Inflammatory "Adiposopathy" in Major Amputation Patients

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Abstract

Background—Much ado has been made about obesity's health impact, largely founded on simple patient weight and circulating adipose-derived mediator levels. Paradoxically, a "healthy obese" state exists, but substantial knowledge gaps also exist regarding human adipose-phenotype determinants. Surgical major amputation (AMP) patients are the "sickest-of-the-sick". Conversely, elective knee-replacement (TKR) is reserved for patients who expect continued health and longevity. To delineate human adipose biology variability and clinical determinants, we studied fresh subcutaneous adipose from AMP patients, using TKR patients as controls. We hypothesized that AMP patients would display a pro-inflammatory adipokine signature, and that certain clinical conditions (diabetes, hypertension, hyperlipidemia, high BMI, uremia) would independently drive elevated adipose inflammation.

Methods—AMP (n=29) and TKR (n=20) adipose and clinical data were collected prospectively, and protein was isolated and analyzed for eight adipose-related mediators. Statistical analyses included Wilcoxon-rank sum, Fischer's exact and multiple linear-regression modeling of clinical parameter predictors of mediator expression.

Results—IL-6, IL-8, leptin, resistin, and PAI-1 were differentially expressed (up to 200-fold) between AMP/TKR cohorts. Key clinical parameters which associated with protein levels of adipose-phenotype included age, sex, hypertension, hyperlipidemia, congestive heart failure, cerebrovascular disease, renal disease, and warfarin, statin, and insulin use, with simple BMI failing to be predictive.

Conclusions—AMP-patients display adiposopathy, with a pro-inflammatory adipose-phenotypic signature compared to TKR-controls. BMI fails to predict phenotype, yet other clinical conditions such as age, hyperlipidemia, and renal insufficiency do drive adipokine expression. Understanding human adipose-phenotypic determinants stands as a fundamental priority when future studies dissect the interplay between adipose biology and surgical diseases/outcomes.

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Introduction

Adipose tissue has emerged as a pivotal effector of mammalian homeostasis beyond its historic role as an inert energy depot.[1, 2] While classical clinical evaluation of adiposity utilizes total fat volume (e.g. body mass index (BMI), percent body fat, distribution, etc.), these measurements do not always correlate well with clinical phenotypes.[3] In recent years the term "adiposopathy" has been coined to represent immune and metabolic derangements in adipose tissue.[4] Substantial knowledge gaps exist regarding the dynamics of adipose phenotype and the true role of adipose-related signaling networks in disease. The literature to date largely builds on serum circulating biomarker levels or animal models of human disease, and direct interrogation of clinically relevant human adipose tissue has been relatively limited.[5–9]

Patients progressing to clinical need for major amputation are considered the "sickest of the sick", usually with advanced stages of diseases such as diabetes, renal insufficiency, and atherosclerosis.[10] Few surgical procedures rival major lower extremity amputation for thirty day morbidity and mortality.[10–12] Conversely, elective orthopedic procedures for osteoarthritis (such as hip and knee replacements) are offered to selected similarly aged patients who are well enough to withstand such elective surgical procedures, and who are predicted to maintain overall health and longevity sufficient to derive benefit from surgery.

Thus to advance understanding of the spectrum and determinants of human adipose biology, we compared key lower extremity adipose tissue protein components from major amputation patients with fat collected from patients undergoing elective orthopedic procedures. Use of these real-world clinical specimens offers insights into the variability and clinical determinants of human adipose phenotypes. We hypothesized that there would be more variation between the two patient cohorts than within the groups, and that patients undergoing leg amputation would display a relatively higher pro-inflammatory adipokine signature and lower levels of anti-inflammatory marker adiponectin. Finally, we hypothesized that clinical conditions such as diabetes, hypertension, hyperlipidemia, body mass index, and uremia would correlate positively with adipose inflammation.

Materials and Methods

Patients undergoing lower extremity major amputation (below knee or above knee) or elective orthopedic total knee replacement at a single institution were prospectively identified via procedures approved by the local institutional review board (IRB). Informed consent was obtained from the control elective orthopedic cohort. The amputation patients were enrolled under an IRB approved protocol that allowed us to collect de-identified medical information and tissue from the amputated limb without informed consent. Indications for amputation included unreconstructable critical limb ischemia and non-salvageable foot. All patients in the control cohort underwent elective knee replacement for osteoarthritis.

All samples were collected intraoperatively by trained surgeons. Two grams of subcutaneous adipose tissue were collected from the amputated limb and immediately flash frozen in liquid nitrogen, then stored at −80 °C until the time of analysis. The adipose samples were from the proximal end of the specimen, i.e. near a level where the clinical evaluation supported enough blood supply for healing, and remote from ischemic and infected tissues. Proteins were isolated from the samples in ice-cold Dulbecco's phosphate buffered saline with Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN), homogenized, and centrifuged ($2,000g \times 5$ minutes) to remove gross debris. The homogenates were next centrifuged once more ($10,000g \times 10$ minutes). The supernatant was

then collected for quantitative protein analysis via multiple antigen flow microparticle bead assay Luminex (Luminex Corporation, Austin, TX) according to the manufacturer's instructions, for eight key biologic mediators: interleukin-6 (IL-6), interleukin-8 (IL-8), leptin, tumor necrosis factor-α (TNF-α), adiponectin, resistin, and plasminogen activator inhibitor-1(PAI-1). Quantities of the biologic mediators were normalized to total protein as determined via a Bradford protein assay.

Demographic and clinical data were collected retrospectively from patients' online medical records. Endpoints recorded included age at surgery, gender, race, body mass index, diagnoses of diabetes mellitus, hypertension, hyperlipidemia, coronary artery disease, congestive heart failure, cerebrovascular disease, renal disease, or pulmonary disease, any history of current or former tobacco use, and use of the following pharmaceutical agents: anti-platelet therapy, warfarin, calcium channel blockers, beta blockers, ACE inhibitors/ angiotensin receptor blockers, HMG-CoA reductase inhibitors, insulin, and oral steroids.

All statistical analyses were performed with the Stata 12 software package (StataCorp, College Station, TX). Patient demographics as well as sample mediator levels from the two groups were compared for significance using Wilcoxon-rank-sum tests. A linear regression model was utilized to determine which clinical parameters were predictive of the mediators examined and assess the relative strength of the predictive ability (denoted by the coefficient β). Significant results were defined as those with α 0.05.

Results

Adipose samples were collected from 29 patients who underwent lower extremity amputation and 20 patients who underwent total knee replacement. Patient demographics and baseline characteristics as collected at the time of surgery are listed in Table I. Diabetes mellitus, hypertension, hyperlipidemia, coronary artery disease, congestive heart failure, cerebrovascular disease, renal disease, and pulmonary disease were defined as documented diagnosis in clinical records. The amputation cohort was older than the control cohort overall and had a greater incidence of diabetes mellitus, cardiac, cerebrovascular, renal, and pulmonary disease. In addition, this cohort took calcium channel blockers, beta blockers, statins, and insulin at higher frequencies and displayed a lower overall BMI and a more frequent history of smoking.

Representative Masson-stained microscopic sections from the two cohorts are shown in Figure I. While some fields in the amputation group demonstrated overt fat necrosis, most fields examined showed virtually normal adipose tissue, with scattered mild chronic inflammation and reactive adipocyte changes identified. Tissue from knee replacement patients showed virtually no histological evidence of pathology.

Protein analysis of adipocyte-related mediators revealed significant differences between groups in IL-6, IL-8, leptin, resistin, and PAI-1 (Table II). Levels of TNF-α, MCP-1, and adiponectin were similar. IL-6 was 200-fold higher in the amputation cohort, and IL-8 was 150-fold higher. Resistin and PAI-1 were also significantly higher in amputees, and leptin was significantly decreased.

Linear regression analysis was performed to link clinical parameters to the measured adipose-derived mediator levels as summarized in Table III. This analysis demonstrated significant relationships between multiple clinical parameters and the mediators. Age was found to be negatively predictive of IL-6, IL-8, and TNF-α. Male sex was negatively predictive of adiponectin and hypertension was positively predictive of PAI-1. Hyperlipidemia was negatively predictive of leptin, resistin, TNF-α, and adiponectin. Congestive heart failure was positively predictive of resistin and PAI-1. Cerebrovascular

disease was positively predictive of resistin. Renal disease was positively predictive of IL-6, IL-8, resistin, and TNF-α. Warfarin was negatively predictive of MCP-1. Statin and insulin use were found to be positively predictive of TNF-α; additionally, insulin negatively predicted adiponectin. Race, diabetes mellitus, hypertension, coronary artery disease, pulmonary disease, smoking history, anti-platelet agent, calcium channel blocker, and beta blocker use, ACE inhibitor/ARB use, oral steroid use, and importantly BMI were not found to be significant predictors of any of the examined markers of adipose phenotype.

Discussion

Utilizing fresh adipose samples, we describe dramatic phenotypic differences between two similarly aged real-world patient cohorts undergoing surgery. The two groups did display substantially different characteristics prior to surgery. In general, the amputation cohort had more chronic disease processes, was more likely to be on medications such as calcium channel blockers, beta blockers, statins, and insulin, and they were leaner (lower BMI). This was expected given that the amputation patients are recognized to be a very ill population with a high incidence of significant comorbidities and corresponding use of medications.[13]

Evidence assembled in recent years unequivocally links inflammation with metabolic diseases and implicates molecular mediators as active participants in this process.[14] Adipocytes appear to play a central role in the dyslipidemia, hypertension, insulin resistance, and prothrombotic/proinflammatory states of the metabolic syndrome.[15–18] Adiposopathy has also been directly linked to endothelial dysfunction and promotion of atherosclerosis.[4] Bariatric surgery may ameliorate cardiovascular disease risk through a partial recovery from adiposopathy (improved adiponectin and leptin/adiponectin ratio).[19] Much of the prior knowledge relating these mediators to clinical parameters is based on serum samples.[20–22] Our findings add to the growing pool of knowledge about adipose as a distinct tissue type and specifically to adipose in patients with advanced medical conditions such as diabetes and peripheral vascular disease necessitating major amputation.

Subcutaneous adipose is negatively correlated with atherogenic metabolic risk factors, but little is understood about differences between subcutaneous depots across different patient groups when stratified by disease profile.[23] Our study is among the first to define the differences in this regard and to establish the pro-inflammatory nature of subcutaneous adipose tissue in a patient cohort with advanced limb ischemia.

Major amputation patients displayed upregulation of proinflammatory cytokines IL-6, IL-8, PAI-1, and resistin, and downregulation of the adipose-derived hormone leptin. Our findings are consistent with a previous study showing higher PAI-1, IL-6, and IL-8 levels in subcutaneous adipose tissue of patients with metabolic syndrome as compared with controls.[7] IL-6 plasma levels drawn from the portal vein have been demonstrated to be higher than radial artery levels in obese individuals undergoing open gastric bypass surgery, suggesting increased secretion by visceral fat depots which drain into the portal circulation of the liver[24], and increased circulating concentrations of IL-6 have been shown to correlate with increased incidence of diabetes mellitus and cardiovascular disease.[25] Plasma IL-8 has been shown to be an independent predictor of cardiovascular events in patients with stable coronary artery disease[26], and subcutaneous adipose tissue expression has been demonstrated to decrease significantly after gastric bypass surgery.[27] Additionally, PAI-1 shows evidence of higher expression in subcutaneous adipose tissue in patients with cardiovascular disease than in patients without.[28]

Analysis of adipocyte-derived mediators in adipose tissue and comparison with clinical data yielded a number of interesting insights. We discovered that a number of clinical

parameters, including age, male sex, hyperlipidemia, congestive heart failure, cerebrovascular disease, renal disease, antiplatelet therapy, warfarin, calcium channel blocker, beta blocker, statins, and insulin use are predictive of the adipose associated mediators. Other factors including obesity, race, diabetes mellitus, hypertension, coronary artery disease, pulmonary disease, smoking history, ARB/ACE inhibitors, and steroid use did not predict adipose phenotype under the conditions of this cross-sectional study. The findings reported reflect the single time point end-product of a number of adipose phenotype determinants, and further work is required to individually isolate the impact of these factors. Although the relationship between visceral adipose mass and metabolic syndrome has been well established, it has also been demonstrated that adipose phenotype is not necessarily predicted by adipose volume alone, which is in our agreement with our finding that BMI was not predictive.[29] Prior research has shown that obese individuals without an inflamed adipose phenotype display a more favorable clinical profile; we did not stratify our groups by inflamed/non-inflamed phenotype, but rather looked at each cohort as a whole.[30]

Clear limitations to the dataset and research strategy are acknowledged. We hypothesized that certain clinical conditions would independently drive elevated adipose inflammation. While we define clear associations, it is possible that the adiposopathy may be an underlying cause of some diseases. Our study included a limited number of patients, and the amputation and control cohorts were not perfectly matched for a number of clinical characteristics. Certainly some of the amputation cohort specimens may have had confounding tissue-level ischemia, but we strived to harvest adipose from the proximal, better perfused portion of the limb at which normal wound healing was expected to occur. Furthermore, the adipose tissue analysis employed here does not indicate whether the phenotypic signatures observed were due to, causative, or merely associated with of the numerous medical conditions present in the patient cohorts. The results are essentially the net effect of a number of factors, some of which may be competing. Similarly, the correlations that we found between clinical parameters and tissue biomarker levels also do not necessarily imply a cause-and-effect relationship. Finally, we did not assay serum biomarkers, which are known to mediate biologic events, and cannot comment on the exact relationship between tissue and circulating levels. However, the relationship of circulating mediator levels with clinical parameters has been previously reported.[21, 22, 31, 32]

Conclusion

In conclusion, this work establishes important BMI-independent inflammatory phenotypic differences between subcutaneous adipose tissue in patients with end-stage peripheral vascular disease undergoing major amputation and a control patient cohort. As adipose biology is increasing linked to surgical diseases and outcomes, adipose phenotypic differentiation represents a fertile field for accelerated exploration.

Acknowledgments

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Figure 1.
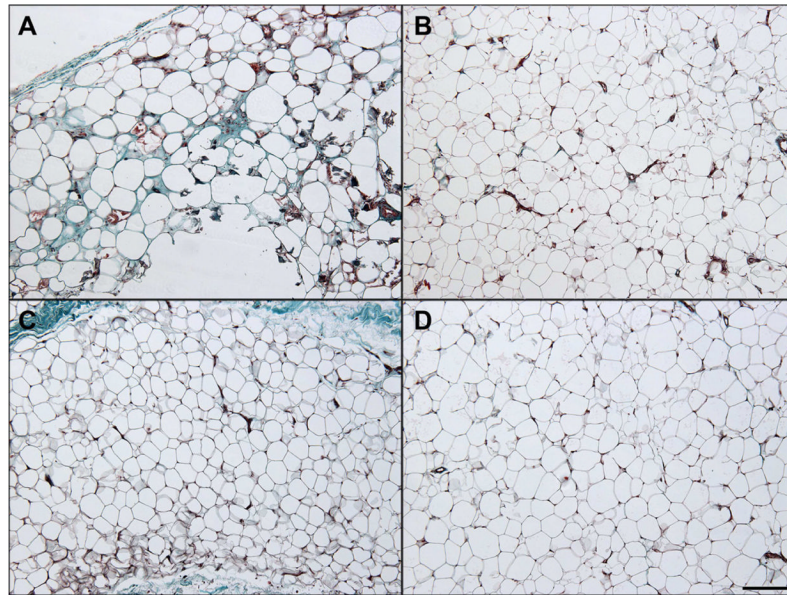Representative Histology, Masson stained. Two representative fields (100X magnification) from trichrome stained sections from each cohort are shown. Tissue from amputation patients (panels A and B) showed rare fat necrosis (panel A) amidst relatively normal adipose tissue (panel B). Tissue from control patients (panels C and D) did not show fat necrosis in the sections examined. Scale bar = 200 µm

Table I

Descriptive statistics; *P* by Rank Sum or Fisher's exact test

	Mean or Weighted Frequency (SE)		*P*
	Amputation (*n* = 29)	Control (*n* = 20)	
Age	70.7 years (±2.7)	63.5 years (±2.2)	**0.029**
Race:			0.596
White	16 (55.17%)	14 (70.00%)	
Black	8 (27.59%)	4 (20.00%)	
Hispanic	5 (17.24%)	2 (10.00%)	
Female	9 (31.03%)	11 (55.00%)	0.140
BMI	24.7 kg/m^2 (±1.2)	34.2 kg/m^2 (±1.8)	**<0.0001**
Diabetes Mellitus	19 (65.52%)	5 (25.00%)	**0.006**
Hypertension	27 (93.10%)	15 (75.00%)	0.087
Hyperlipidemia	20 (68.97%)	10 (50.00%)	0.149
CAD	17 (58.62%)	3 (15.00%)	**0.002**
CHF	7 (24.14%)	0 (0.00%)	**0.018**
CVA	8 (27.59%)	0 (0.00%)	**0.010**
Renal Disease	12 (41.38%)	0 (0.00%)	**0.001**
Pulmonary Disease	9 (31.03%)	0 (0.00%)	**0.005**
Smoking History	18 (62.07%)	5 (25.00%)	**0.006**
Antiplatelet Therapy	22 (75.86%)	14 (70.00%)	0.446
Warfarin	9 (27.58%)	11 (55.00%)	0.051
Calcium Channel Blocker	10 (34.48%)	1 (5.00%)	**0.015**
Beta Blocker	24 (82.76%)	3 (15.00%)	**<0.0001**
ACE Inhibitor/ARB	12 (41.38%)	12 (60.00%)	0.161
Statin	22 (75.87%)	9 (45.00%)	**0.029**
Insulin	18 (62.07%)	3 (15.00%)	**0.001**
NSAID	23 (79.31%)	17 (85%)	0.455
Steroid	3 (10.34%)	0 (0.00%)	0.198

Table II

Comparative subcutaneous adipose protein concentrations

	Amputation (*n* = 29)	TKA (*n* = 20)	*P*
	Mean pg/mg total protein ± SEM		
IL-6	293.10 ± 234.10	1.50 ± 2.20	**<0.0001**
IL-8	245.46 ± 234.24	1.68 ± 0.15	**0.0005**
Leptin	1635.66 ± 444.69	3434.29 ± 586.24	**0.0032**
TNF-α	1.08 ± 0.29	0.68 ± 0.07	0.6545
MCP-1	173.46 ± 38.30	93.78 ± 14.50	0.2147
Adiponectin	438818.40 ± 85487.62	416429.90 ± 33316.05	0.0838
Resistin	2930.30 ± 603.87	637.41 ± 116.27	**0.0001**
PAI-1	447.37 ± 111.32	74.51 ± 9.01	**0.0008**

Table III

Association of clinical factors with key mediators

	IL-6		IL-8		Leptin		TNF-α		MCP-1		Adiponectin		Resistin		PAI-1	
	Beta	P	Beta	P	Beta	P	Beta	P	Beta	P	Beta	P	Beta	P	Beta	P
Age	-0.5493	0.026	-0.5352	0.035	-0.2006	0.372	-0.6250	0.005	-0.3297	0.183	-0.3731	0.125	-0.1896	0.332	-0.2620	0.253
Sex	0.0690	0.707	0.0924	0.626	-0.2169	0.220	-0.1052	0.515	-0.2822	0.146	-0.4319	0.027	-0.1623	0.289	-0.0589	0.739
Race	0.0289	0.875	0.0450	0.812	0.1473	0.402	0.1710	0.295	0.1210	0.527	0.1535	0.413	0.0379	0.803	-0.0216	0.903
BMI	-0.3552	0.173	-0.3481	0.194	0.0339	0.889	-0.2535	0.265	0.2011	0.450	-0.1887	0.468	-0.0486	0.818	-0.2070	0.404
DM	0.2352	0.574	0.1865	0.664	0.3793	0.342	0.4276	0.249	0.3745	0.389	0.7117	0.101	0.4364	0.211	0.3907	0.335
HTN	0.2330	0.308	0.2233	0.342	0.0110	0.959	0.2196	0.274	0.1506	0.521	0.1023	0.655	0.1061	0.570	0.4666	0.040
HLP	-0.2685	0.228	-0.2693	0.240	-0.5246	0.018	-0.5273	0.011	-0.1352	0.553	-0.5118	0.028	-0.3929	0.038	-0.2263	0.290
CAD	0.3750	0.188	0.3240	0.267	0.3074	0.253	0.3134	0.209	0.1522	0.600	-0.0232	0.935	0.2856	0.222	0.4660	0.093
CHF	0.1901	0.320	0.1684	0.391	-0.0891	0.621	0.3355	0.052	0.2423	0.223	0.2862	0.143	0.4042	0.015	0.3916	0.040
CVA	0.1534	0.514	0.0996	0.680	-0.0317	0.886	0.2485	0.233	0.3736	0.132	0.2975	0.216	0.4245	0.035	0.2936	0.200
Renal Disease	0.6544	0.012	0.6397	0.016	-0.1370	0.554	0.5954	0.009	0.3900	0.130	-0.2392	0.335	0.4185	0.045	0.4599	0.058
Pulm Disease	0.2209	0.268	0.2511	0.223	-0.0043	0.982	0.1392	0.424	-0.1173	0.566	-0.0880	0.659	0.0474	0.770	-0.2469	0.200
Smoking	-0.0982	0.624	-0.0901	0.662	0.1484	0.436	0.0745	0.671	0.0324	0.875	0.3241	0.117	-0.1225	0.459	-0.0448	0.816
Anti-platelet	0.3378	0.340	0.3912	0.284	0.5417	0.113	0.3686	0.238	-0.4758	0.198	0.2229	0.531	-0.0453	0.875	-0.2551	0.453
Warfarin	0.2766	0.329	0.3456	0.238	0.1741	0.515	0.1569	0.526	-0.5937	0.049	-0.2723	0.342	-0.3393	0.151	-0.2787	0.307
CaCh Blocker	-0.2986	0.176	-0.2977	0.190	-0.1556	0.452	-0.3845	0.052	-0.4506	0.053	0.2417	0.276	-0.3079	0.094	-0.3102	0.146
Beta Blocker	-0.1512	0.674	-0.2131	0.565	0.4604	0.184	0.3163	0.320	0.7397	0.055	0.6435	0.085	0.4028	0.180	0.1861	0.591
ACEI/ARB	-0.2358	0.312	-0.2417	0.314	0.1683	0.444	-0.2268	0.269	-0.2520	0.296	-0.2112	0.369	-0.0236	0.901	-0.4384	0.057
Statin	0.3828	0.165	0.4015	0.157	0.1987	0.440	0.4878	0.048	0.2543	0.366	0.2667	0.333	-0.0132	0.953	0.1342	0.606
Insulin	-0.5661	0.278	-0.4999	0.350	-0.9100	0.072	-1.1788	0.014	-0.8871	0.105	-1.1738	0.032	-0.6214	0.152	-0.8692	0.089
Steroid	-0.1145	0.553	-0.1072	0.589	0.0584	0.749	0.0183	0.914	0.1123	0.574	0.1713	0.382	0.0823	0.604	0.0234	0.899
Amputation	-0.2700	0.477	-0.2247	0.564	-0.2757	0.444	-0.3099	0.354	-0.2957	0.452	-0.5031	0.195	-0.2078	0.506	-0.2015	0.580
NSAID	0.1562	0.582	0.1114	0.703	-0.5404	0.053	0.0489	0.844	0.3257	0.272	-0.0756	0.792	0.1797	0.443	0.3785	0.173