analyses of ALDH activity and co-expression of cell surface markers was done using the Student's t test through GraphPad Prism software and presented as mean ± SEM. ELISA data were quantified using a SpectraMax M5 Multi-Mode Microplate reader and analyzed using the Student's t test on GraphPad Prism software and presented as mean ± SEM. The Fisher's exact test was conducted for the categorical variables because some cells used for the analysis had a value of 0, making this test the most appropriate. Normality was assessed using the Shapiro-Wilk normality test on GraphPad Prism software and thus, analyses using the Student's t test was deemed most accurate based on our data. Details for all statistical analyses are found in Table and Figure Legends.

ADDITIONAL RESOURCES

This study has been registered on ClinicalTrials.gov to provide another resource in which individuals can view our study protocol and/or validate our findings. We did not initially register the study since it did not fit the ICMJE definition of a clinical trial (i.e., it did not "prospectively assign people or a group of people to an intervention") and the ICMJE guidelines specify that registration is not required for studies as this in which the "exposure or intervention is not dictated by the researchers." Regardless, the registry provides a description of the study, its design, the outcome measures and related literature pertinent to this study. ClinicalTrials.gov Identifier: NCT04132531.