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Epigenetic alterations of AKT1 orchestrate a metabolic reprogramming in advanced lipedema: translational insights from an integrated multi-omics study

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Table of Contents

1 Abstract **3**

2 Introduction **4**

2.1 Lipedema *4*

2.2 Epidemiology *6*

2.3 Etiology *6*

2.3.1 Hormonal 6

2.3.2 Genetic 7

2.3.3 Vascular & Lymphatic 8

2.4 Symptoms *9*

2.4.1 Clinical symptoms 9

2.4.2 Psychological symptoms 10

2.4.3 Comorbidities 10

2.5 Diagnostic criteria *11*

2.6 Classification *12*

2.7 Differentiation Lipedema, Lymphedema, Obesity and Lipohyperthrophy *14*

2.8 Current potential biomarker candidates and potential novel biomarkers *15*

2.9 Therapy *17*

2.9.1 Conservative treatment 17

2.9.2 Surgical treatment 18

3 Questions and Hypothesis **19**

3.1 Goals and Objectives *19*

3.2 Specific Aims and Impacts on the National Healthcare System *20*

4 Material and methods **22**

4.1 Population study *22*

4.2 Description of two cohorts and criteria *22*

4.3 Tissue sampling *22*

4.4 DNA extraction and Genome-wide methylation array *23*

4.5 Protein extraction, western blot and antibodies *23*

4.6 RNA isolation and RNA sequencing analysis *24*

4.7 Metabolic extraction and NMR metabolomics *24*

4.8 NMR spectra acquisition and assignment *25*

4.9 NMR Statistical analysis *26*

4.10 Bioinformatics analysis *26*

4.11 Statistical methods *27*

5 Results **28**

5.1 Characteristics of the participants *28*

5.2 Genome-wide methylation profiling highlighted a dysregulated receptor tyrosine kinase and phospho-metabolism signaling pathways in advanced stage lipedema patients. *29*

5.3 Transcriptome profiling of advanced stage lipedema patients confirmed mitochondrial and metabolic disfunctions and highlighted sirtuin signaling alteration *30*

5.4 Multi-omics data integration revealed the RAC (Rho family) -alpha serine/threonine-protein kinase AKT1 alteration as novel biomarker for lipedema patients *32*

5.5 Trans-Omic integration highlights AKT1-linked metabolic signatures in advanced lipedema ... *33*

6 Discussion **37**

6.1 Translational Implications *38*

7 Conclusion **39**

7.1 Limitations of the study *39*

7.2 Ethics approval and consent to participate *40*

7.3 Availability of data and materials *40*

8 Acknowledgements **41**

8.1 Funding *41*

9 Supplementary Material **41**

10 List of tables, figures and references **46**

11 List of abbreviations **47**

12 Bibliography **48**

1 Abstract

Background

lipedema is a chronic, progressive adipose disorder predominantly affecting women, characterized by painful, symmetrical subcutaneous fat accumulation, and typically resistant to lifestyle interventions. The pathophysiology of advanced-stage lipedema remains poorly defined, and no validated biomarkers or targeted therapies are currently available.

Methods

in this observational study, we applied a comprehensive multi-omics approach to dissect the molecular and metabolic alterations underlying late-stage lipedema.

Results

Genome-wide DNA methylation profiling identified over 5,000 differentially methylated CpG sites affecting genes involved in receptor tyrosine kinase signaling, phospho-metabolism, and immune pathways. Transcriptomic analysis revealed profound downregulation of mitochondrial functions, including oxidative phosphorylation, the TCA cycle, and fatty acid β-oxidation, alongside disruption of the sirtuin pathway and extracellular matrix remodeling. Integrative analysis pinpointed AKT1 as a central regulatory node: its promoter region was hypomethylated, correlating with increased gene expression and protein phosphorylation. Metabolomic profiling confirmed AKT1-linked metabolic dysregulation, including altered levels of L-arginine, NADP+, ATP, guanosine, glycerol, and glutamate, indicating impaired redox balance and energy metabolism. Trans-omic network analysis positioned AKT1 at the intersection of multiple dysregulated pathways, suggesting its key role in advanced-stage lipedema.

Conclusions

the consistent enhancing of AKT pathway signaling across omic layers highlights its potential not only as a biomarker for disease stratification but also as a putative druggable target for therapeutic intervention. These findings offer new mechanistic insights into lipedema pathophysiology and provide a rationale for future personalized treatment strategies guided by AKT1-centric molecular profiling.

2 Introduction

2.1 Lipedema

Lipedema is a chronic disease, which almost exclusively affects women in phases of hormonal changes such as puberty, pregnancy or menopause [1,2]. The condition presents as a maldistribution of adipose fat tissue between the extremities and trunk of the patient, typically identifiable by the bilateral symmetrical increase of subcutaneous adipose tissue of the limbs [3-5]. Symptomatically patients usually complain of moderate pain in affected areas, easy bruising and orthostatic edema [6]. In more progressed stages, lipedema patients usually suffer from chronic pain and often psychological comorbidities due to the emotional distress [7]. Whilst lipedema patients usually do not complain of an increase in abdominal adipose tissue, it is the symmetrical growth of nodular subcutaneous adipose connective tissue in upper and lower extremity, most notably in the lower extremity sparing the feet (and hands), that effects wide ranging discomforts [8,9].

The pathology was first described by two physicians Allen and Hines in the 1940's in the United States [2]. About a decade later in 1951, the first diagnostic criteria was published providing the first tool to classify the pathology. Over the past years the disease has been put into classification and multiple attempts to investigate the pathophysiology have been undertaken, however the definite pathologic mechanisms remain unclear [4]. This lack of knowledge creates a big issue for patients who suffer from lipedema, due to the delayed diagnosis and in many cases no or a belated treatment [9]. A common challenge patients are also confronted with a misdiagnosis, commonly as "habitual" obesity or lymphedema[9], or even that it is solely regarded as an "aesthetic problem" [10]. The current diagnostics are generally focused on a clinical approach, predominantly based upon on three indicative components: the typical population, timing of appearance of symptoms and location of adipose tissue accumulation [11]. This vague approach however creates a wide range of sources of error.

The aim of this study is to investigate purely the molecular pathophysiology of lipedema using liquid chromatography-mass spectrometry (LC-MS), an analytical chemistry technique that can separate mixtures (tissues) and identify separated components. Therefore, the study is designed to surgically extract adipose tissue from two cohorts (suspected lipedema and a control group) and by aid of LC-MS analyze the proteomic profile of the

samples. Biomarkers are characteristic biologic features that may be genetic, anatomic, physiologic, or biochemical and may be helpful as a reference for physiologic processes and pathologies [12]. Previous attempts to research the pathology as a whole have already established possible links to genetics and possible marker-candidates derived from exosomes, cytokines and other profiling studies [4].

The ongoing dilemma of no universally accepted biomarker or diagnostic test that can reliably diagnose and distinguish lipedema from similar conditions, leads research to attempting to explore such biomarkers. The main research questions and questions of interest are: What molecular and/or metabolic alterations in subcutaneous adipose tissue characterize lipedema? Can reliable biomarkers be identified, that distinguish lipedema from other adipose tissue disorders? And could such findings pave the way for reliable earlier diagnosis and potentially even a personalized therapy? The hypothesis of this prospective study suggests that the subcutaneous adipose connective tissue of lipedema patients shows distinguishable metabolomic patterns to healthy patients, possibly allowing specific biomarkers to be established.

This study aims to take the current ground of data to a next level and focuses entirely on profiling subcutaneous adipose connective tissue on a molecular-biological level comparing samples of lipedema patients to healthy patients and investigating the differences and commonalities on a molecular tier. Ultimately, it must be a collective endeavor to investigate and assess the etiology and pathogenesis of lipedema and specially to raise a certain standard of awareness in order to provide better care for lipedema patients in the future.

2.2 Epidemiology

Based on the vague diagnostic criteria, information about the prevalence of lipedema varies in literature. Lipedema is a disease that nearly exclusively affects women, and only very few male cases have been documented, which have strongly been linked conditions such as growth hormone deficiency, hypogonadism and other forms of testosterone depletion and/or signs of estrogen excess such as in severe liver disease [13-15]. The prevalence of lipedema is estimated to be around 7-9,7%, some sources even reporting numbers as high as 18% in European countries and other authors reporting a "rare disease"[16-18]. Generally the consensus throughout most papers is that lipedema is likely a common disorder, that combats with its rate of underdiagnosis [11,17,19]. However due to the frequent misdiagnosis of lipedema, numbers are thought to be even higher than classically presumed [6,20]. Interestingly, the disease or the identification of the constellation of lipedema symptoms is not very well understood by practicing physicians often leading to incorrect diagnosis and in further consequence mistreatment [1,21]. A British survey from 2014 showed that only 9% of British health professionals diagnosed their patients correctly at first visit and only 5% of general practitioners, indicating a very low understanding of the pathology [22].

2.3 Etiology

The etiology of lipedema remains unclear to the present time and the likely multifactorial pathophysiologic mechanisms are not fully investigated [4,5,20]. Current approaches to study the etiology of lipedema primarily focus on hormonal, genetic and vascular components, yet factors such as disturbances in adipocyte metabolism and cytokine production (inflammatory processes) are also object of investigation [11,15,23,24].

2.3.1 Hormonal

One of the first concrete assumption about the etiology of lipedema was hormonal changes/factors, since the disorder nearly exclusively affects females in times of hormonal changes [23]. Also, the direct link of estrogen modulating estrogen receptors in adipose tissue is well established knowledge [11]. Adipocytes are part of a human body's endocrine system, responding to a variety of molecules associated with lipedema [25]. Estrogen is a steroid hormone that mainly sources in the ovary, placenta during pregnancy and adipose tissue. It exists in three forms, called estradiol, estrone and estriol, with estradiol being the most potent form and estriol the least potent form, respectively. Gene expression of estrogen is regulated

via intracellular receptor signaling pathways called estrogen receptor-α and estrogen receptor-β and it is commonly adopted knowledge that ER-α and ER-β act in direct opposition to each other [26,27]. Most research has been conducted on ER-α, the predominant form.[11] The exact role of estrogen in adipose tissue regulation remains controversial, since early investigations on mice suggested that estrogen receptors (primarily ER-α), can inhibit adipose connective tissue growth, whereas later human studies counter these outcomes and show that estrogen increases body adipose tissue, as commonly perceived by the current state of human physiologic research [11,28,29]. Adipose tissue has shown to be a non-homogenous tissue throughout the body, showing different patterns of growth, morphology, receptor expression and reaction to stimuli at different locations [30]. Estrogen shows to promote adipose tissue growth in the gluteal/femoral region, indicating a higher sensitivity of adipose tissue in this region to certain (steroid) hormones [30]. Different estrogen-mediated signaling processes of both estrogen receptors ER-α and ER-β, such as regulation via Lipoprotein Lipase (LPL), Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ), adrenergic receptors (α and β), GLUT 4 transporter and angiogenesis via Vascular endothelial growth factor (VEGF) have been investigated [11]. The role of both estrogen receptors remains controversial, however it is pretty clearly established that ER-α and ER-β have distinct functions in the body. ER-α is primarily associated with reproductive functions, cell proliferation and oncologic conditions whereas ER-β seems to oppose the effects by promoting anti-proliferative and protective actions [31]. A recently adopted approach to explain the hormonal approach of lipedema etiology is due to a higher ER-α/ER-β ratio that lead to dysregulated adipose tissue behavior.

2.3.2 Genetic

The genetic predisposition for acquiring lipedema has shown to have a significant impact on its etiology. A study conducted in 2009 by Child et al. investigated the genetical component of lipedema and demonstrated a highly likely genetic linkage, however the exact forms of genetic inheritance are not fully explicable [15]. The major Child et al. study detected a familial incidence of 15% that had at least one first-degree relative with confirmed lipedema. In total, 67 family pedigrees have been plotted in the study by Child A. and the pattern showed a consistency with an X-linked dominant inheritance, or an autosomal dominant inheritance with sex limitation, but could also be oligogenic. Even though sex-limited dominant conditions remain rare genetic cases, an additional genetic blood workup of the

same study excluded all markers on the X-chromosome, indicating autosomal dominant inheritance (with sex limitation) to be more likely. Examples of genes that have been recently identified in a context of clinical criteria for lipedema are the AKR1C1 gene encoding for Aldo-keto reductase and a mutation in the PIT1 gene, which encoded for the pituitary-specific positive transcription factor 1, involved in growth- and sex hormone expression in patients with lipedema-like symptoms [24,32].

2.3.3 Vascular & Lymphatic

The vascular and lymphatic hypothesis is mainly based on findings that show microvascular damages and dysfunctions in blood- and lymphatic capillaries and lymphatic fluid accumulation in the interstitium, causing edema [5,21,33]. It is also a controversial subject of how the lymphatic and vascular system trigger or contribute to the formation of lipedema, since there is noticeable evidence for and against this hypothesis. Older studies rather suggest an impairment of the lymphatic system for example by microlymphatic aneurysms in lipedema patients, an abnormal lymphoscintigraphic pattern with a marked slowness of the lymphatic system and an indication of a subclinical status of lymphedema of lipedema patients [34,35]. More recent studies have though shown that lymphatic/vascular insufficiency might not be as important in the etiology of the disorder as previously presumed. According to a German publication of 2020 by Bertsch et al., the paradigm of lipedema being associated with edema should be reconsidered and the authors refer to the first lipedema study of 1951, where only 24% of 119 participants had shown signs of orthostatic edema [36]. MRI imaging studies partially confirmed this hypothesis by demonstrating that T1- and T2- weighted MR-scans of lipedema patients could not verify the presence of edema in the lower limbs [37]. With the current evidence of the vascular/lymphatic dysfunction in lipedema, it is possible to say that edema formation in some patients may indicate a vascular and/or lymphatic malfunction. The increased expression of CD-90 (mesenchymal marker) and CD-146 (endothelial marker) are suggestive of capillary injury, leading to a higher rate of cell turnover [23]. However, due to a rather large inconsistency throughout lipedema patients and lacking knowledge of exact pathological mechanisms, the vascular/lymphatic component seems to play a rather small role in the etiology of lipedema, especially in beginning stages of the disorder. More likely appears the assumption that tissue expansion in early stages of lipedema is due to adipocyte hypertrophy and hyperplasia with minimal edema, however due to yet uninvestigated reasons [25].

2.4 Symptoms

2.4.1 Clinical symptoms

The symptoms of lipedema are chronically progressive and neither the course of the condition, nor the severity of symptoms at different stages of disease can yet be predicted for individual patients [13]. Initially, the clinical symptoms of lipedema have been described as a disproportionate enlargement of adipose connective tissue in the buttock region and lower extremities by Allen and Hines in 1951 [38]. In the past decades, the variety of symptoms has increased, mainly due to the observations of the disease oftentimes manifesting in the upper extremity as well [4]. The most indicative symptom physicians should look at today is the bilateral and symmetrical enlargement of subcutaneous adipose tissue in upper and lower extremity, whereby the lower extremity type presents a higher incidence [2,18]. Isolated lipedema in the upper extremity has shown to appear in only very rare cases [13].

The first and most abundant symptoms to watch out for are pain that can be triggered by pinching of the skin in affected areas, the presence of palpable subcutaneous nodules in the skin that can range from smooth to granular and nodular, suprapatellar and shin fat pads and the disappearance of the retro malleolar indentation [25,39]. Many patients also report of easy bruising in affected areas, which is linked to the increased capillary permeability resulting in increased fluid shift from the vasculature into the interstitial compartment [13,15]. In further course of the disorder, the sensation of leg heaviness, fatigue and chronic unspecific pain in affected regions are dominant [21,40]. The pain, which is described in lipedema patients is worsened by warm weather and exercise and does typically also not disappear when the affected limbs are elevated or compressed, indicating that the enlargement of the legs is rather depended on fatty deposits than the edema [21,38]. Important symptoms that may help to distinguish lipedema from other adipose tissue disorders sparing of hands and feet, where the onset of excess adipose connective tissue is consistent abruptly above the medial and lateral malleoli [21]. Physical complications of lipedema are primarily chronic pain conditions and orthopedic complications. Chronic pain conditions in a polish lipedema study of 2021 by Dudek et al. were affirmed by over 80% of patients, half of which claimed moderate pain and the other half to have severe pain [40]. The orthopedic complications originate due to the excess tissue accumulation, especially in the lower extremity that can result in osteoarthritis of the knee due to mechanical axis shifts and imbalanced stress in the

knee joint [13]. Knee pain as a result of lipedema was also recorded in up to 55% of patients, as a study from 2009 by Child et al. found out [15].

2.4.2 Psychological symptoms

The disorder generally has a very high impact on patients' mental health and quality of life. According to a large lipedema study that has been conducted in the UK, approximately 85% of patients report psychological complications and a massively reduced quality of life since living with the diagnosis [22,41]. Conditions such as depression and anxiety are very common among patients with lipedema [17]. A recent research article, investigating quality of life of lipedema using the PHQ-9 assessment to assess depression severity in Poland, shows that more than half of lipedema patients reported a severity compatible with depression and 11% even indicated a level compatible with severe depression, a PHQ-9 score of over 20 [40]. One of the major psychological stressors' patients experiences are the aesthetic changes the disorder causes. The large 2014 lipedema UK survey study by Fetzer et al. showed that for example 95% of patients reported difficulty buying clothes, 86% reported low self-esteem, 60% reported personal restrictions in social life due to discomfort and 45% even reported eating disorders because of lipedema diagnosis [22]. The psychological adverse effects that arise consequently are likely explainable due to the long lapse of time to diagnosis, the repeated counseling of physicians, nutrition- and exercise experts, the to a large extent ineffective treatment options and the patients despair of the general unawareness of the condition. In a US study by Fife et al. of 2010, researchers found out, that women with lipedema revealed a higher severity of depression than paralysis patients [42].

2.4.3 Comorbidities

Comorbidities primarily occur in patients with advanced age. An exploration by Romeijn et al. showed more than half of lipedema patients in a study had comorbidities, the mean age of patients being 46,8 years [19]. The most common comorbidities in the provided cohort were cardiac pathologies, followed by thyroid disease, fibromyalgia, diabetes mellitus and bowel disease. The dispersion of occurrence of comorbidities suggests, that lipedema is not actively responsible for causing comorbidities, rather they occur by the natural process of ageing.

2.5 Diagnostic criteria

The diagnosis of lipedema is a clinical diagnosis, which is currently based on a physician's individual anamnesis, accurate surveying of the medical history and a clinical investigation.[5] Very rarely, additional procedures such as genetic testing or histological analysis are applied due to the complexity and expenses of these methods [43]. The WHO has categorized lipedema in stages in their ICD-10 classification of 1994, however only in 2019 the WHO has introduced lipedema in the newer ICD-11 classification as a separate clinical condition, in category "Certain noninflammatory disorders of subcutaneous fat" [23,40]. It is marked as condition ICD EF02.2 and described as following: "Lipoedema is characterized by non-pitting diffuse "fatty" swelling, usually confined to the legs, thighs, hips and upper arms. It may be confused with lymphoedema. Lipoedema may also occur in the scalp" [43].
Originally the establishment of diagnostic criteria dates back to 1951 to Wold, Allen and Hines who analyzed 119 cases and provided a diagnostic criteria for lipedema, however recent modifications, mainly by Herbst, have established what is used at present time [2,7,38]. The anamnesis and examination should include following components, as listed in *figure 1*.

Clinical criteria lipedema diagnosis:

- Almost exclusive appearance in women (A&H)
- Bilateral and symmetrical manifestation with minimal involvement of the feet
- Minimal pitting edema
- Pain, tenderness on pressure and easy bruising
- Persistent enlargement after elevation of extremities or weight loss
- Increased vascular fragility
- Negative Kaposi-Stemmer sign*
- Arms are affected 30% of the time*
- Hypothermia of the skin*
- Swelling worsens with orthostasis in summer*
- Unaffected by caloric restrictions*
- Telangiectasias*

Figure 1: Clinical criteria lipedema diagnosis. The first 6 points date back to 1951 and have been established by Wold, Allen and Hines. The points marked with a "*" are modifications and have more recently been added to the list of criteria.

Apart from the clinical diagnostic criteria, factors such as the timing of first appearance as well as genetic background and family history should be considered due to its high indicative correlation [9,24]. As already indicated, the onset of lipedema seems to strongly correlate

with changes in the hormonal household, particularly estrogen [11]. The phases to closely monitor are puberty, pregnancy and menopause, but also when starting or discontinuing hormonal contraception, whereby the age of puberty notes down the greatest incidence of new cases of lipedema with one study by Child et al. even showing an incidence of 55% during puberty [15,22]. The genetic component is also important since several studies have shown that patient pedigrees suggest a inheritable pattern, particularly X-linked dominant or more likely autosomal dominant with sex limitation [21,23].

2.6 Classification

Several classifications for lipedema exist, but there is no international consistently used and accepted universal classification. One of the first widely used classification was the ICF-classification (International Classification of Functioning, Disability and Health), which was established together with the WHO in 2014 [10,11]. It was designed in a matter of two parts, one part that describes function and disability of lipedema patients and a second part that is based on environmental- and personal factors. In other studies such as the "Lipedema-3" study by Marshall et al., the authors propose a sonometric approach to diagnose lipedema, where the thickness of the cutis and subcutaneous tissue measured at the medial malleolus, determines the degree of disease [16]. The most commonly used and accepted classification in literature is that in types (I-V) and stages (1-4) [25]. The tables are mainly adapted from the Lipedema foundation, which created the classification in types and stages for lipedema. Five types of the lipedema classification describe the location of accumulation of adipose subcutaneous tissue. These are divided in:

Table 1. Classification of lipedema types

Type 1	Pelvis, buttocks, hips
Type 2	Buttocks to knees, possibly with folds of fat around the inner side of the knee
Type 3	buttocks to ankles
Type 4	a) upper arm b) lower arm c) whole arm
Type 5	knees to ankles

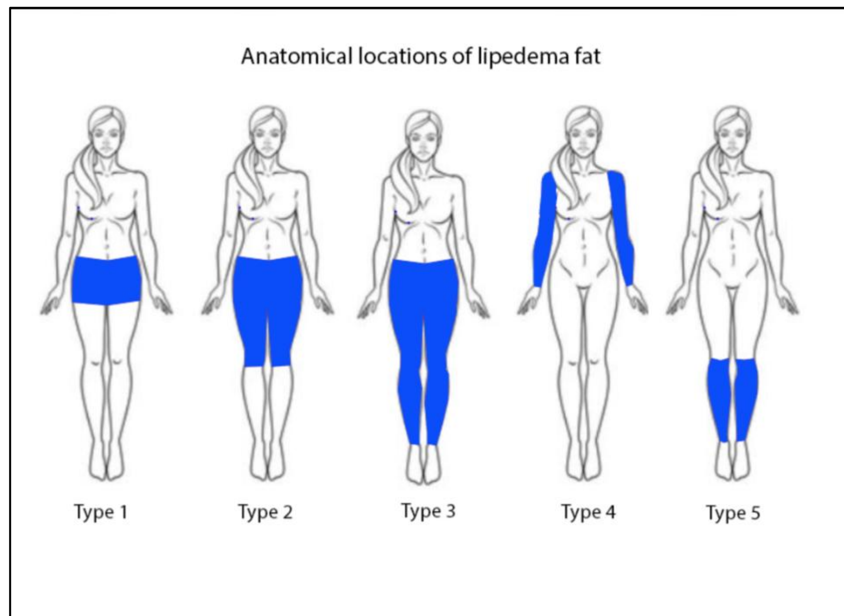


Figure 2: Classification of lipedema types

The four stages of lipedema are indicating the texture and composition of the skin, as well as certain individual properties.

Table 2. Classification of lipedema stages

Stage 1	Normal smooth skin surface with enlarged subcutaneous tissue and soft fat tissue with noticeable small nodules
Stage 2	Uneven skin with enlarged subcutaneous tissue and indentations in the fat[25], larger adipose tissue nodules palpable
Stage 3	Large extrusions of tissue causing visible deformations on skin surface, especially on the thighs and around the knees
Stage 4	Lipedema with progress to lymphedema (Lipolymphedema)

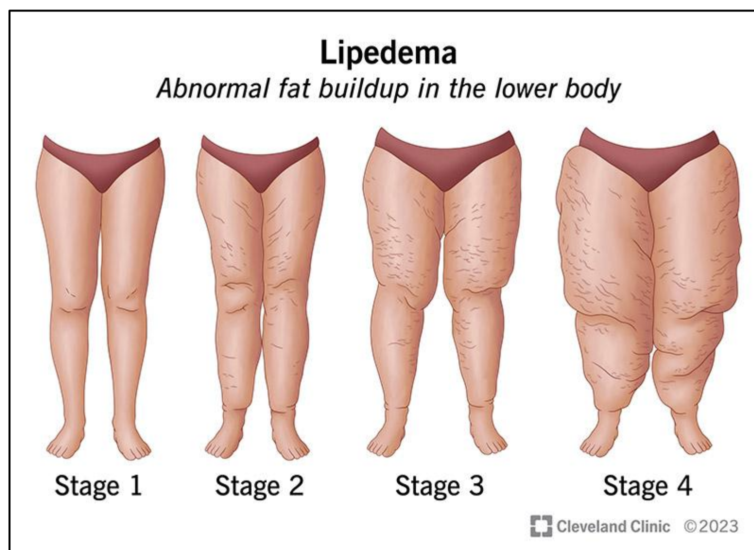


Figure 3: Classification of lipedema stages

2.7 Differentiation Lipedema, Lymphedema, Obesity and Lipohyperthrophy

Lipedema does show a distinctive pattern of symptoms that with correct assessment of patient history and clinical examination enable educated physicians to make a correct diagnosis. However, due to the common unawareness, lipedema is often overseen or misdiagnosed [21]. Oftentimes the disorder is mistaken for common obesity or nearby conditions, stating the importance to familiarize the most common differential diagnoses of lipedema [15]. To combat the common misdiagnosis of lymphedema, a study conducted by Naouri et al. demonstrated the possibility of utilizing high-resolution ultrasound to effectively distinguish between lipedema and lymphedema[44]. Naouri et al. showed that dermal thickness, the echogenicity and the ability to identify of the dermo-epidermal junction were normal in patients with lipedema. In patients with lymphedema however, dermal diameter was increased, reduced dermal echogenicity visible and the dermo-epidermal junction was not fully identifiable, likely due to accumulation of edematous fluids in the dermis [44].

Especially due to the great increase of lifestyle-obesity in the past decades, the enormous prevalence of obesity causes semblance of lipedema to appear less frequently in comparison to obesity. In the US, obesity rates are as high as to affect one third of adult population over 20 and overweight affects almost 60% of European adults and nearly 30% of European children [7,45].

Table 3: Major differential diagnoses to lipedema.

	Lipedema	**Lymphedema**	**Lipohyperthrophy**	**Obesity**
Gender	Women (very rarely males)	Women and men	Women (very rarely males)	Women and men
Age of onset	Puberty, Pregnancy Menopause	Anytime	Anytime	anytime
Family history	Relevant, in about 15% of cases	Relevant, in about 20% of cases	Possible, not evident	Common
Edema	Nonpitting, not reduced by elevation & compression	Pitting, reduced by elevation & compression	No edema	No edema
Symmetry	symmetrical	asymmetrical	symmetrical	symmetrical

Pain of affected areas	pain	No pain	No pain	No pain
Easy bruising	present	absent	Possible, seems to not be linked to LHT, but other factors	absent
Stemmer sign	negative	positive	negative	negative
Most affected parts	Extremities (lower>upper)	Extremities (lower>upper)	Extremities	Entire body
Response to diets	Very low/none	Even loss of trunk and extremities	Even loss of trunk and extremities	Even loss of trunk and extremities

2.8 Current potential biomarker candidates and potential novel biomarkers

Lipedema faces the major issue that a unified and coherent diagnosis is not yet possible, and diagnostic clarity based on specific "markers" is currently not yet available. In the last few years lipedema research has moved away from pure subjective clinical testing to a molecular approach, investigating the disorder on a molecular, cell biological and histological tier. Several studies have already tried to examine potential biomarker candidates using various techniques with limited success so far [4,46,47]. Biomarkers are defined by the WHO as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [12]. They can exist in various forms such as metabolic- (metabolites), protein-, genetic- (genes), immunologic- (cytokines) and other forms of biomarkers. Current candidates of potential biomarkers for lipedema taken from current literature include Platelet factor 4, various cytokines, lipids and their metabolites, sodium, extracellular vesicles and contents, and various amino acids [4,46].

Platelet factor 4 is a plasma circulating signaling protein (chemokine), primarily expressed in phases of thrombocyte aggregation (wound healing and inflammatory processes) that was recently discovered to appear in elevated levels in lipedema patients suggesting the hypothesis that PF4 may be associated in lymphatic/vascular disorders [48].

Inflammatory processes appear to be a relevant part of lipedema pathophysiology. Several studies have shown significant increases in inflammatory markers such as IL-6, IL-8, IL-11, IL-28A and IL-29, as well as CD68+ macrophages and many more [47,49-52]. IL-29 has especially been linked to obesity-induced inflammation suggesting an inflammatory component of lipedema. Supporting the hypothesis of inflammatory processes involved in

lipedema, a recently published study by Vasella et al. showed that lipedema patient tissue samples also had increased expression of inflammatory proteins macrophage migration inhibitory factor (MIF) [53]. The expression of MIF-1 and CD74, a common receptor for MIF-1 and MIF-2, were significantly elevated in lipedema patients, while MIF-2 expression did not appear different [51]. The inflammatory components of lipedema subcutaneous adipose tissue shown in various studies helps to distinguish lipedema from other adipose-tissue related disorders such as obesity and lipohypertrophy.

The lipid profile of lipedema patients has been reported differently by various sources. While an investigation of basic serum lipid markers such as total cholesterol, triglycerides, LDL and ApoB have shown to be increased in lipedema patients compared to healthy controls, an analysis of three cohorts (lipedema, healthy normal weight and healthy obese) has not found any differences in basic serum LDL or total cholesterol [54]. Metabolomic research has yet shown that specific subfractions of lipid markers do show differences in lipedema patients. Significant increases were shown to appear in LDL-6 TG, significant decreases were observed in LDL-2 cholesterol, LDL-2 free cholesterol, LDL-2 phospholipid, LDL-2 ApoB and LDL-2 particles [46].

Sodium is a chemical element which constitutes as an electrolyte with an extracellular concentration of 140mmol/l. Its main functions are to maintain the membrane potential of human cells and regulate the exchange of water between intra- and extracellular spaces. Sodium has shown to appear in larger concentrations in skin and muscle tissue of lipedema patients and the concentrations of sodium correlate with the degree of symptoms, discomfort and pain. It is believed that the impaired venous and/or lymphatic system contribute to an imbalance of the sodium homeostasis, leading to higher levels of Na primarily in the lower extremity due to the gravitational strain [55].

Extracellular vesicles are membrane particles, which are released by almost every cell, can be taken up by almost every cell and can exist in various forms such as exosomes, microvesicles and apoptotic bodies and can contain DNA, mRNA, miRNA's and specific proteins [4]. The attention of extracellular vesicles has considerably increased in the past years, due to their expressiveness of healthy- and ill states in diseases such as diabetes, various forms of cancer, neurodegenerative diseases, Parkinson's disease and more [56]. A recent study looking at extracellular vesicles of the stromal vascular fraction (SVF) in lipedema has found that small extracellular vesicle miRNA profiles were significantly different in lipedema patients than in healthy controls [57]. The alteration of these markers may indicate a role of extracellular vesicles and their contents in lipedema.

The profile of amino acids and other metabolites is depicts an interesting approach to identifying biomarkers for lipedema, since disparities between lipedema patients and control patients has been established [46]. According to a metabolomic analysis, amino acids histidine and phenylalanine showed to be statistically significantly lower to two control cohorts (normal weight and obese), whereas pyruvic acid appears at significantly higher concentrations in lipedema patients than control groups [46]. Leucine, Glycine and Glutamine showed at least a significant reduction in comparison to the lean control group, however not to the obese control group.

Biomarkers of all facets are relevant to developing lipedema diagnosis and treatment. Especially the belated- and inconsistent schemes of diagnosis constitute for a major handicap for affected patients. A collective effort in understanding and identifying consistent and reliable biomarkers may provide a sustainable solution for patients suffering from the disorder.

2.9 Therapy

The therapy of lipedema remains a controversial topic, and the window of therapeutic options is narrow. A causal etiological therapy has not been established due to the lack of knowledge of its pathogenesis and possible points of application. Currently the approaches aim to reduce physical and psychological complaints, improve patients' overall condition, as well as to prevent secondary complications by conservative and in more severe cases surgical treatment [10,25].

2.9.1 Conservative treatment

The main goal of conservative treatment are the management of symptoms and improvement of quality of life [5]. Conservative treatment should always be initially applied before surgical approaches are taken [10,13,58]. Treatment options include manual lymph drainage, compression therapies and kinesio taping, physiotherapy and exercise therapies, dietary counseling and lifestyle adaptions and patient education or cognitive behavioral therapy [41]. According to the Lipoedema 2014 UK Survey by Fetzer et al., MLD and compression were the most frequently applied treatments [22]. Dieting and dietary supplements are also a major area of investigation, however no specific diet has shown to be superiorly effective [59]. Studies have investigated mainly Mediterranean diets, various anti-inflammatory diets and ketogenic diets for its potential to treat lipedema, however even though all diets have shown

to have effective approaches, there is not enough data to proof its efficacy yet [60,61]. An important component of conversative treatment not to obliviate is psychological counseling due to the common mental health issues emerging by the disorder [61].

2.9.2 Surgical treatment

If symptoms of lipedema persist after conservative treatment, surgical procedures may be envisaged. The most frequently conducted operation is liposuction under general or local anesthesia [25]. Liposuction should be performed as tumescent liposuction (where large volumes of tumescent solutions are pumped into subcutaneous adipose tissue) or water-assisted liposuction (where only small amounts of water are used to aspirate adipose tissue) in comparison to dry liposuction, since several articles have shown dry liposuction might lead to lymphedema due to destruction of lymphatic vessels [62,63]. Even though surgery was long believed to present the only method to effectively restore the visual appearance, surveys have shown that liposuction and manual lymph drainage show similar outcomes in the way patients perceive their appearance after treatment [5,22]. The therapeutic benefit of liposuction has been investigated and long term results suggest liposuction to be an effective method to reduce symptoms and other treatments required [64]. Other types of surgery, which may also be considered for the surgical treatment of lipedema are bariatric surgeries and reductive (resection) surgeries, however are less frequently performed as they target obesity patients, rather than lipedema patients [25].

A possible association of lipedema with varicose veins has been established, due to the questionable vascular component of lipedema etiology. Kamamoto et al. reported that pre-operative treatment of varicose veins is important to reduce post-operative complications of lipedema surgery (liposuction) such as bleeding, hematoma and phlebitis [43]. Treatments for varicose veins often resemble conservative treatment options for lipedema, including exercises, lifestyle changes, compression hoses of the affected legs and phlebectomy.

3 Questions and Hypothesis

The aim of this project is to investigate the molecular pathophysiology of lipedema thought the study of metabolomic profile. Lipedema is an adipose tissue disorder characterized by the disproportionate increase of subcutaneous fat tissue in the lower and/or upper extremities. The underlying pathomechanism remains unclear and no molecular biomarkers to distinguish the disease exist, leading to many undiagnosed and misdiagnosed patients. Since the underlying pathophysiological mechanisms remain unclear, identifying biomarkers would facilitate timely diagnosis and treatment of affected patients at an early stage. Therefore, this work investigates adipose tissue in women with lipedema, focusing on alterations in local protein composition, and changes in protein profile by comparing the results obtained with subcutaneous adipose tissue from healthy subjects, and metabolic phenotype to gain insight into the pathophysiology of lipedema and potentially identify reliable biomarkers. The final aim of this project is to build a panel to help in the diagnosis of lipedema by identifying protein/peptides specific for lipedema but also targets for tailored treatments.

The hypothesis of this prospective study suggests that the subcutaneous adipose tissue of lipedema patients shows distinguishable metabolomic analysis to healthy patients, possibly allowing specific biomarkers to be established.

3.1 Goals and Objectives

- Performing innovative and advanced procedures (metabolomic, epigenomic, proteomic type), aimed at identifying biomarkers that highlight early symptoms or still reversible dysfunctional situations, which could help to better understand the complex metabolic mechanisms that can lead to damage to health and, consequently, contribute to the identification of predictive markers of the development of metabolic complications in obesity people, such as type 2 diabetes and Lipedema.

- The results obtained will be employed to reveal the role of biomarkers and biochemical pathways in metabolomic/epigenomic/proteomic studies and to design nutritional supplements clinical trials.

- Therefore, it is envisaged to develop innovative methods and tools for the acquisition, analysis and archiving of large aggregates of data to provide useful tools for the

epidemiological study of the chronic pathologies (covered by the project) and which allow sociological and preventive aspects to control the distribution of these pathologies.

3.2 Specific Aims and Impacts on the National Healthcare System

1. To study the potential differences between constitutionally thin and obese people with the clinical-translational aim of suggesting a potential target for the development of an anti-obesity drug and nutritional supplements;

2. Identify potential targets that allow classifying obese patients who will develop type 2 diabetes in the future from those who do not have the potential to develop metabolic complications of obesity;

3. Allow early diagnostic classification for dysmetabolic pathologies, based on innovative and precognitive molecular markers;

4. To study the biochemical/molecular mechanisms underlying the role of proteome in patients with Lipedema, on adipose tissue with the clinical-translational aim of using the modulation of these proteins as a clinical tool for the treatment of pathology;

5. To use the obtained results to reveal the role of biomarkers and biochemical pathways in metabolomic/epigenomic/proteomic studies and to design nutritional supplements clinical trials. Develop health "big data" that can be aggregated in different ways to provide multidimensional graphical representations allowing the identification of the correlations existing between them.

This research and development program will therefore favor the technological transfer from the structures that deal with research to clinical practice with a clear benefit in terms of process innovation and organizational innovation with the following improvements both in production and organization:

I. Identification of new therapeutic pathways of greater and proven efficacy, with the consequent reduction of local health expenditure and with a significant reduction in costs of the health service of investigations and hospitalization of

patients;

II. Identification and application of targeted therapeutic treatments with greater and more lasting efficacy over time as well as any choices for the prevention of certain chronic-degenerative pathologies;

III. Reduction of the onset of complications from chronic pathologies (obesity, diabetes, etc.);

IV. Improvement of the management of healthcare facilities by implementing a model of medicine better focused on the analysis of large amounts of data for the search for personalized treatments by carrying out predictive analyses and defining metrics to measure performance.

4 Material and methods

4.1 Population study

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the ethics committee of the Land Salzburg. All study participants gave written informed consent and were informed about the study (EK Nr: 1023/2024). The study is designed to be an experimental non-therapeutic biomedical study investigating the molecular basis of lipedema. All operative interventions to collect tissue sample were conducted in the clinic Dr. Papp & Papp in Salzburg, Austria.

4.2 Description of two cohorts and criteria

The study included two cohorts, each consisting of relatively equal groups of female patients, a cohort of lipedema patients and a healthy control group.
The inclusion criteria for the study participants were explicitly chosen:
- 19 to 70 years old;
- Women with Lipedema stage III (cohort 1) and without lipedema who are receiving an alternative surgical procedure, as liposuction and biopsy (cohort 2).

The exclusion criteria for the study participants were:
- Patients under 18 years of age or did not consent to the study or wanted to withdraw at any given time of the study;
- Female with other lipid disorder;
- Use of any immunosuppressive or corticosteroid pharmacotherapy.

The conventional surgical procedure employed to remove lipedema tissue is liposuction from the extremities (arms and legs), with reductive plastic surgery of the extremities being utilised in exceptional cases of severe lipedema. Tissue samples from non-lipedema patients were collected from other types of reductive surgical operations from body regions such as breast (breast reduction surgery) or abdomen (abdominoplastic surgery).

4.3 Tissue sampling

The period of sample collection was about 12 months from 03/24 until 03/25. Subcutaneous adipose tissue samples were collected from female patients grouped into two cohorts, one lipedema cohort and a control-cohort. The tissue samples were taken during operations and placed directly into sterile Eppendorf plastic cryogenic vials or aspiration syringes, which

were at once flash-frozen in a container of liquid nitrogen and transferred to a −80°C freezer. In an Eppendorf Cyrocube© freezer, the samples were stored for a couple of weeks until transported to the laboratory facilities on dry ice.

4.4 DNA extraction and Genome-wide methylation array

Genomic DNA was extracted from approximately 100 mg of adipose tissue using a combination of mechanical disruption and commercial kits. Briefly, frozen tissue samples were pulverized in liquid nitrogen using a mortar and pestle, followed by DNA isolation with the Genomic DNA Isolation Kit (Norgen Biotek, Cat. 24700), according to the manufacturer's instructions. The extracted DNA was subsequently cleaned and concentrated using the DNA Clean & Concentrator-5 kit (Zymo Research, Cat. D4003) following the recommended protocol. DNA purity was assessed using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific), while DNA concentration was determined with the Qubit™ Fluorometer (Life Technologies, Monza, Italy) using the Quant-iT™ dsDNA High Sensitivity Assay Kit. For each sample, 500 ng of genomic DNA was subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research), as previously described [65]. Subsequently, 250 ng of bisulfite-converted DNA was hybridized to the Infinium MethylationEPIC v2.0 BeadChip array (Illumina, Cat. 20087706), which interrogates approximately 930,000 unique methylation sites. BeadChips were scanned with the Illumina iScan platform. The genome-wide methylation array data are available in the EBI ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) with accession number E-MTAB-15365 [66].

4.5 Protein extraction, western blot and antibodies

Total proteins extraction was performed as described in An et al. [5]. Protein concentrations were determined using Bradford assay and their expression was analysed by western blotting. Briefly, protein sample extracts were denatured and separated on 10% polyacrylamide, 0.1% SDS (SDS-PAGE), and transferred onto a nitrocellulose blotting membrane (GE Healthcare, Milan, Italy). Following blocking with 5% nonfat dry milk in TBST buffer (0.01 m Tris-HCl, pH 8.0, 0.15 m NaCl, and 0.1% Tween 20), membranes were immunoblotted with different primary antibodies. In details, the antibodies used for western blot experiments were Pan-Akt Polyclonal Antibody (E-AB-30471, Elabscience), Phospho-

Pan-Akt (Ser473) Polyclonal Antibody (E-AB-20802, Elabscience) and anti-α-Tubulin (sc-32293, Santa Cruz Biotechnology). Then, primary Abs were detected by horseradish peroxidase-conjugated secondary Abs (GE Healthcare) and revealed by chemiluminescence and autoradiography. Densitometry was performed by ImageJ software analysis [67].

4.6 RNA isolation and RNA sequencing analysis

Total RNA was isolated from adipose tissue samples obtained from 8 patients (4 with lipedema and 4 healthy individuals). Tissues were snap-frozen in liquid nitrogen at once after collection and stored at −80 °C until further processing. Prior to RNA extraction, samples (~150 mg each) were cryopulverized using the CP02 cryoPREP® Automated Dry Pulverizer (Covaris). RNA was extracted using TRIzol™ Reagent (Invitrogen, #15596026) according to the manufacturer's protocol. Briefly, 1 ml of TRIzol was added to ~150 mg of pulverized adipose tissue, followed by homogenization and phase separation. RNA was quantified with Qubit 2.0 fluorimeter using Qubit RNA HS assay kit (Termo Fisher Scientifc, USA), and the assessment of nucleic acids integrity (RNA Integrity Number) was performed with Agilent 4150 TapeStation System (Agilent Technologies, USA). Indexed libraries were prepared starting from 200 ng total RNA according to TruSeq Stranded Total RNA Library Prep Gold (Cat. 20020599, Illumina, San Diego, California, USA) and sequenced on the Novaseq 6000s4 v 1.5 platform (Illumina Inc.) using 2 × 100 bp paired end mode as previously described [68,69]. RNA sequencing primary data have been deposited in the EBI ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) with accession number E-MTAB-15370.

4.7 Metabolic extraction and NMR metabolomics

Adipose tissue samples were collected according to standard operating procedures (SOPs) [70]. Metabolic extraction was carried out using a two-step protocol. To each tissue sample collected from the limbs, weighing between 40 and 60 mg, 1 mL of H2O and 2 mL of cold methanol were added with the aim of halting enzymatic metabolism (quenching). Subsequently, the samples were subjected to vortexing to ensure uniform dispersion of the material and were then sonicated for 15 minutes, utilising an on/off mode under temperature-controlled conditions of 3°C [70]. The amplitude was set at 23% (referred to as 23 A.U. in the ultrasonicator operating system) to facilitate complete cell lysis and tissue

homogenization. Following this, 4 mL of methyl-t-butyl ether (MTBE) was added initially. After maintaining the samples on dry ice for one hour, 2 mL of water was added to promote the separation of polar and apolar phases [70,71]. Subsequently, all samples were subjected to centrifugation at 1500 rpm for 15 minutes at 4°C, facilitating a clear delineation of the two phases: the upper (lipophilic) phase, which contains the apolar metabolites, and the lower (hydrophilic) phase, which comprises the polar metabolites of interest. Three millilitres were taken from each phase, and the extraction was repeated on the original samples that still contained the pellet, adding four millilitres of MTBE and two millilitres of H_2O. The samples were mixed by vortexing and then subjected to centrifugation at 1000 rpm for 5 minutes at 4°C to re-establish separation of the two phases. Afterwards, 1 ml was taken from each phase and added to the respective phases collected during the previous extraction. After removing the solvents from the polar phases using an SP Genevac EZ-2 series 4.0 centrifugal evaporator, the lyophilised extracts were resuspended in 500 µL of a tissue-specific phosphate buffer containing 50 mM Na_2HPO_4, 1 mM 2,2,3,3-d_4-trimethylsilyl-propionic acid, sodium salt (TSP-d4), 50 µL of D2O, and 2 mM sodium azide (NaN3) as an antibacterial agent. The resultant samples were ultimately transferred into 5-mm NMR tubes for spectra acquisition utilising ^{1}H-NMR spectroscopy. TSP-d4 at a concentration of 0.1% in D2O served as an internal standard for the alignment and quantification of NMR signals [72].

4.8 NMR spectra acquisition and assignment

^{1}H NOESY spectra acquisition was conducted with a spectral width of 12 ppm, 20,000 data points, presaturation during the relaxation delay, and a mixing time for water suppression along with gradient spoilage, a 5-second relaxation delay, and a mixing time of 10 ms [73-75]. Topspin version 3.0 (Bruker Biospin) was utilised for spectrometer control and data processing. Analysis of the NMR spectra was conducted using a targeted metabolomic approach. Consequently, each metabolite was identified prior to statistical analysis with Chenomx NMR-Suite v8.0 software (Chenomx NMR suite, v8.0, Edmonton, AB, Canada), which combines advanced analysis tools with a compound library. Quantitative analysis of the NMR spectra was conducted using NMRProcFlow, and the obtained data matrix was subjected to statistical analysis [76].

4.9 NMR Statistical analysis

Partial least squares discriminant analysis (PLS-DA) was performed on the normalised dataset using MetaboAnalyst 6.0 (http://www.metaboanalyst.ca/). The model derived from the supervised methodology underwent validation through the 10-fold cross-validation technique, considering the Q2 and R2 indices as well as accuracy. The contribution of individual variables to the separation of clusters was assessed using Variable Importance Projection (VIP), with only those metabolites exhibiting VIP values greater than 1.3 being significant [77,78].

Univariate analysis was performed on the adipose tissue metabolomic profiles using the T-test and Fold Change, with the results displayed in a Volcano plot, setting the threshold at p-value < 0.05 and fold change ±1 [79]. The hierarchical clustering analysis was performed using MetaboAnalyst 6.0, taking into account the median metabolomic profile to provide a comprehensive depiction of the quantitative variations in metabolites and to ascertain the similarities between the adipose tissue metabolomic profiles obtained from various anatomical sites. Heatmaps were generated from normalized data, average group concentration, and Euclidean distances [9]. The meta-analysis, conducted with Metaboanalyst 6.0, employed integrated principal component analysis (iPCA) using both the sites of sampling for pathological tissue (leg, arm) and healthy tissue (abdomen and breast) as discriminatory variables, in addition to the presence or absence of lipedema [80]. Pathway Topology analysis was carried out using MetPa. Pathways with more than two Hits, i.e. metabolites belonging to the biochemical pathway and a p-value of less than 0.05, were deemed significant. To assess the impact of individual pathways on the examined clusters, the Pathway Impact (PI) value was calculated. This was achieved by combining path centrality and enrichment results [81].

4.10 Bioinformatics analysis

Infinium MethylationEPIC v2.0 analysis was performed using Chip Analysis Methylation Pipeline (ChAMP) package [82,83]. The analysis was performed by comparing lipedema tissues with control tissues. Only the CpG associated with a p-value adjusted ≤ 0.01 and DeltaBeta ($|\Delta B|$) cutoff set to the first quartile value ($|DB| \geq 0.15$) of DB distribution were considered differentially methylated. Genomic annotation of CpGs was performed using the information available in Infinium MethylationEPIC v2.0 manifest file. In detail,

transcriptional start site (TSS)200 refers to CpGs between 0 and 200 bases upstream of the TSS; TSS1500 refers to CpGs between 200 and 1500 bases upstream of the TSS; 5'UTR refers to those GpGs within the 5' untranslated region, between the TSS and the ATG start site; gene body refers to CpGs between the ATG and stop codon, regardless of the presence of introns and exons. Promoter region includes TSS1500, TSS200, 5'UTR, and 1st exon regions. Functional annotation analyses were performed using Enrichr tool [82]. For RNA-Seq data analysis was performed as previously described [84]. In brief, raw sequencing reads were assessed for quality using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adapter sequences were trimmed using Trimmomatic [85]. Reads were aligned to the human genome reference (GRCh38.p13, Gencode Release 41) using STAR v2.7.10a with default parameters [86]. Transcript quantification was carried out using FeatureCounts, and differential expression analysis was performed using DESeq2 [87,88]. The analysis compared lipedema versus non-lipedema patients. Transcripts were considered differentially expressed if they showed an absolute fold change ($|FC|$) ≥ 1.5 and an adjusted p-value ≤ 0.05 (Benjamini–Hochberg correction). Functional enrichment analysis was conducted using Ingenuity Pathway Analysis (IPA, QIAGEN) and Gene Set Enrichment Analysis (GSEA) [89].

4.11 Statistical methods

All statistical analyses were performed using R software (version 4.0.2). Data are presented as mean ± standard deviation (SD). Statistical significance was assessed using an unpaired two-sample t-test, unless otherwise specified. A p-value ≤ 0.05 was considered statistically significant. For genome-wide methylation array, RNA-Seq analyses and metabolic profiling, specific statistical approaches and thresholds are described in the corresponding Methods sections.

5 Results

5.1 Characteristics of the participants

The anthropometric parameters of participants and the collected tissue regions are presented in Table 4. The average age of the subjects as a collective group was 42.29 (±13.07) years. The average body weight and BMI were 69.24 (±9.43) and 24.38 (±3.6), respectively. Concerning lipedema, all patients were at stage 3.

Table 4. Characteristics of the participants and anatomical regions of collected tissue samples. Data are presented as mean ± standard deviation (SD) or percentage.

Variable	Lipedema group n= 12 (±SD)	No-lipedema group n= 9 (±SD)
Age	41.6 (±11.3)	43 (±15.8)
BMI (kg/m^2)	69.2 (±9.4)	24.3 (±3.6)
Body-region-tissue		
Abdomen	-	4 (44.4%)
Arms	6 (50%)	-
Breast	-	5 (55.6%)
Upper leg	6 (50%)	-

All samples were subjected to multi-omics analyses according to the experimental workflow depicted in figure 4. We integrated epigenetics, transcriptomics, and metabolomics profiling to garter deep insight lipedema pathophysiology and development.

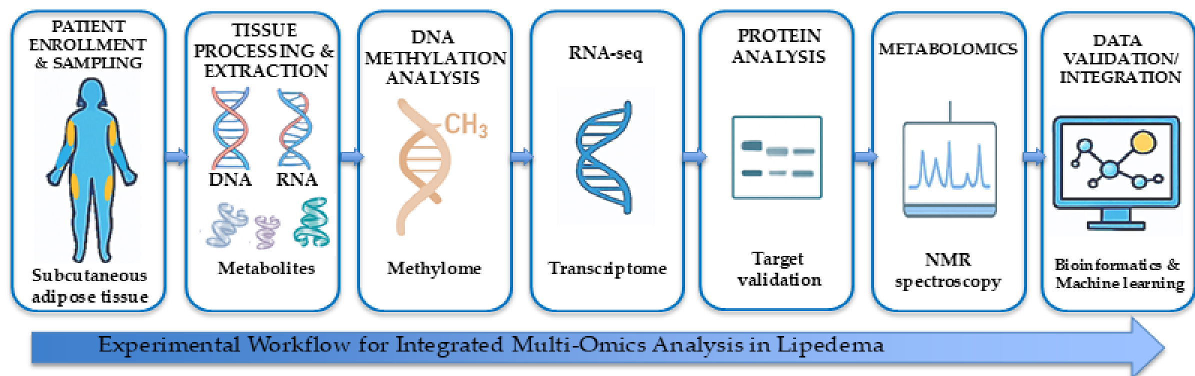


Figure 4. Experimental plan. Schematic overview of the experimental workflow involving multi-omics profiling in lipedema patients and control tissues.

5.2 Genome-wide methylation profiling highlighted a dysregulated receptor tyrosine kinase and phospho-metabolism signaling pathways in advanced stage lipedema patients.

Genome-wide methylation profiling highlighted a dysregulated receptor tyrosine kinase and phospho-metabolism signaling pathways in advanced stage lipedema patients. Epigenetic modifications, able to alter gene expression without changing the underlying DNA sequence, are increasingly recognized to play essential roles in the development and progression of several diseases including possibly lipedema. These modifications, such as DNA methylation, can influence how genes involved in fat metabolism, inflammation, and other relevant pathways are deregulated and thus influence disease initiation and progression. To gain insight into the epigenetic mechanisms underlying lipedema pathogenesis, a genome-wide DNA methylation analysis was performed on subcutaneous adipose tissue samples collected from two cohorts of patients. By using high density DNA methylation array with single CpG site resolution we were able to detect a total 5,484 CpGs plotted in the heatmap reported in Fig. 2A. According to the methylation beta value that clearly distinguished between the two groups, with a with a statistically significant difference in the methylation level ($\Delta\beta \leq -0.15$ and $\Delta\beta \geq 0.15$; padj ≤ 0.1) between lipedema and healthy tissue; in particular, as reported int the volcano plot in Fig. 2B, we found 3,343 CpG sites are hypermethylated, whereas 2,140 are hypomethylated, respectively (Supplementary Table 1). This analysis revealed a clear pattern of differential methylation across the genome with greatest piking in promotor region and last methylation in the 3′ UTRs (untranslated regions) and gene bodies as shown in Fig. 1C, despite the overall pattern resulted quite similar for both Hyper- and Hypo-methylated lipedema specific CpGs. Given the impact of methylation changes observed we further functionally annotated these profiles to gather insight into deregulated pathways affected by observed methylation changes. As reported in Fig. 2D we interestingly found that methylation profiling significantly impacted receptor tyrosine kinase and phospho-metabolism signaling pathways with also a concomitant action on Interleukin and PD-1 signaling, highlighting the impact that DNA methylation deregulation could have on cell communication and other vital cellular processes like growth, differentiation and metabolism, but also on immune responses, as already hypothesized for the genesis an progression of this disease.

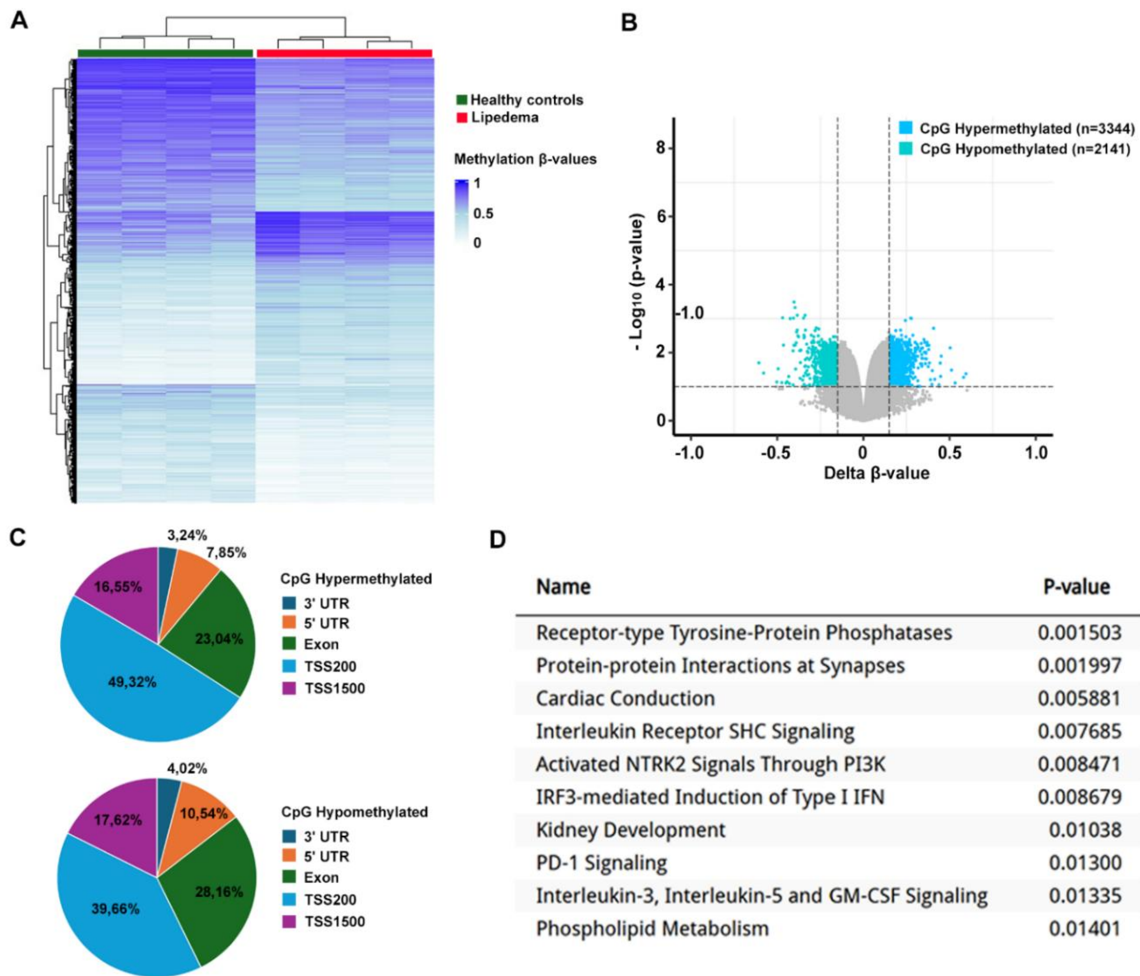


Figure 5. Genome-wide DNA methylation profiling in lipedema patients. **A)** Hierarchical clustering heatmaps of methylation levels CpG sites in healthy control and lipedema expressed as β-value. Color gradient from dark blue (β-value=1) to white (β-value=0) represents hypermethylation to hypomethylation. **B)** Vulcano plot depicting differentially methylated CpGs between control and lipedema tissues ($|\Delta B| \geq 0.15$ and $p < 0.05$). C**)** Pie chart showing the distribution of differentially methylated CpGs (Hypermathylated; upper panel and Hypomethylated; lower panel) on different element of the transcriptional unit. **D)** Table showing statistically enriched pathway according to "Enrichr" functional annotation tool considering genes harbouring differentially methylated CpGs.

5.3 Transcriptome profiling of advanced stage lipedema patients confirmed mitochondrial and metabolic disfunctions and highlighted sirtuin signaling alteration

Together with DNA methylation profile, transcriptome reprogramming has emerged to have a significant on impact cellular behaviour and to contribute to disease development or progression. Thus, the understanding these changes is crucial for the developing of new diagnostic strategy and potential therapeutic approaches. Considering these premises, we

employed total-RNA sequencing for gene expression profiling comparing lipedema and healthy patient tissues. As shown in Fig. 3A, we observed a marked deregulation of gene expression program in lipedema samples compared to control tissues that highlighted 668 up- and 266 down-regulated genes, respectively (Supplementary Table 2). Transcriptome deregulation functional annotation (Fig. 3 B and C) revealed an impact on oxidative phosphorylation mitochondrial protein degradation as well as TCA cycle and Fatty acid beta oxidation in coherence with already published data [90]. However, our results highlighted also an up-regulation of estrogen signaling as well as an impairment of extracellular matrix organization and sirtuin signaling pathway. This resulted particularly interesting considering possible additional epigenetic influence of lipedema progression.

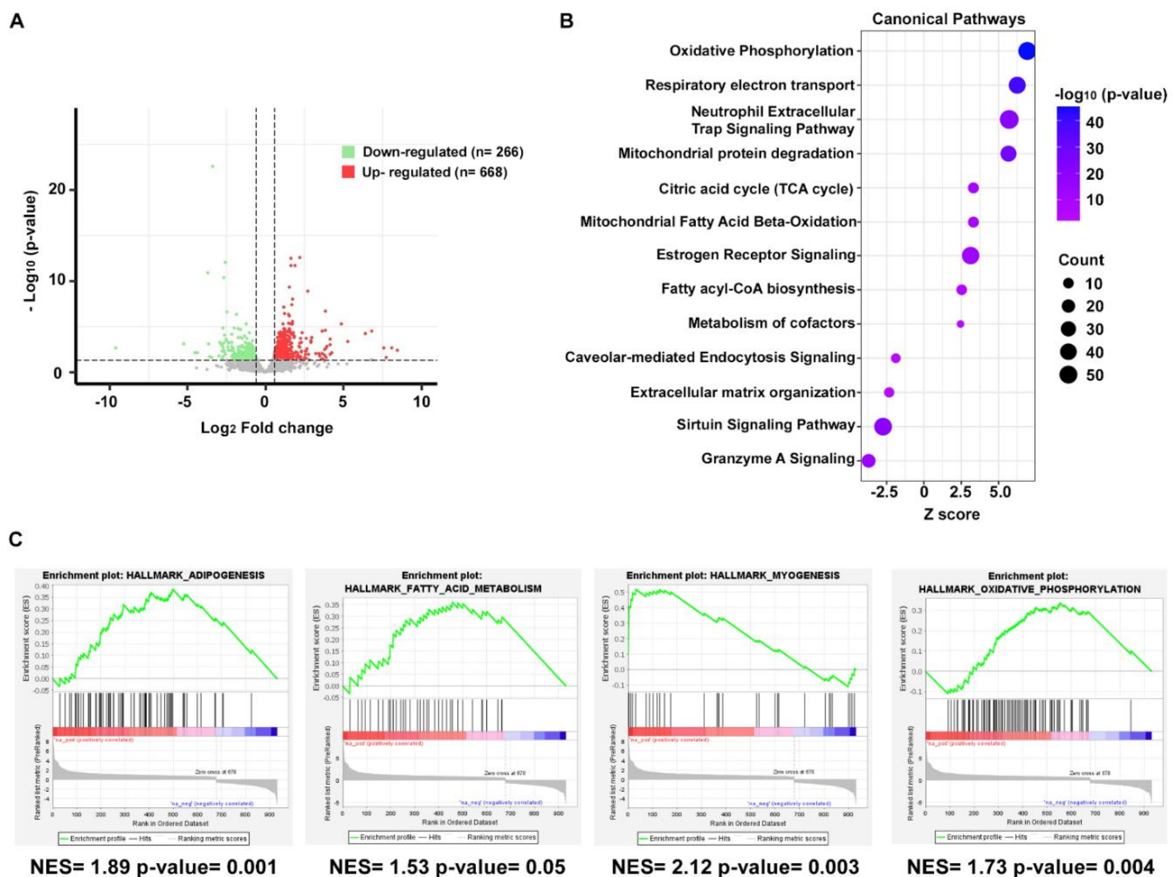


Figure 6. **Lipedema transcriptome profiling. A)** Vulcano plot depicting differentially expressed transcripts between control and lipedema tissues (|FC|) ≥ 1.5 and an adjusted p-value ≤ 0.05. **B)** Dot plot chart showing statistically enriched pathway according to IPA functional annotation tool according to activation Z-score. **C)** Statistically significant functions highlighted by Gene Set Enrichment Analysis (GSEA) in lipedema transcriptome.

5.4 Multi-omics data integration revealed the RAC (Rho family) -alpha serine/threonine-protein kinase AKT1 alteration as novel biomarker for lipedema patients

Multiomics offers a comprehensive perspective on biology, driving breakthroughs in human disease research. By integrating multiple genomics layers like transcriptomics and epigenetics, this approach enables a deeper understanding of gene expression, regulation, and protein activity. In this context we employed an integrative analysis of epigenetic (DNA-methylation) and transcriptomics deregulation in advanced stage lipedema tissues. Results obtained indicate that concerning deregulated pathways, obtained from this integration, there is a major influence on Metabolic pathways, in general, and on Pyruvate metabolism, in particular, that resulted the top enriched one (Fig. 4A). We then restricted our focus to transcriptional charges couplet to concomitant and opposite methylation alterations as shown in Fig. 4B (Supplementary Table 3). These further analyses revealed that among deregulated transcripts the RAC (Rho family)-alpha serine/threonine-protein kinase AKT1 resulted particularly interesting. This is due to not only the involvement of this protein kinase in regulating cell growth, survival, and proliferation for the direct impact on cell cycle progression and apoptosis, but especially for the role it has in cellular metabolism, influencing glucose uptake and the conversion of glycogen back to glucose [91]. Looking at the methylation influence on AKT1 expression we found a strong hypo-methylated region in the 5’ UTR of the protein able to influence its abundance in lipoedema patients (Fig. 4C). To validate our finding, we performed an analysis of protein expression levels comparing healthy controls and disease samples. Results obtained and showed in Fig. 4D clearly demonstrate an AKT1 and phospho-AKT1 increase in lipedema tissues highlighting this enzyme as a new possible biomarker for this pathology to translate to the clinical management of patients affected by this disease.

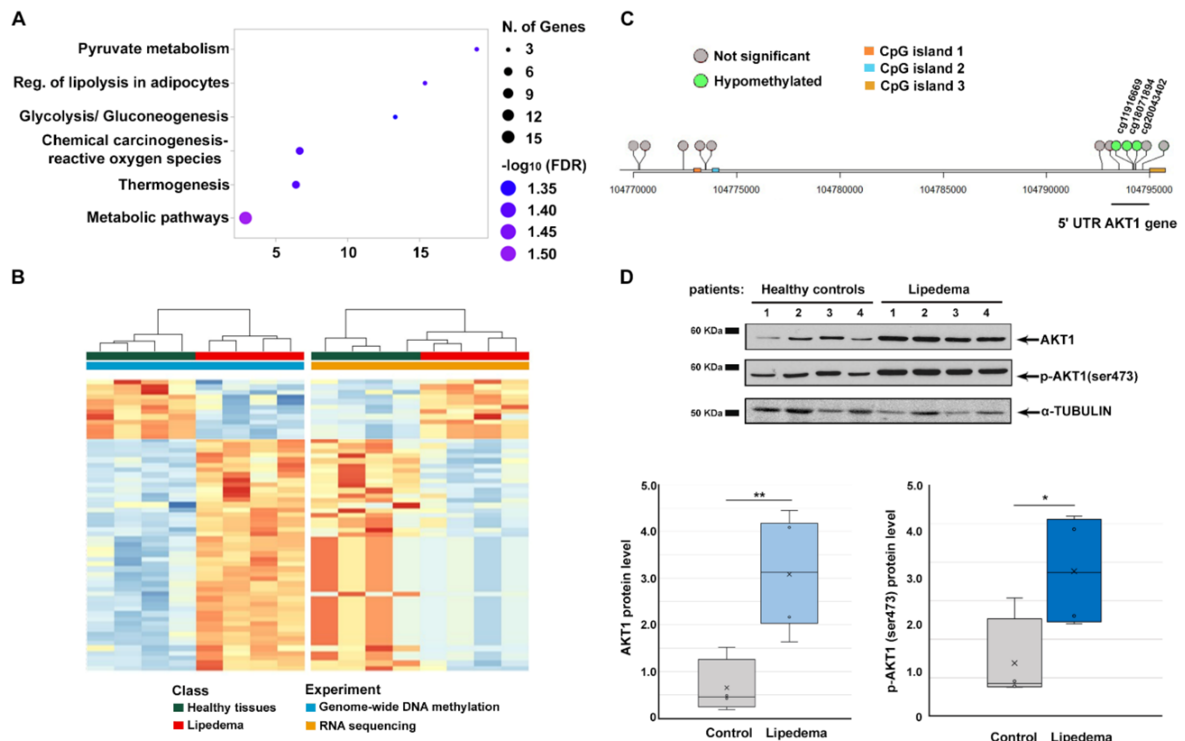


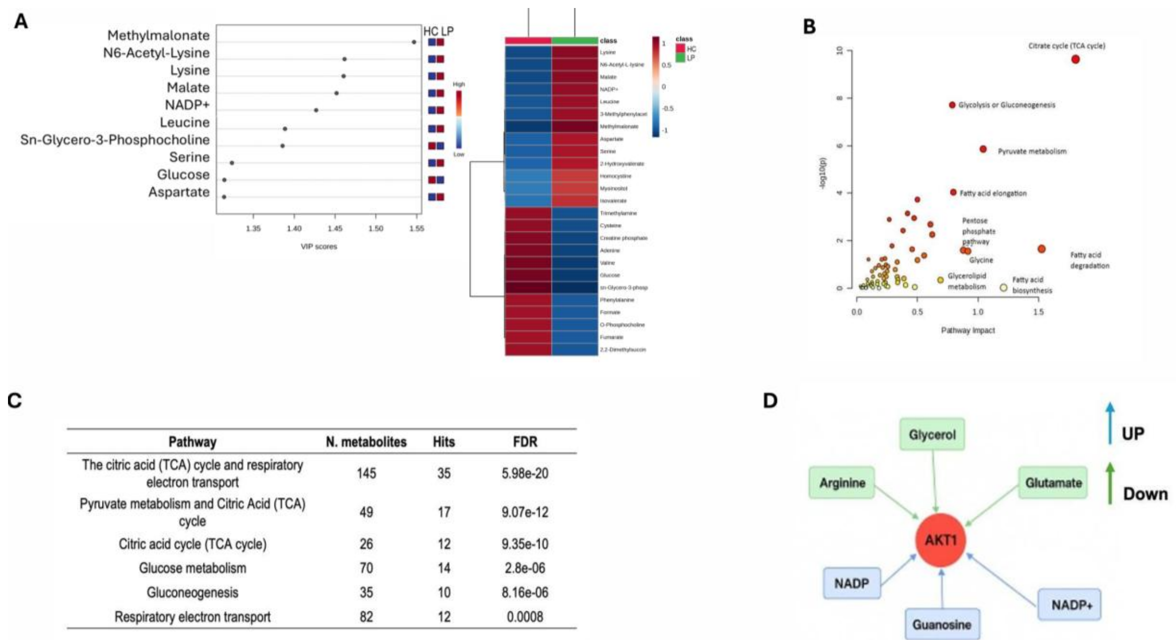
Figure 7. **Multi-Omics profiling integration in lipedema. A)** Dot plot chart showing statistically enriched pathway according to "ShinyGO" functional annotation tool considering all differentially methylated CpG and the corresponding differentially expressed transcript obtained as described in methods sections. **B)** Heatmap depicting differentially expressed CpGs and transcripts between control and lipedema tissues showing opposite behaviours **C)** Lollipop graph depicting differentially expressed CpGs harboured in AKT1 transcription unit **D)** Western blot (upper) and box plot (lower) showing AKT1 protein and phosphoprotein levels comparing healthy control and lipedema patients. The box blot shows the average of band intensity relative to control or lipedema

5.5 Trans-Omic integration highlights AKT1-linked metabolic signatures in advanced lipedema

Given the broad metabolic impact of the phosphoinositide 3-kinase (PI3K)-AKT signaling pathway, whose aberrant activation can modulate multiple metabolic processes through direct and indirect mechanisms, we next investigated the metabolic alterations in lipedema using untargeted metabolomics [92,93]. The adipose tissue samples were subjected to a double-phase extraction, both polar and apolar. Samples containing the polar phase were prepared by adding deuterium-enriched phosphate buffer, and one-dimensional ^{1}H NOESY spectra were acquired using a 600 MHz NMR spectrometer. NMR spectra were analyzed using Chenomx software, resulting in the identification of 56 metabolites (Supplementary Fig. S1A). An analogous procedure was followed for the adipose tissue of the healthy

subjects. Accordingly, we obtained two data matrices: one containing metabolite concentrations for each lipedema patient (LP) and the other containing metabolite concentrations for each healthy control (HC) subject. The data matrices were compiled and analysed using both univariate and multivariate statistical methods on the MetaboAnalyst 6.0 online platform. Supervised Partial Least Squares Discriminant Analysis (PLS-DA) revealed that the tissue metabolomic profiles of patients with lipedema are significantly distinct from those of the control group (PC'1 accuracy: 1.0; Q2: 0.95) (Supplementary Fig. S1B). Variable Importance Projection (VIP) analysis identified abnormal metabolite levels in LP adipose tissues: (i) increased concentrations of several amino acids, including lysine, N6-acetyl-lysine, leucine, serine, and aspartate; (ii) changes in energetic metabolites, with upregulation of methylmalonate and malate, and a decrease in glucose; (iii) additionally, higher methylmalonate levels and lower sn-glycerol-3-phosphocholine levels were observed (Fig. 5A, left panel). The univariate approach utilising the Volcano plot (Supplementary Fig. S1C), together with the heatmap representation displaying the initial 25 discriminative variables (Fig. 5A right panel), confirmed the energetic dysregulation caused by higher concentrations of NADP+, malate, and lower concentrations of succinate and glucose. Additionally, it corroborated the amino acid dysregulation, evidencing a downregulation of valine and an upregulation of serine, leucine, and lysine in the pathological tissue. Subsequently, an analysis of dysregulated pathways was carried out utilising the Metaboanalyst 6.0 software with the MetPa tool. The findings indicate that the onset of lipedema contributes to dysregulation of energy metabolism, involving changes in the metabolic pathways. Pathways analysis identified dysregulation in bioenergetics, indicating disruptions in glyoxylate and dicarboxylate metabolism, pyruvate metabolism, and the citrate cycle (TCA). Additionally, amino acid dysregulation depends on changes in arginine biosynthesis and the metabolism of alanine, aspartate, glutamate, glycine, serine, threonine, cysteine, and methionine. We also highlighted a dysregulation in BCAA metabolism. Lipedema tissue is notably marked by an alteration in a single carbon pool through folate pathways, suggesting a disruption in vitamin B synthesis and homocysteine regulation. Lastly, the identification of an imbalance in the redox environment was recognised because of the upregulation of glutathione metabolism (Fig. S1D and E). To investigate the functional crosstalk between gene expression and metabolic alterations in advanced lipedema, we performed a trans-omic network analysis by integrating untargeted metabolomics data with bulk RNA sequencing profiles derived from adipose tissue biopsies. This approach aimed to uncover coordinated molecular interactions linking metabolic dysfunction with

transcriptional remodeling in the diseased tissue. Initial pathway-level interrogation of the metabolomics dataset using the MetaboAnalyst 6.0 platform and the MetPa enrichment tool revealed prominent alterations in bioenergetic circuits, including the TCA cycle, glycolysis/gluconeogenesis, pyruvate metabolism, and fatty acid elongation (Fig. 5B). These pathways exhibited high statistical significance and impact scores, underscoring a generalized disruption in central carbon metabolism in lipedema. A quantitative summary of the most significantly enriched metabolic routes, along with matched metabolite counts and corresponding false discovery rates (FDR) (Fig. 5C). The enrichment of pathways associated with mitochondrial respiration and redox balance further supported a scenario of increased energetic and oxidative burden in lipedema tissue. To mechanistically link these metabolic alterations with gene expression dynamics, we constructed a trans-omic network model integrating differentially expressed genes (pink nodes) and metabolites (blue squares) through known biochemical associations and literature-curated interactions (Fig. 5D). This network pinpointed AKT1, a serine/threonine kinase, as a central transcriptional hub significantly overexpressed in lipedema and exhibiting strong connectivity to altered metabolites such as $NADP^+$, glutamate, glycerol, arginine, and guanosine. Further analysis revealed that AKT1 is also highly associated with key metabolic nodes, including adenine nucleotides (ATP, AMP), glutamic acid, and intermediates of the TCA cycle, suggesting a rewiring of the metabolic landscape toward increased energy demand, redox activity, and utilization of carbon and nitrogen substrates. These patterns are consistent with a model of metabolic reprogramming driven by chronic stress, mitochondrial dysfunction, or compensatory responses during adipose tissue remodeling in late stage lipedema. Notably, these findings align with AKT1's well-established role in regulating glycolysis, glucose uptake, and TCA cycle flux via downstream effectors such as mTOR, GSK3, and FOXO1 [94-96]. Together, these results reinforce the concept that AKT1 acts as a key integrator of metabolic stress and transcriptional reprogramming in lipedema, orchestrating alterations in energy production, amino acid metabolism, and antioxidant defence.



Pathway	N. metabolites	Hits	FDR
The citric acid (TCA) cycle and respiratory electron transport	145	35	5.98e-20
Pyruvate metabolism and Citric Acid (TCA) cycle	49	17	9.07e-12
Citric acid cycle (TCA cycle)	26	12	9.35e-10
Glucose metabolism	70	14	2.8e-06
Gluconeogenesis	35	10	8.16e-06
Respiratory electron transport	82	12	0.0008

Figure 8. Metabolomic profiling and data integration. A) The VIP score plot displays the most relevant metabolites for distinguishing between groups, arranged according to their statistical contribution to the PLS-DA model (VIP > 1.3 is considered relevant) (left panel). Heatmap show the top 25 altered metabolites in lipedema tissue are compared to healthy control adipose tissue. The colour of each section corresponds to the concentration value of each metabolite, calculated through a normalised concentration matrix (red indicating upregulated; blue indicating downregulated) (right panel). **B)** The analysis of metabolic pathways was conducted utilising MetaboAnalyst 6.0 with the application of the MetPa tool. Bubble color indicates pathway significance (p-value/-$\log_{10}$(p)), and bubble size is proportional to pathway impact derived from topology analysis, with large red circles signifying highly altered pathways. Prominent dysregulated routes include the TCA cycle, glycolysis/gluconeogenesis, pyruvate metabolism, and fatty acid elongation. **C)** The table presents the discriminant biochemical pathways associated with the metabolic profiles of pathological and control adipose tissue. "Hits" refer to the number of matched metabolites, while the False Discovery Rate (FDR) reflects the expected proportion of false positives. Pathway impact values combine topological centrality and enrichment scores, with higher values indicating stronger influence within the metabolic network. **D)** Trans-omic network analysis integrates transcriptomic and metabolomic data to reveal key interactions centered around overexpressed AKT1. The network connects AKT1 to several differentially regulated metabolites including $NADP^+$, glutamate, glycerol, arginine, and guanosine. Blue and green arrows indicate directionality of change (up= light blue or down= green), supporting a model of AKT1-driven metabolic rewiring involving redox imbalance, amino acid metabolism, and altered energy flux in lipedema adipose tissue.

6 Discussion

In this study, we employed an integrative multi-omics approach to delineate the molecular and metabolic landscape of advanced-stage lipedema, a disabling adipose tissue disorder characterized by chronic pain, inflammation, and progressive fat deposition. By combining genome-wide DNA methylation profiling, transcriptomic analysis, and untargeted metabolomics in subcutaneous adipose tissue, we found a central regulatory role for AKT1, linking epigenetic deregulation to mitochondrial dysfunction and metabolic reprogramming. These findings offer novel mechanistic insight into late-stage lipedema pathophysiology and highlight a potential molecular target for disease stratification and therapeutic intervention. Our data show extensive epigenetic remodeling in lipedema adipose tissue, with over 5,000 differentially methylated CpG sites affecting pathways related to receptor tyrosine kinase signaling, immune regulation, and phospho-metabolism. This aligns with previous findings suggesting chronic low-grade inflammation, vascular dysregulation, and hormonal sensitivity as core features of lipedema pathogenesis [4,97,98]. Notably, we identified a hypomethylated regulatory region in the promoter of AKT1, corresponding with its overexpression and increased phosphorylation, indicating epigenetically driven activation of this key signaling node. Transcriptomic profiling further revealed marked downregulation of mitochondrial genes involved in oxidative phosphorylation (OXPHOS), fatty acid β-oxidation, and the TCA cycle. These alterations suggest impaired mitochondrial bioenergetics, consistent with previous reports showing reduced mitochondrial activity and altered lipid metabolism in lipedema adipose tissue [11,99]. In line with these findings, Straub et al. demonstrated reduced expression of mitochondrial proteins in early-stage lipedema using proteomic profiling, though they did not identify upstream regulatory nodes or link the observed changes to DNA methylation [90]. Our study extends these observations by proving a mechanistic connection between epigenetic deregulation and mitochondrial dysfunction in the context of advanced disease. Metabolomic profiling supported these transcriptomic and epigenomic changes, revealing altered levels of several metabolites associated with AKT1-driven pathways, including arginine, NADP, glycerol, guanosine, and glutamate. These metabolites are critical regulators of redox homeostasis, energy balance, and amino acid metabolism—key processes that are frequently disrupted in chronic metabolic diseases. A previous study by Al-Ghadban et al. [8] also reported elevated oxidative stress and inflammation in lipedema tissue, and our metabolomic data support the existence of a dysregulated redox environment potentially sustained by aberrant AKT1

activation. Together, these findings suggest that AKT1 may serve as a central metabolic integrator that drives adipose tissue dysfunction and disease chronicity in lipedema. The functional convergence of multi-omic layers on AKT1 is particularly notable given its central role in regulating insulin signaling, nutrient sensing, and cell survival [91,92]. In the context of obesity, AKT1 activation is known to promote adipocyte hypertrophy, lipid storage, and macrophage recruitment, but its involvement in non-obesity-related disorders such as lipedema has not been previously characterized [100-102]. Our findings suggest that AKT1 may represent a shared pathological mechanism across distinct adipose tissue disorders, with lipedema presenting a unique epigenetically driven pattern of activation. Compared to the study by Straub et al., which analyzed early-stage disease using transcriptomics, proteomics, and cytokine profiling, our approach emphasizes functional integration across omics layers and focuses on advanced-stage pathology [90]. While Straub's study provided valuable insight into early molecular alterations, it did not resolve mechanistic regulators or explore metabolic outputs. By incorporating DNA methylation and NMR-based metabolomics in a tissue-specific context, our study uncovers a regulatory axis that may contribute to the transition from early to advanced disease stages. Importantly, the convergence of methylation, expression, and metabolite changes on AKT1 provides a strong rationale for its future evaluation as a biomarker and druggable target. Among the strengths of our study are the use of clinically and histologically validated tissue samples from patients with stage III lipedema, the simultaneous profiling of methylome, transcriptome, and metabolome in the same samples, and the application of integrated bioinformatic tools for cross-layer analysis. This comprehensive design enables a systems-level understanding of disease progression and identifies molecular candidates with translational relevance.

6.1 Translational Implications

This study offers critical translational insights into the molecular pathogenesis of advanced-stage lipedema, highlighting AKT1 as a central integrator of epigenetic, transcriptomic, and metabolic dysregulation. Our multi-omics approach uncovered a hypomethylated regulatory region in the AKT1 promoter, leading to increased gene expression and protein phosphorylation. These findings suggest that AKT1 activation is driven by stable epigenetic changes, rather than somatic mutations, underscoring its potential as a modifiable molecular target. Given AKT1's established role in insulin signaling, mitochondrial function, and energy metabolism, its upregulation may explain key pathological features of lipedema, including impaired oxidative phosphorylation, chronic inflammation, and altered adipocyte

metabolism. Moreover, the identification of AKT1-linked metabolic signatures, such as changes in NADP+, ATP, glutamate, and amino acid profiles, supports the development of metabolomics-based biomarkers for non-invasive disease monitoring. From a systems biology perspective, these findings position AKT1 at the intersection of signaling networks that govern adipose tissue homeostasis and disease progression. Therapeutically, our results suggest that pharmacological modulation of AKT1 or its downstream effectors may represent a novel strategy for intervention in late-stage lipedema. Importantly, AKT inhibitors are already in clinical development for other metabolic and oncologic disorders, potentially accelerating drug repurpose efforts. Overall, our integrative analysis not only elucidates a previously unrecognized regulatory axis in lipedema but also provides a mechanistic framework for biomarker discovery and targeted therapy development, aligning with the core translational goals of precision medicine.

7 Conclusion

To our knowledge, this is the first multi-omics study in advanced-stage lipedema to suggestes a epigenetically regulated AKT1 activation and coordinated metabolic reprogramming, offering both mechanistic insights and translational targets. Our data provide compelling evidence that AKT1 functions as a central epigenetically regulated node in advanced lipedema, integrating signals across molecular and metabolic layers. These findings expand current understanding of lipedema pathophysiology and open new avenues for biomarker discovery and mechanism-based therapeutic development. Moreover, our study highlights the utility of multi-omics integration in identifying disease-driving mechanisms in underexplored adipose tissue disorders. Taken together, these findings position AKT1 not only as a molecular hallmark of advanced lipedema but also as a promising therapeutic target that warrants further validation in preclinical models and pharmacological intervention studies.

7.1 Limitations of the study

However, several limitations should be acknowledged. First, the sample size was modest due to the clinical challenges associated with obtaining large adipose tissue biopsies from late-stage patients. While the molecular alterations identified were robust and internally consistent, validation in larger, independent cohorts will be important to confirm our findings and assess inter-individual variability.

Second, functional experiments (e.g., AKT1 knockdown or overexpression) were not performed; therefore, further mechanistic studies are required to establish a causal role for AKT1 in mediating the observed phenotypes. Third, although we focused exclusively on female patients in line with the epidemiology of lipedema, future studies should consider potential sex-specific differences and include appropriate controls for hormonal status, menopausal state, and body mass index (BMI).

In addition, while our untargeted metabolomics approach provided a comprehensive overview of metabolic alterations, more refined techniques, such as targeted fluxomics or lipidomics, may yield deeper insights into lipid processing and redox cycling. That said, it is noteworthy that portions of our transcriptomic and metabolomic profiles are consistent with previously published findings, providing an independent layer of validation [77]. Moreover, the role of AKT1 and its associated pathways was supported by orthogonal evidence from integrated omics layers, underpinned by robust statistical power. Finally, AKT1 dysregulation and phosphorylation were independently confirmed through validation assays.

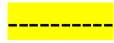
7.2 Ethics approval and consent to participate

The study was reviewed and approved by the ethics committee of the Land Salzburg. All study participants gave written informed consent and were informed about the study (EK Nr: 1023/2024).

7.3 Availability of data and materials

The data supporting the findings of this study are available within the main text and from the corresponding authors upon reasonable request.

8 Acknowledgements



8.1 Funding

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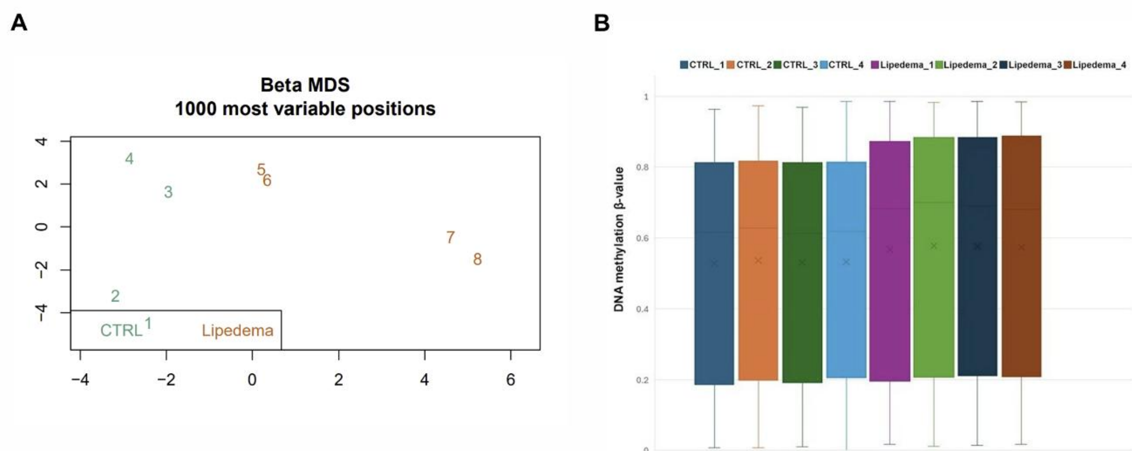
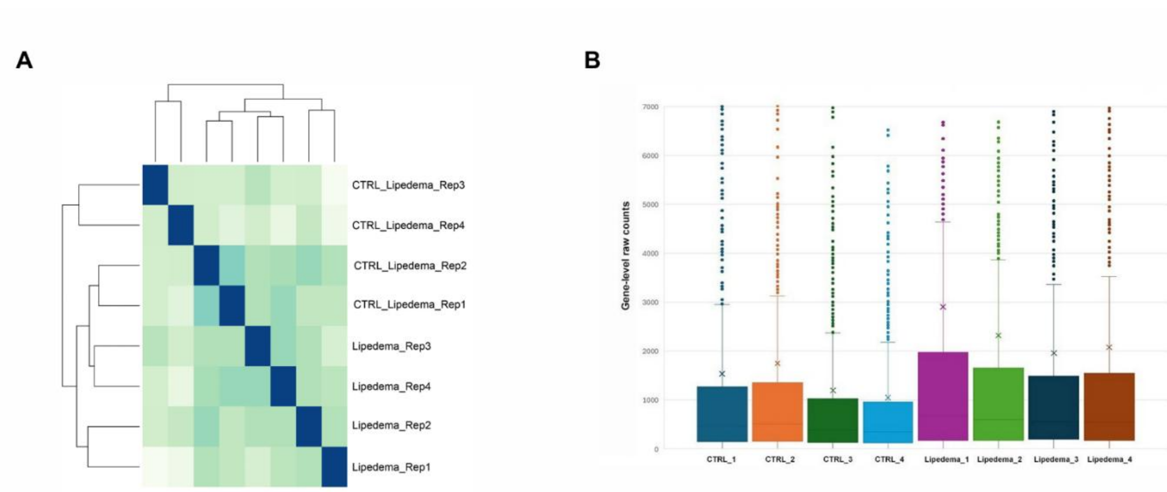
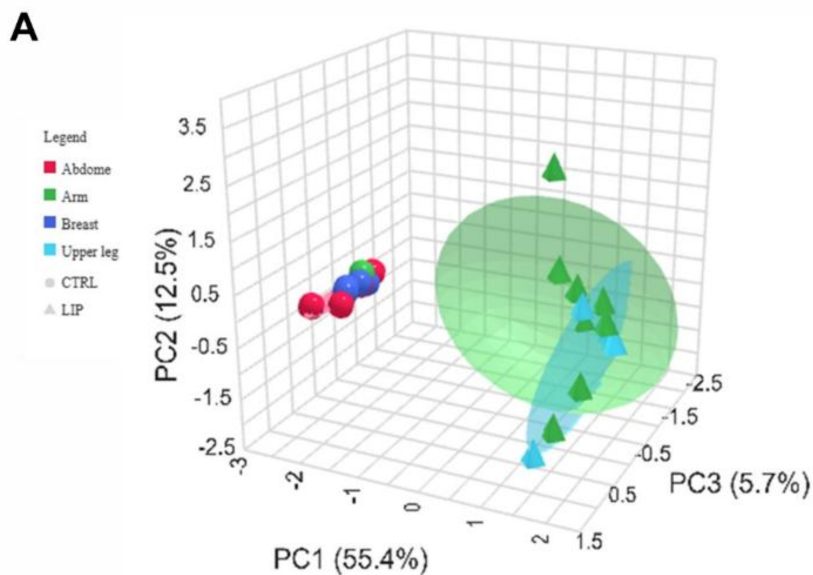
9 Supplementary Material



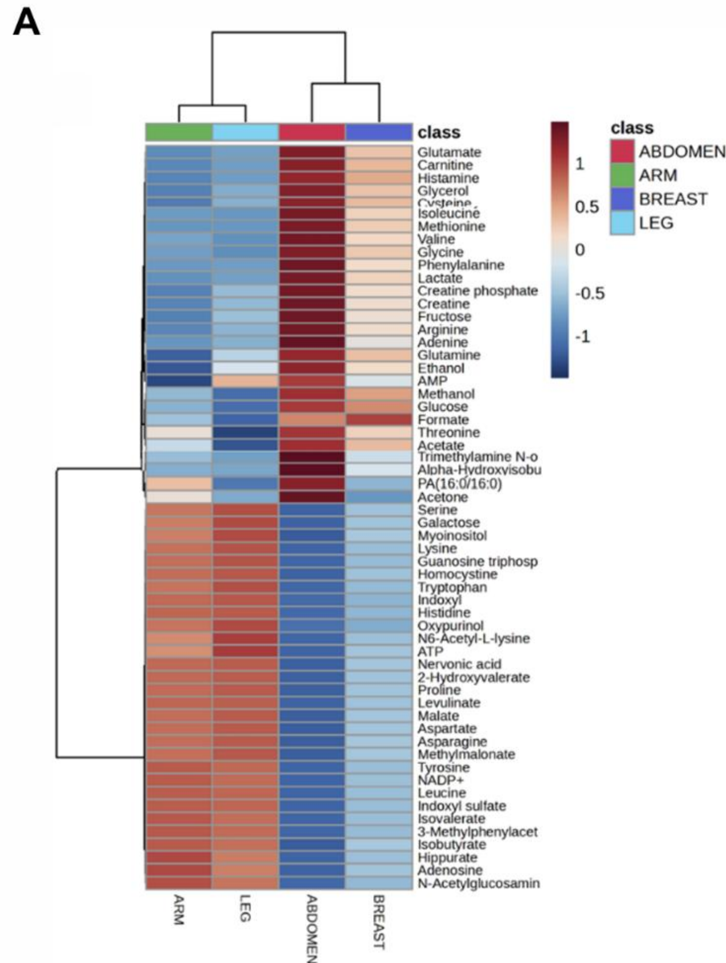
Supplementary Figure 1. DNA methylation QC and sample similarity assessment. **A)** Multidimensional scaling (MDS) plot of methylation β-values based on the 1,000 most variable CpG positions after preprocessing/normalization, used to assess sample similarity and detect potential outliers or gross batch-driven structure; samples are colored by group (CTRL vs lipedema). **B)** Global distribution of normalized DNA methylation β-values (0–1) across samples shown as boxplots (median, interquartile range, and 1.5×IQR whiskers; “×” indicates the mean). Together, the MDS and β-value distributions support the absence of evident outlier samples and no gross technical shifts between groups.



Supplementary Figure 2. RNA-seq QC and sample similarity assessment. **A)** Heatmap of pairwise sample–sample similarity (Pearson correlation) of RNA-seq expression profiles with hierarchical clustering, used to identify outlier samples and potential batch-driven clustering. Darker shading indicates higher similarity. **B)** Per-sample distribution of gene-level read counts shown as boxplots (median, interquartile range, 1.5×IQR whiskers; “×” indicates the mean; highly expressed genes appear as upper outliers).



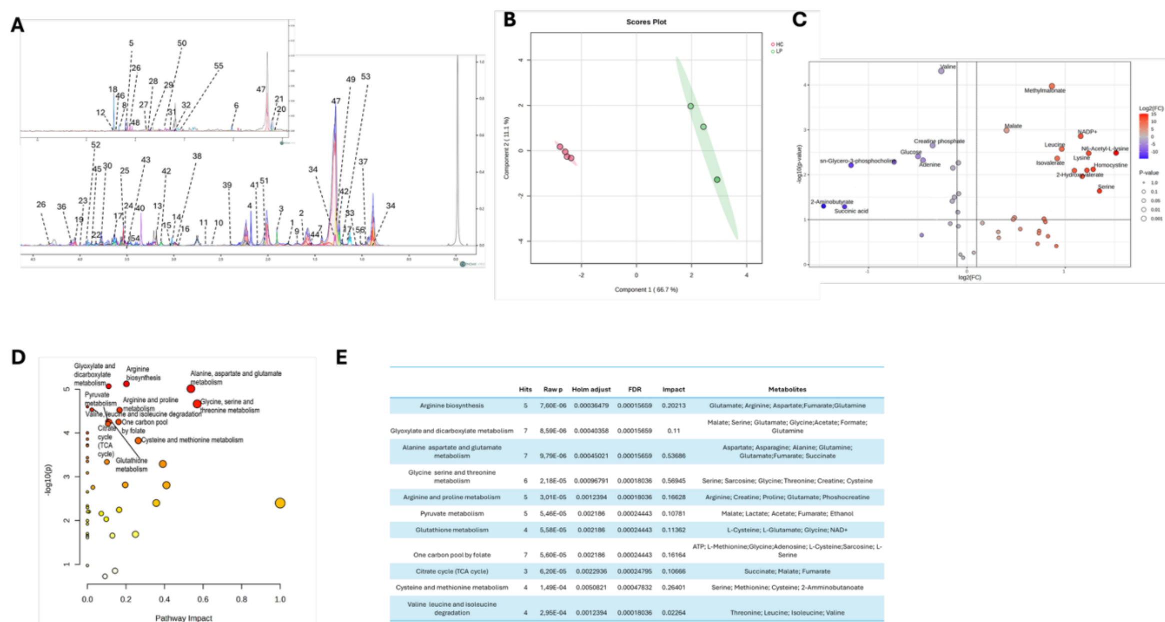
Supplementary Figure 3. A) iPCA the score plot illustrates the separation of metabolic profiles based on the presence or absence of pathology and the anatomical site of tissue sampling. Cluster analyses are represented in Cartesian space, described by principal components PC1: 55.4%, PC2: 12.5%, and PC3: 5.7%. Triangles denote pathological metabolic profiles, while circles represent those of controls. Anatomical sites are identified by colour: red for abdomen, green for arm, blue for breast, and light blue for leg.



Supplementary Figure 4. A) Heatmap of site-specific metabolomic profiles. Heatmap showing relative metabolite levels across adipose tissue sampling sites (ARM, LEG, ABDOMEN, BREAST). Rows represent metabolites and columns represent anatomical sites; hierarchical clustering is applied to both metabolites and sites. The top annotation bar indicates the sampling site ("class"). Colors represent standardized (z-score) metabolite abundance (red = higher than the mean, blue = lower than the mean; scale −1 to +1).

Index	Name	P-value	Adjusted p-value
1	Metabolism	3.492e-10	1.035e-7
2	Aerobic Respiration and Respiratory Electron Transport	6.293e-10	1.035e-7
3	Respiratory Electron Transport	0.000007326	0.0008034
4	Mitochondrial Protein Degradation	0.00002352	0.001934
5	Complex IV Assembly	0.00008571	0.005640
6	TP53 Regulates Metabolic Genes	0.0005063	0.02776
7	VEGFA-VEGFR2 Pathway	0.0008105	0.02846
8	CD28 Dependent PI3K Akt Signaling	0.0008298	0.02846
9	Immune System	0.0008453	0.02846
10	Signaling by VEGF	0.001027	0.02846

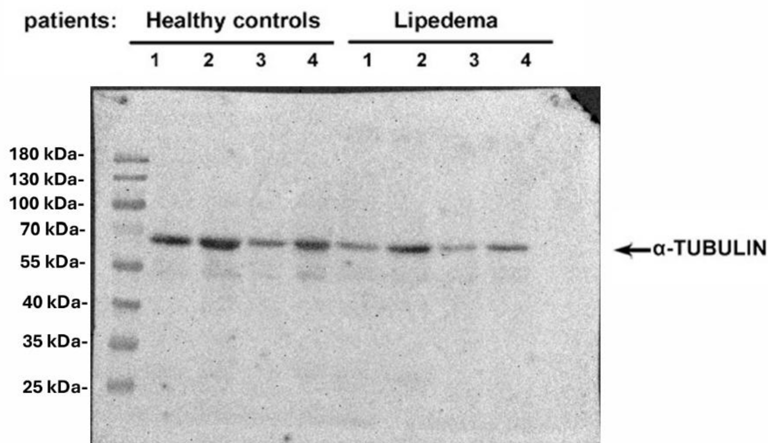
Supplementary Figure 5. Top enriched pathways from the integrative analysis restricted to genes showing hypomethylation ($\Delta\beta < 0{,}15$) together with increased expression in RNA-seq ($\log2FC > 1{,}5$). The table reports nominal enrichment p-values and Benjamini–Hochberg adjusted p-values.

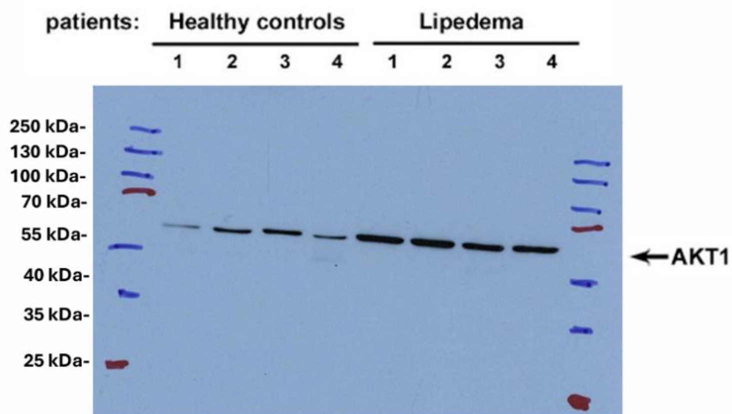


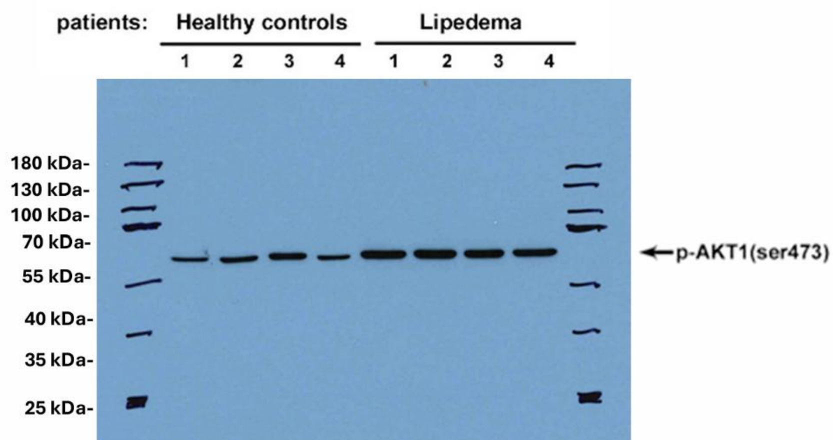
	Hits	Raw p	Holm adjust	FDR	Impact	Metabolites
Arginine biosynthesis	5	7,60E-06	0.00036479	0.00015659	0.20213	Glutamate; Arginine; Aspartate;Fumarate;Glutamine
Glyoxylate and dicarboxylate metabolism	7	8,59E-06	0.00040358	0.00015659	0.11	Malate; Serine; Glutamate; Glycine;Acetate; Formate; Glutamine
Alanine aspartate and glutamate metabolism	7	9,79E-06	0.00045021	0.00015659	0.53686	Aspartate; Asparagine; Alanine; Glutamine; Glutamate;Fumarate; Succinate
Glycine serine and threonine metabolism	6	2,18E-05	0.00096791	0.00018036	0.56945	Serine; Sarcosine; Glycine; Threonine; Creatine; Cysteine
Arginine and proline metabolism	5	3,01E-05	0.0012394	0.00018036	0.16628	Arginine; Creatine; Proline; Glutamate; Phoshocreatine
Pyruvate metabolism	5	5,46E-05	0.002186	0.00024443	0.10781	Malate; Lactate; Acetate; Fumarate; Ethanol
Glutathione metabolism	4	5,58E-05	0.002186	0.00024443	0.11362	L-Cysteine; L-Glutamate; Glycine; NAD+
One carbon pool by folate	7	5,60E-05	0.002186	0.00024443	0.16164	ATP; L-Methionine;Glycine;Adenosine; L-Cysteine;Sarcosine; L-Serine
Citrate cycle (TCA cycle)	3	6,20E-05	0.0022936	0.00024795	0.10666	Succinate; Malate; Fumarate
Cysteine and methionine metabolism	4	1,49E-04	0.0050821	0.00047832	0.26401	Serine; Methionine; Cysteine; 2-Amminobutanoate
Valine leucine and isoleucine degradation	4	2,95E-04	0.0012394	0.00018036	0.02264	Threonine; Leucine; Isoleucine; Valine

Supplementary Figure 6. Metabolomics analyses A) 1H NOESY spectra associated with the tissue adipose extract. 1:2 Amminoadipate; 2:2 Hydroxyvalerate; 3:Acetate; 4:Acetone; 5:Adenine; 6:Adenosine; 7:Alanine; 8:AMP; 9:Arginine; 10:Asparagine; 11:Aspartate; 12:ATP; 13:Carnitine; 14:Creatine; 15:Creatine phosphate; 16:Cysteine; 17:Ethanol; 18.Formate;19:Fructose; 20:Galactose;21:Glucose; 22:Glutamate; 23:Glutamine; 24.Glycerol; 25:Glycine; 26:GTP ;27:Hippurate; 28:Histamine; 29:Histidine; 30: Homocysteine; 31:Indoxyl; 32:Indoxyl-sulfate; 33:Isobutyrate; 34:Isoleucine; 35:Isovalerate; 36:Lactate; 37:Leucine; 38:Lysine; 39:Malate; 40:Methanol; 41:Methionine; 42:Methylmalonate; 43:Myo-inositol; 44:N6-Acetyllysine; 45:N6-Glucosammine; 46:NADP+; 47:Nervonic acid; 48:Oxopurinol;49: PA(16:0/16:0); 50:Phenylalanine; 51:Proline; 52.Serine; 53:Threonine; 54:Tryptophan; 55:Tyrosine; 56:Valine; 57:TMAO. **B)** PLS-DA score plot. The metabolomic profile of pathological adipose tissue (n:4) and control tissue (n:4) is shown. Cluster analyses are shown in Cartesian space described by principal components PC1:66.7% and PC2:11.1%. PLS-DA was validated by cross-validation (CV) methods with accuracy values of 1.0% relative both PC1 and PC2, and Q2 indices of 0.95 and 0.96 for the first and second components, respectively. **C)** The volcano plot illustrates metabolic changes in the polar extracts of pathological adipose tissue. Each dot on the graph is determined by both p-value and fold-change values, which are set at 0.05 and 2.0, respectively. Red dots represent up-regulated metabolites, while blue dots denote down-regulated metabolitese). **D)** The analysis of metabolic pathways was conducted utilising MetaboAnalyst 6.0 with the application of the MetPa tool. The color and size of each circle represent the p-value and impact score, respectively, with large red circles signifying highly altered pathways. **E)** Table presents the discriminant biochemical pathways associated with the metabolic profiles of pathological and control adipose tissue. The Hits represent the corresponding number of metabolites derived from the user-uploaded data. The p-value is determined through the enriched analysis. The False Discovery Rate (FDR) quantifies the proportion of false positives exceeding the user-specified score threshold. Pathway impact constitutes a synthesis of centrality and pathway enrichment outcomes. This metric is computed by aggregating the importance measures of each matched metabolite and subsequently dividing this sum by the total importance measures of all metabolites within each pathway. Pathways exhibiting an impact value approximating 1 are those that most effectively differentiate the analyzed clusters.

Uncropped Western Blot







10 List of tables, figures and references

Figure 1: Clinical criteria lipedema diagnosis

Figure 2: Classification of lipedema types

Figure 3: Classification of lipedema stages

Figure 4: Experimental plan

Figure 5: Genome-wide DNA methylation profiling in lipedema patients

Figure 6: Lipedema transcriptome profiling

Figure 7: Multi-Omics profiling integration in lipedema

Figure 8: Metabolomic profiling and data integration

Supplementary Figure 1: DNA methylation QC and sample similarity assessment

Supplementary Figure 2: RNA-seq QC and sample similarity assessment

Supplementary Figure 3: Cluster analyses

Supplementary Figure 4: Heatmap of site-specific metabolomic profiles

Supplementary Figure 5: Top enriched pathways from the integrative analysis

Supplementary Figure 6: Metabolomics analyses

Supplementary Material: Uncropped Western Blot

Table 1: Classification of lipedema types

Table 2: Classification of lipedema stages

Table 3: Major differential diagnoses to lipedema

Table 4: Tissue samples collected from 2 cohorts

11 List of abbreviations

- AKT1: RAC-alpha serine/threonine-protein kinase
- BMI: Body Mass Index
- CpG: Cytosine-phosphate-Guanine dinucleotide
- DESeq2: Differential Expression analysis based on the Negative Binomial distribution
- DNA: Deoxyribonucleic Acid
- DMSO: Dimethyl Sulfoxide
- FDR: False Discovery Rate
- FC: Fold Change
- GSEA: Gene Set Enrichment Analysis
- GO: Gene Ontology
- GRCh38: Genome Reference Consortium Human Build 38
- IPA: Ingenuity Pathway Analysis
- KEGG: Kyoto Encyclopedia of Genes and Genomes
- LP: Lipedema Patient
- HC: Healthy Control
- MTBE: Methyl-t-butyl ether
- NMR: Nuclear Magnetic Resonance
- PLS-DA: Partial Least Squares Discriminant Analysis
- RIN: RNA Integrity Number
- RNA: Ribonucleic Acid
- RNA-Seq: RNA Sequencing
- SD: Standard Deviation
- SOP: Standard Operating Procedure
- STAR: Spliced Transcripts Alignment to a Reference
- TCA: Tricarboxylic Acid (cycle)
- TSS: Transcription Start Site
- TSP-d4: 2,2,3,3-d4-trimethylsilyl-propionic acid sodium salt
- UTR: Untranslated Region
- VIP: Variable Importance in Projection

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