



OPEN Pilot intraindividual secretome profiling of adipose-derived stem cells in multiple symmetric lipomatosis

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Multiple symmetric lipomatosis (MSL) is a rare adipose tissue disease characterized by progressive, non-encapsulated, symmetrical fat accumulation. Its pathophysiology is still not completely understood. Here, we performed the first intraindividual comparison of secretomes of human adipose-derived stem cells (hASCs) isolated from affected and unaffected regions of $n = 15$ patients (proteomic discovery cohort $n = 5$). Affected MSL-hASCs tend to show accelerated metabolic signals and higher cell densities with preserved adipogenic differentiation capacity. Secretome transfer of affected MSL-hASCs significantly increased metabolic activity of wt-hASCs after 24 h in 4/7 samples (all explorative Benjamini-Hochberg corrected $p = 0.040$). Bottom-up proteomic analysis identified 811 proteins, of which 220 were classified as secreted. Thirteen proteins exhibited nominally significant dysregulation of expression, including CALR ($p = 0.0061$), LAMC1 ($p = 0.0241$), S100A7 ($p = 0.0281$) and HDGF ($p = 0.0474$), but none remained significant after application of the Benjamini-Hochberg (BH) correction to control the False Discovery Rate (FDR) at 0.05. Transcriptional validation by rt-qPCR revealed a trend toward downregulation of hLAMA4 and hCFB (both $p = 0.0625$). In conclusion, convergent patterns are consistent with extracellular matrix remodeling and PI3K/AKT-ERK-associated proliferative signaling. This model supports a hypothesis of progenitor-level dysregulation rather than a single clonal driver in MSL, which will require further mechanistic validation in larger cohorts.

Keywords Multiple symmetric lipomatosis, Adipose-derived stem cells, Proteomics, Orphan disease, Extracellular matrix, paracrine signaling

Multiple symmetric lipomatosis (MSL) is a rare disease characterized by hyperproliferation of adipose tissue, presenting as a non-tender, progressive increase in adipose tissue volume, predominantly in the neck, but also extending to the shoulder, trunk and proximal extremities. Afflicted patients report a disfiguring aesthetic appearance and associated negative effects on their overall well-being. Initially considered a benign condition, recent reports have indicated a risk of life-shortening symptoms, such as severe airway and major blood vessel compression due to mediastinal mass effects, in addition to the risk of malignant transformation into liposarcoma^{1,2}.

Following first descriptions in the 19th century, MSL has been investigated and classified in several European populations^{2–4}. Initially categorized into 3 phenotypes, MSL was further subclassified by Schiltz et al. in 2018 taking the most commonly affected areas: neck, shoulder, trunk, and proximal extremities into account⁵.

Despite the broad clinical characterization of the phenotypes in MSL, its etiology remains unclear. Consequently, clinical management has rather focused on symptomatic relief, e.g. through liposuction or lipectomy, than on causative treatments⁶. While these surgical interventions offer temporary improvement, recurrence rates are high⁷, and seem to differ depending on the exact site of treatment by lipectomy or liposuction⁷. A comprehensive understanding of MSL pathogenesis might also accelerate the development of more promising therapies that offer improved long-term outcome. Light microscopic examination of removed tissue revealed differences in tissue composition between affected and unaffected areas. Some studies found MSL tissue to be more solid, fibrous, and vascularized, whereas others were unable to confirm this^{8–10}, describing

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rather unencapsulated proliferation and spread of small human adipose-derived stem cells (hASC) into peripheral fat, thus suggesting MSL to be a proliferative rather than hypertrophic disease^{10,11}. Importantly, some authors report that the method chosen for the collection of tissue samples did not show statistically significant differences in the quantity of the retrieved stem cells¹². Various studies have been conducted to shed more light onto the origins of MSL, with both mitochondrial DNA mutations and environmental factors such as alcohol abuse having been proposed^{13,14}. Recent studies examining the MSL stem cell secretome have shown that exposure of healthy human adipose tissue derived stem cells (hASCs) to the MSL secretome resulted in altered growth behavior and surface markers⁸.

Here, we hypothesized that a differential protein secretion profile of the hASCs in affected areas to be responsible for the changes observed by Caponnetto et al., which may be linked to an enhanced proliferative signaling and local remodeling of the extracellular matrix⁸. To test this hypothesis, we conducted a series of experiments including a paired, intraindividual comparison of the secretomes of hASCs isolated from both affected and unaffected areas in the same MSL patients (Fig. 1), to facilitate a better understanding of MSL pathophysiology and to serve as a starting point for extended genetic and proteomic studies.

Results

Isolation, morphological identification and characterization of hASCs

Isolation of human adipose-derived stem cells (hASC) succeeded in all cases after three days of cell culture. Phenotypical stability was ensured by the utilization of samples from the first six passages for subsequent experiments. hASCs from MSL-unaffected and MSL-affected regions displayed the characteristic fibroblast-like, spindle-shaped morphology. However, no apparent morphological differences were detected by light microscopy. The adipogenic differentiation potential of both populations was visually validated. Upon adipogenic differentiation, intracellular lipid droplets formed uniformly in both groups, as confirmed by Oil Red O staining (Fig. 2A–C).

No visual differences between affected and unaffected samples were observed after adipogenic differentiation. Notably, differences in cell densities were observed. At the time of medium change, hASCs that had been isolated from MSL affected tissue formed consistently a denser population of smaller stem cells compared to those derived from unaffected reference tissue.

Functional characterization of hASC metabolic activity in response to secretome exposure

To investigate possible paracrine drivers of the metabolic activity of wildtype hASCs following exposition to the supernatants collected from MSL-ASCs (affected and unaffected sites, respectively), we determined the active

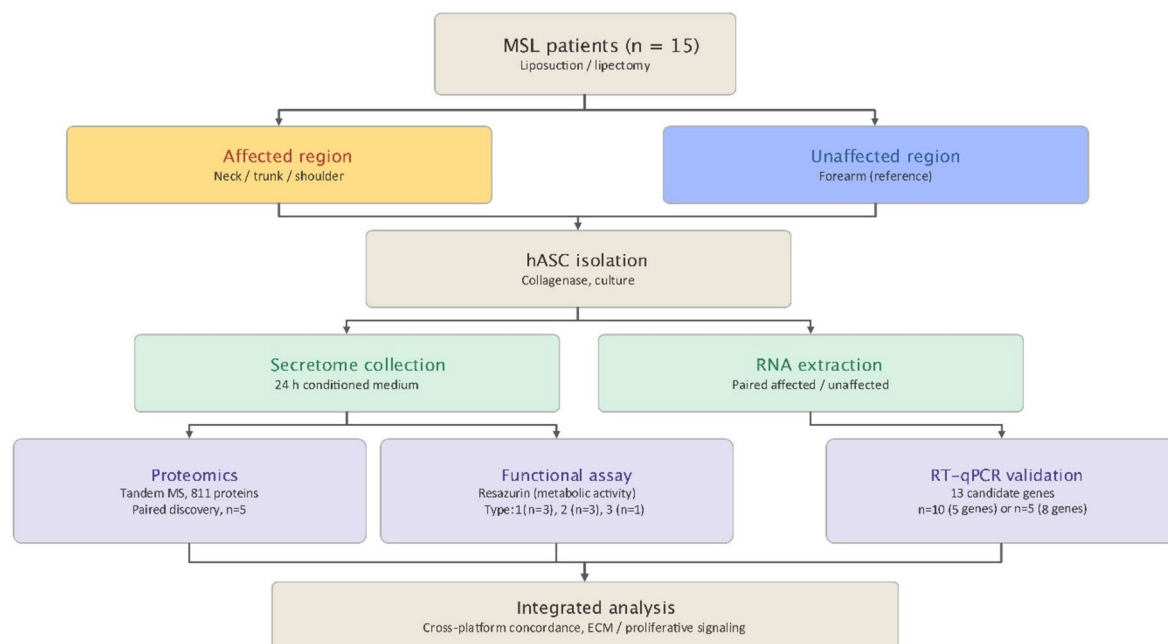


Fig. 1. Overview of experimental design. Diagram reflecting the paired, intraindividual study design with sample collection via liposuction or lipectomy from affected and unaffected, referential regions. Conditioned medium (secretome) was collected after 24 h and used for a functional resazurin assay (metabolic activity; Type 1 $n=3$ pairs, Type 2 $n=3$ pairs, Type 3 $n=1$ pair) and for tandem mass spectrometry-based proteomic profiling (paired discovery cohort, $n=5$, 811 proteins identified). In parallel, RNA was extracted from paired affected and unaffected hASC for rt-qPCR validation of 13 candidate genes. Depending on available RNA, five genes (hS100A7, hCLTA, hHDGF, hHSPB1, hIL6) were assessed in ten paired patients and eight genes (hCFB, hCLCA, hLAMA4, hMMP1, hPAPPA, hPCOLCE2, hPPAR γ , hPTEN) in five.

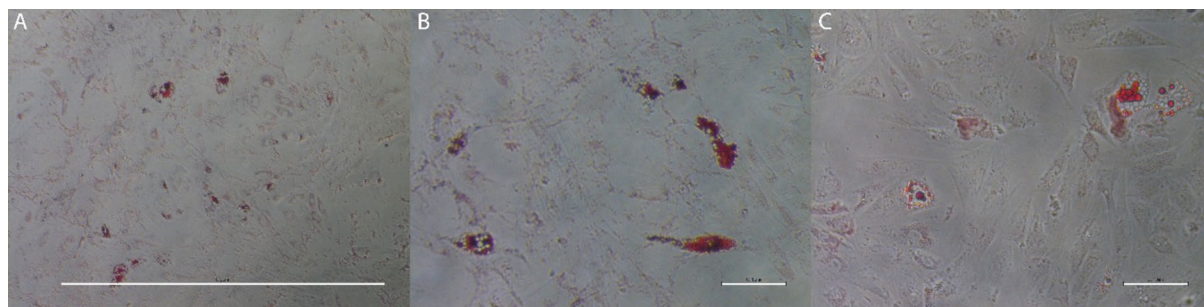


Fig. 2. Adipogenic differentiation of MSL hASC. Representative light microscopy images of MSL type 1-affected hASCs following adipogenic induction. Intracellular lipid droplets stained with Oil Red O are visible as red vesicles (A-C). Scale bars: A = 1 mm; B = 100 µm; C = 100 µm.

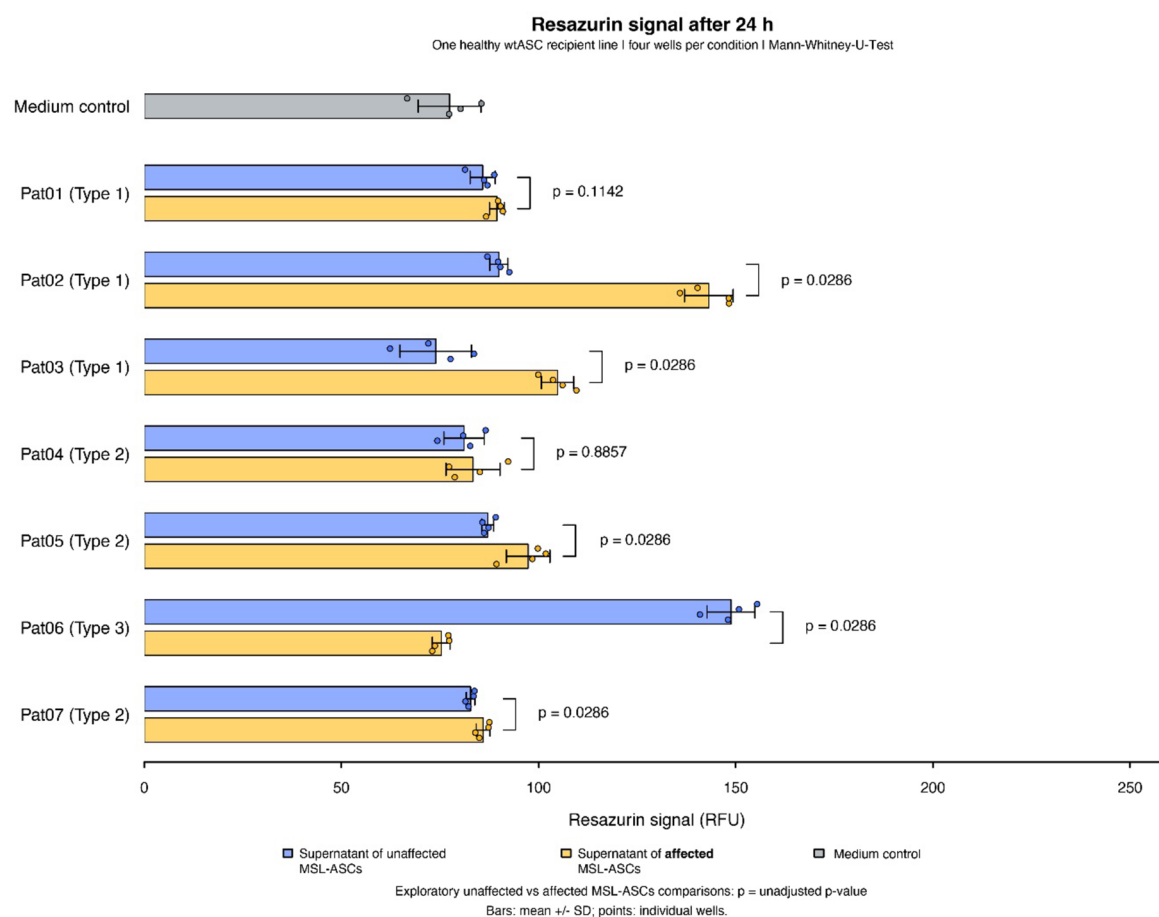


Fig. 3. Resazurin signals from healthy wild-type hASCs after 24-hour exposure to patient-derived MSL supernatants. Bars represent mean fluorescence intensity (RFU) $\pm$ SD. The dots represent the four individual wells. Y-axis: patient and MSL type; X-axis: RFU. Blue: supernatants from unaffected MSL-hASCs; gold/orange: supernatants from affected MSL-hASCs; gray: descriptive medium control. Reported p-values: explorative unadjusted exact Mann-Whitney-U-Test.

enzymatic reduction of the non-fluorescent resazurin to the red-fluorescent product resorufin after 24 h of cell culture. The experiment design is similar to that of Caponnetto et al.⁸.

Seven sample pairs were evaluated after 24 h and treated as explorative findings. The results indicate differential responses towards the supernatant of choice. In 4 patients (MSL Type 1 $n = 2$ and MSL Type 2 $n = 2$), we observed a significantly elevated resazurin signal under exposure to the supernatant from affected MSL-hASCs (each unadjusted $p = 0.029$, and BH-corrected $p = 0.0400$, Fig. 3). Two patients did not show any significant difference between the tested supernatants. The only MSL Type 3 patient revealed an inverse

constellation: higher resazurin signals in presence of unaffected supernatant from MSL-hASCs (148,81 vs. 75,30 RFU, respectively, Fig. 3). These results reflect only a testing of technical replicates and should be viewed explorative only. A summary of these findings is further represented in the Supplementary Table S1. To decipher the molecular composition driving these functional observations, we performed sequential window tandem mass spectrometric analysis of the supernatants of MSL affected and unaffected hASCs in this ($n = 5$ paired samples) using a previously published protocol¹⁵.

Combined analysis of all secretomes identified a total of 811 proteins. Initial fold-change based classification revealed a similar expression in both MSL affected and unaffected supernatants for 91.7% ($n = 744$) of the proteins identified, while 5.8% ($n = 47$) were enriched ($FC > 1.5$) in samples from affected tissue, and 2.5% ($n = 20$) were enriched in samples from unaffected tissue (i.e. reduced in affected tissue, $FC < 0.5$) (Fig. 4A).

Information about subcellular localization could be assigned to 88.7% ($n = 719$) of identified proteins. The remaining 92 proteins were excluded because no annotation about subcellular localization could be retrieved from UniProt. Since UniProt annotations are not mutually exclusive; hence, a protein can be assigned to more than one subcellular compartment. As a result, these categories may sum up to more than 100%. Out of 719 annotated proteins, intracellular proteins accounted for 76.4% ($n = 549$), membrane-associated proteins for 27.4% ($n = 197$), and secreted proteins for 30.6% ($n = 220$) (Fig. 4B). The latter are hereafter referred to as the secretome.

Differential protein expression

To identify specific regulatory drivers in the secretome, we analyzed differentially abundant proteins (DAPs) at a nominal significance threshold of $p < 0.05$. To account for the cohort size, we additionally visualized nominal significance ($-\log_{10}$ p-value) and effect size ($\log_2$ fold change) in a volcano plot (Fig. 5A). Thirteen proteins were nominally significant, representing 5.9% of the total secretome (Fig. 5A), but none remained statistically significant after Benjamini-Hochberg correction for multiple testing (min. adjusted $p = 0.554$), consistent with the limited cohort size.

Four and nine proteins were upregulated, and downregulated, respectively, in the affected (Mad) group compared to unaffected controls (Fig. 5B). Among upregulated proteins, calreticulin (CALR) featured the lowest p-value ($p = 0.00609$; $\log_2 FC = +0.335$; FC magnitude = 1.26), while among downregulated proteins, Scavenger receptor cysteine-rich domain-containing protein (SSC5D/SRCRL) showed the lowest p-value ($p = 0.016$, $\log_2 FC = -0.782$; FC magnitude = 1.72). The 20 most significant proteins are summarized in a Supplementary Table 3, while nominally significant proteins ($p < 0.05$) are visualized in paired boxplots in Fig. 5B to illustrate the change for each patient. A complete overview of all 220 secreted proteins and their differential abundance is provided in Supplementary Table S4.

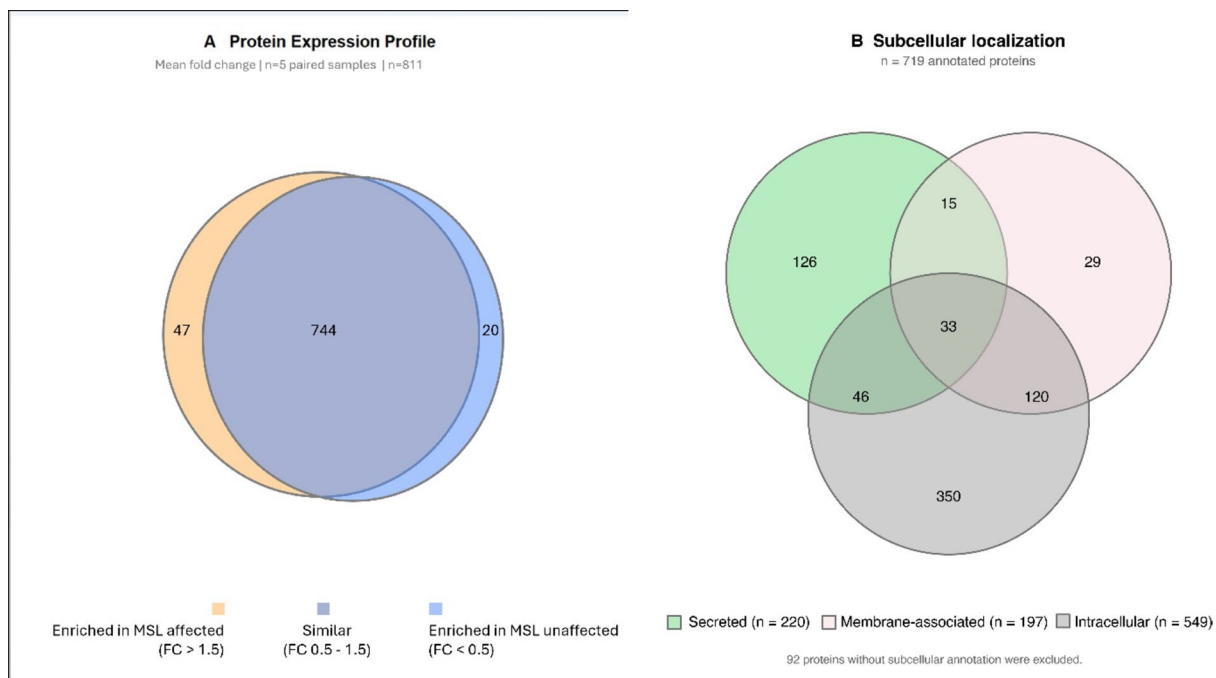


Fig. 4. Global proteome analysis of MSL hASC supernatant. (A) Fold change-based classification of 811 identified proteins comparing MSL affected and unaffected hASC supernatant across five paired patient samples. Enriched proteins in affected tissue ($FC > 1.5$, $n = 47$), enriched in unaffected tissue ($FC < 0.5$, $n = 20$), similarly expressed in both conditions ($FC 0.5 - 1.5$, $n = 744$). (B) Subcellular localization based on UniProt cell component analysis of 719 annotated proteins. Categories: Secreted ($n = 220$), membrane-associated ($n = 197$), Intracellular ($n = 549$). 92 proteins lacked subcellular annotation and had to be excluded.

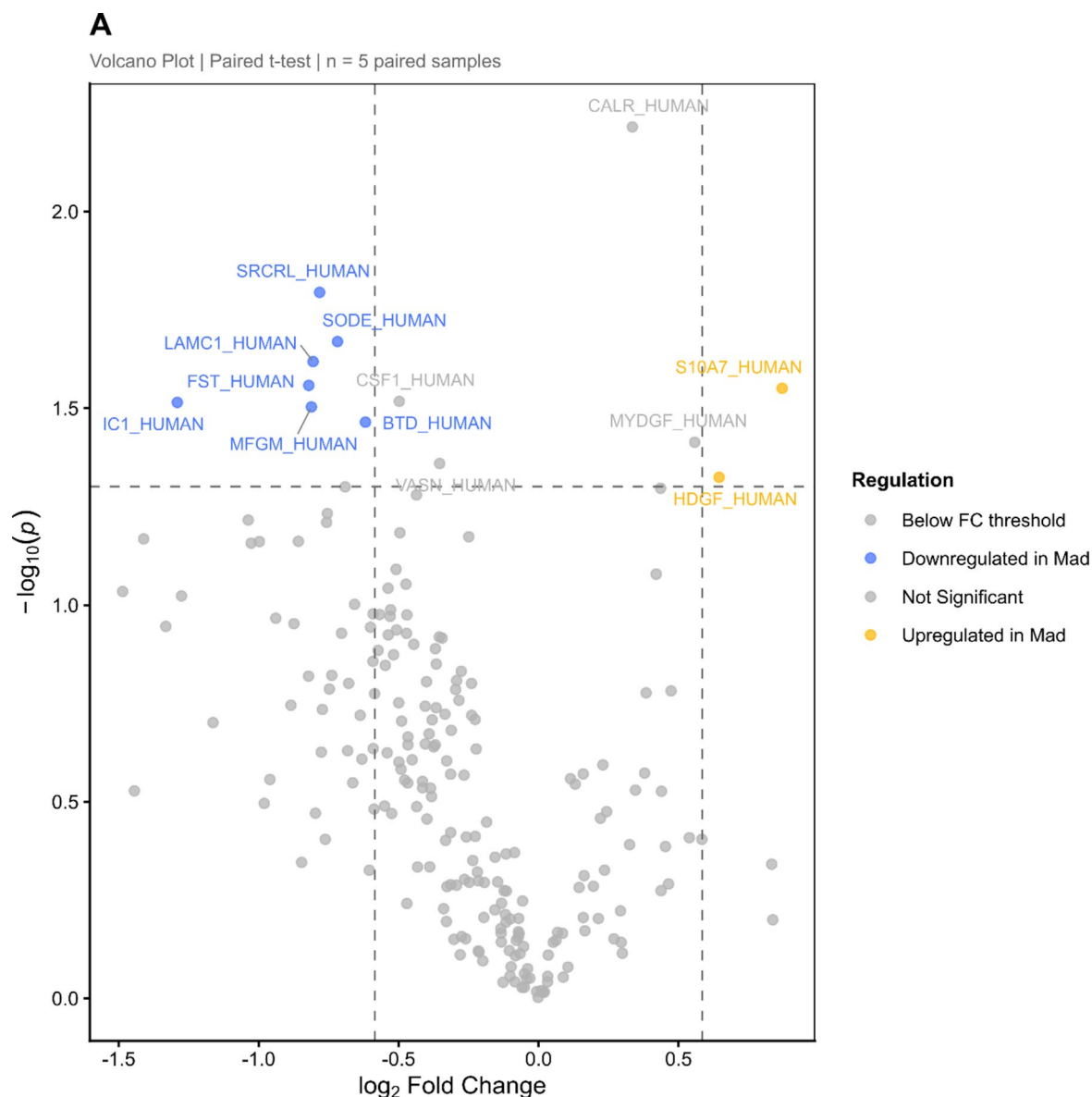
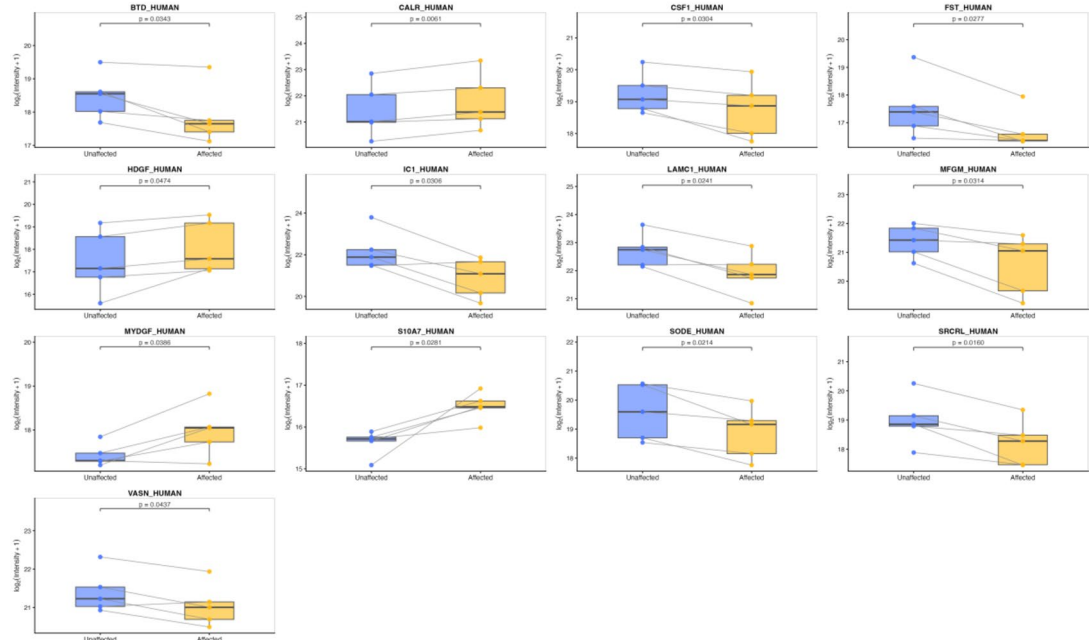


Fig. 5. MSL secretome comparison of unaffected and affected hASC. **(A)** Volcano plot of secreted proteins from hASCs derived from unaffected and affected (Mad) tissue (x-axis: $\log_2$ -fold change (affected vs. unaffected), y-axis: $-\log_{10}(\text{unadjusted } p\text{-value})$). Proteins with $p \leq 0.05$ and $|\log_2 \text{FC}| > \log_2(1.5)$ are highlighted. Orange indicates proteins upregulated in affected tissues, blue indicates proteins downregulated in the same affected MSL-tissue. Grey denotes the remaining proteins. Dashed lines mark the nominal significance threshold at $-\log_{10}(0.05)$ and the fold-change cutoffs at $\pm \log_2(1.5)$ (ca. ± 0.585). **(B)** Paired per-patient abundance profiles for the nominally significant proteins depicted as boxplots of its $\log_2(\text{Intensity} + 1)$ with paired lines connecting intraindividually matching samples.

Among the nominally significant DAPs, three biologically coherent clusters were identified, each of which referred to survival signaling, ECM remodeling and structural integrity, as well as to oxidative stress and lipid metabolism.

More specifically, MSL-affected hASCs showed upregulation of secreted mitogens, including Hepatoma-derived growth factor (HDGF; $p = 0.047$; $\log_2 \text{FC} = +0.645$; FC magnitude = 1.56), Myeloid-derived growth factor (MYDGF; $p = 0.039$; $\log_2 \text{FC} = +0.558$; FC magnitude = 1.47), and the stress- and proliferation-associated protein S100-A7 (Psoriasin; $p = 0.028$; $\log_2 \text{FC} = +0.870$; FC magnitude = 1.83). In marked contrast, several ECM-associated proteins were significantly downregulated in MSL-affected hASCs. These included the basement membrane component Laminin subunit gamma-1 (LAMC1; $p = 0.024$; $\log_2 \text{FC} = -0.806$; FC magnitude = 1.75), the Plasma protease C1 inhibitor (SERPING1; $p = 0.031$; $\log_2 \text{FC} = -1.291$; FC magnitude = 2.45), the anti-fibrotic glycoprotein Lactadherin (MFG8; $p = 0.031$; $\log_2 \text{FC} = -0.812$; FC magnitude = 1.76) and Follistatin (FST; $p = 0.028$; $\log_2 \text{FC} = -0.821$; FC magnitude = 1.77). Colony-stimulating factor 1 (CSF1; $p = 0.030$; $\log_2 \text{FC} = -0.498$; FC magnitude = 1.41) and Vasorin (VASN; $p = 0.044$; $\log_2 \text{FC} = -0.353$; FC magnitude = 1.28), were additionally

B**B Paired Abundance Profiles - Nominally Significant Proteins ($p < 0.05$)**log₂(intensity + 1) | Paired t-test | $n = 5$ paired samples | 13 proteins | Unadjusted p -values**Fig. 5.** (continued)

reduced in the MSL secretome. Extracellular superoxide dismutase (SOD3; $p = 0.021$; $\log_2FC = -0.718$; FC magnitude = 1.65) was significantly downregulated in MSL-affected hASCs. Biotinidase (BTG; $p = 0.034$; $\log_2FC = -0.619$; FC magnitude = 1.54), was similarly reduced.

Transcriptional validation

To validate the secretome findings at the transcriptional level, RT-qPCR was performed for 13 candidate genes in paired MSL-affected/unaffected samples ($n = 5$ or $n = 10$ per gene, depending on triplicate data availability). Gene expression differences ($\Delta\Delta Ct$) were assessed using the paired Wilcoxon signed-rank test, applied uniformly to all genes. Shapiro-Wilk results are reported for transparency only (Fig. 6, Supplementary Table S1) but were not used to select the statistical test, therefore only non-parametric testing was applied. No gene reached nominal significance ($p \geq 0.05$, and none remained significant after Benjamini-Hochberg FDR correction, Fig. 6). Exploratory trends toward down-regulation were observed for CFB ($\log_2FC = -1.75$, $p = 0.063$, FDR = 0.406) and LAMA4 ($\log_2FC = -2.11$, $p = 0.063$, FDR = 0.406), followed by PAPPB ($\log_2FC = -2.16$, $p = 0.125$, FDR = 0.542) and MMP1 ($\log_2FC = -3.03$, $p = 0.188$, FDR = 0.609), which showed the greatest fold-change magnitude among all genes tested. The remaining genes showed no relevant regulation: PTEN ($\log_2FC = -0.62$, $p = 0.312$), PCOLCE2 ($\log_2FC = -1.97$, $p = 0.438$), HDGF ($\log_2FC = -0.82$, $p = 0.557$), CLTA ($\log_2FC = -0.11$, $p = 0.625$), HSPB1 ($\log_2FC = -0.47$, $p = 0.625$), IL-6 ($\log_2FC = -0.08$, $p = 0.770$), CLCA ($\log_2FC = -0.39$, $p = 0.812$), and PPAR γ ($\log_2FC = -0.05$, $p = 1.000$). S100A7 was the only gene trending toward up-regulation ($\log_2FC = +0.39$, $p = 0.625$).

Cross-platform comparison revealed post-transcriptional discordance for HDGF, which was upregulated in the secretome (FC magnitude = 1.56, $p = 0.047$) but showed a non-significant trend toward reduced mRNA expression ($\log_2FC = -0.82$, $p = 0.557$). S100A7 showed directionally concordant but non-significant upregulation at both the mRNA ($\log_2FC = +0.39$, $p = 0.625$) and protein level (secretome: FC magnitude = 1.83, $p = 0.028$). The non-significant trend towards reduction of the laminin $\alpha 4$ chain (LAMA4) at the mRNA level ($p = 0.0625$) was consistent with the significant downregulation of the laminin $\gamma 1$ chain (LAMC1) in the secretome ($p = 0.024$).

Discussion

Multiple symmetric lipomatosis is an orphan and progressive disease of the subcutaneous fat tissue with common relapses following even established treatments such as liposuction or lipectomy^{6,7,16}. Entailing in detrimental effects on the functional and psychosocial health of affected patients. A better understanding of its pathophysiology has been the focus of most of the studies conducted to date on MSL¹⁷. Our study represents the first intraindividual analysis of hASC secretomes of affected and unaffected regions from the same patients, combining functional assays with shotgun proteomics and qPCR.

Central hASC characteristics were biologically confirmed for MSL hASC by the formation of typical fibroblast-like morphology and comparable adipogenic differentiation in both sample types, as shown with Oil-Red-O staining. Consistent with observations of previous experiments, MSL-affected cells exhibited higher cell densities, observed by qualitative microscopy. Affected hASCs reached higher cell densities earlier and showed a smaller phenotype. Much like previous findings by our study group and previous reports, these findings support

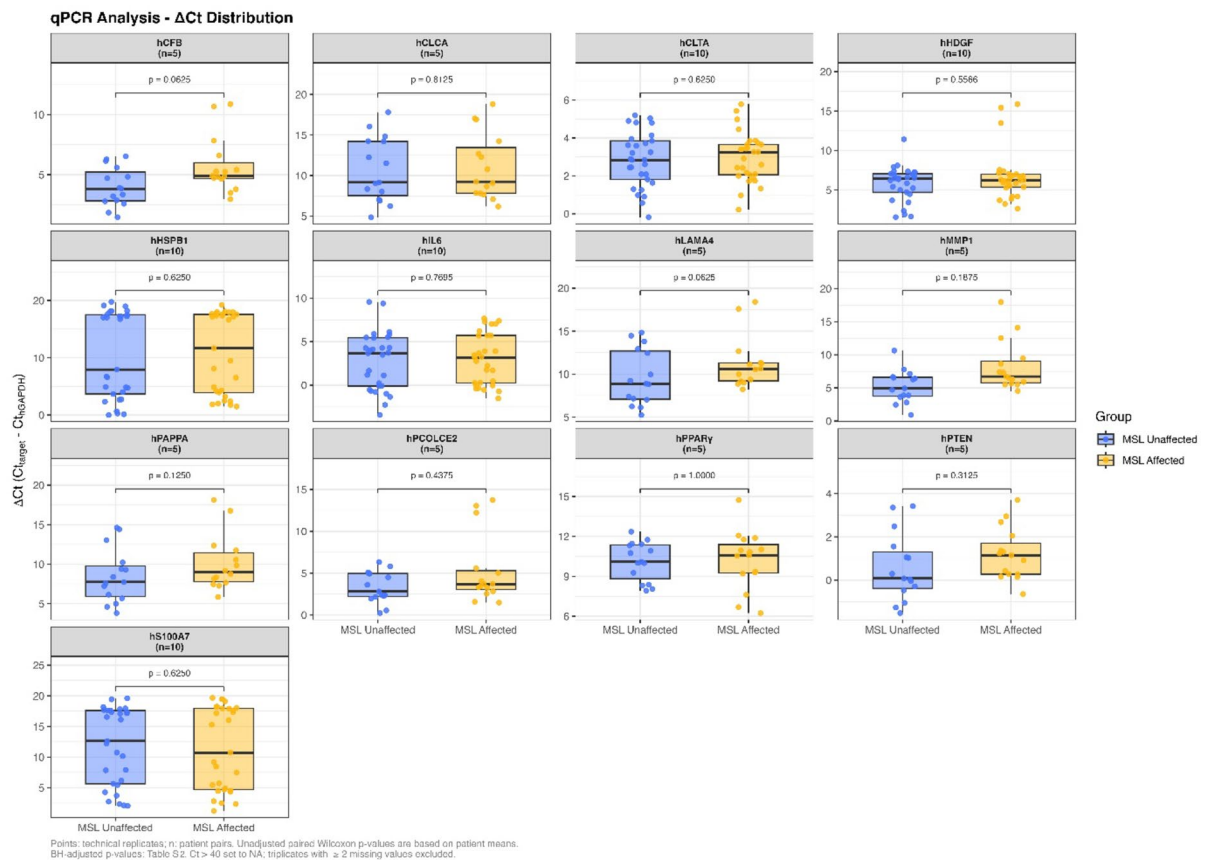


Fig. 6. Rt-qPCR analysis of candidate gene expression in affected and unaffected MSL hASC. Each sample in triplicates. Housekeeping gene hGAPDH. (A) Boxplots of ΔC_t values per primer with individual points (blue: unaffected, orange: affected). Boxes show median and interquartile range, whiskers indicate 1.5 times the interquartile range.

a progenitor-based etiology in the development of MSL^{8,10}. Additionally, earlier reports on beige/brown-like features of MSL-hASCs is consistent with the model of higher cell plasticity and adaptive pathway activation¹⁰.

Compared to previous studies, we investigated secretome effects of affected and unaffected hASC, using a shorter incubation period⁸. The resazurin responses differed between individual patient samples. The small sample size and technical replication do not allow conclusions about differences between MSL types. However, paracrine modulation capacity appears to be most pronounced for affected type 1 and 2 secretomes. Due to the pilot nature of our study, these findings need to be verified in larger cohorts. In the proteomic comparison individual proteins reached nominal significance, however, none of the candidate proteins passed an FDR-correction. We attribute this finding to the relatively small number of included samples, which can be explained by the orphan nature of MSL in the scope of discovery proteomics. Given its pilot nature, our study remains mainly hypothesis-generating with a focus on convergent trends and shared patterns. Within this limitation, our results are consistent with an axis based around extracellular matrix (ECM)/basal laminae, growth/survival signaling and matrix remodeling for the affected hASCs. The most prominent corresponding pattern here is observed for the convergent downregulation of several laminin chains. The LAMC1 ($\gamma 1$), which was shown to be associated with AKT-dependent metabolic reprogramming, was reduced in secretome nominally, whereas LAMA4 ($\alpha 4$) showed a consistent trend in both experiment series (secretome and qPCR-validation)^{18,19}. A consistent directional pattern of downregulation was additionally observed for pregnancy-associated plasma protein-A across both platforms (PAPPA, qPCR: $\log_2 FC = -2.155$, $p = 0.125$). However, it was not significant at the transcriptional level and a $p > 0.05$ in the secretome analysis. This metalloprotease cleaves insulin-like growth factor binding proteins (IGFBPs) and hence regulates local IGF-1 bioavailability. A reduced PAPPA-activity may lead to a decreased cleavage of IGFBP-4 and IGFBP-5, which results in the lowered local IGF-1 bioavailability. Since IGF-1 is responsible for proliferation, adipogenesis and migration in the hASCs, its reduced local bioavailability could reflect a compensatory reaction towards excessive proliferation or, alternatively, a disrupted autocrine feedback^{20–24}. Such a hypothetical mechanism would be consistent with the broader ECM dysregulation observed in this dataset. Even though none of these findings reached conventional significance thresholds, a consistent pattern of its expression allows for the further investigation. Consistently, an upregulation or activation of AKT/ERK-associated cascades has been described for MSL-tissue^{25,26}.

The pathogenesis of MSL demands a clear molecular distinction from clinically similar entities. Lipomas show frequent associations with rearrangement of 12q13–15, overexpression or fusion of HMGA2^{30,31}. This may

influence cell cycle and differentiation genes as a transcript modulator^{27,28}. Their growth is limited by a capsule, unlike in MSL. Here, hASCs show a sustained differentiation capacity with a higher observed cell densities and in some cases higher resazurin signal. It is inconsistent with an HMGA2-driven neoplastic model. Actual evidence behind lipedema, on the other hand, suggests microangiopathy, capillary leaks and inflammatory signaling (e.g., TNF- α , IL-6, VEGF-associated axis) to play the key role in the pathogenesis^{29,30}. Especially the tenderness of the fatty masses is often explained by juxta-vascular inflammation and interstitial fluids. However, little is known and reports remain inconsistent about the role of mTOR-signaling in this entity³¹. In lymphedema (VEGFR3/FLT4-associated) a primary lymphatic dysfunction is considered to be responsible for a subsequent TGF- β -mediated fibrosis and secondary fatty tissue hypertrophy^{32,33}. These patterns suggest remodeling of the basal lamina and a paracrine shift towards survival and proliferation signaling in MSL. Despite its hypothetical and preliminary nature, it is especially plausible when taking into account the findings around the laminin axis (LAMC1, LAMA4), COL4A1 and MFGE8, and the upregulation of HDGF, MYDGF and S100A7. One of the latest multi-omics studies of Santella et al. suggest AKT1-mediated remodeling in progressive lipedema, whereas Cifarelli et al. characterize classic lipedema by pro-inflammatory macrophage infiltration and dense interstitial fibrogenesis^{31,34}. Either way, our observations are neither typical of lymphedema nor lipedema nor neoplastic driven lipoma.

At the molecular level this finding can be supported via several ECM-associated proteins, that had a distinct profile prior to the FDR correction. Aside from the significant finding for LAMC1, our secretome results suggest a subtle but concordant downregulation of basal lamina components: laminin- β 1 (LAMB1, $p = 0.059$), collagen IV (COL4A1, $p = 0.066$) and agrin (AGRN, $p = 0.062$), despite its low statistical power. These components influence proliferation and cell survival directly via regulation of FAK-, PI3K/AKT- and MAPK/ERK- pathways^{35,36}.

Other growth factors, such as HDGF and MYDGF underscore the possibility of PI3K/AKT- and ERK-dependent activation of survival signals^{37–39}. Follistatin (FST) is reported to modulate TGF- β /Activin axis and can, hence, be responsible for the mismatch between differentiation and expansion⁴⁰. Engagement of the microenvironment is otherwise at least indicated by the presence of CSF1, S100A7 and the complement factor C3 in the Top 20 list. Despite its possible interaction with local macrophages and NF- κ B or ERK-dependent signalling, it does not fully express patterns typical for primary inflammatory adipopathy^{41–43}. At the same time, we observed a nominally significant downregulation of the secreted extracellular superoxide dismutase (SOD3) within the MSL secretome. Together with the upregulated calreticulin (CALR), this indicates possible oxidative stress or stress within the endoplasmic reticulum. Taking into account that mitochondrial alterations were previously described in MSL patients, a redox-sensitive mechanism behind pro-proliferative modulation appears plausible^{13,44,45}. In summary, our pilot results are compatible with a hypothetical MSL model, where cell-type-specific changes induce local remodeling of the microenvironment and ECM. In contrast to the similar entities described above, which have partially identifiable drivers, MSL propagation may occur through a pro-proliferative signaling without a clearly distinguishable master-driver. Similar concepts were reported for the stem cell niches within the central nervous system, but it does not suggest a direct comparability^{46,47}.

Another relevant remark is the association with alcoholism. In the literature, MSL is often associated with chronic alcohol abuse with corresponding co-morbidities and medical histories in selected case-reports^{14,48,49}. In addition to previous reports about mitochondrial involvement in MSL, alcohol may also facilitate mitochondrial stress and metabolic imbalance both mechanisms that are topic of a discussion in MSL^{13,49,50}. Moreover, abusive alcohol consumption is reportedly associated with ECM-remodeling in the liver, and ECM-changes can influence systemic or local adipocyte progenitors^{51–54}. Despite not being assessed directly in this study, its role should be examined in future studies.

Consistent with the hypothesis of ECM-remodeling, our results based around MMPs (e.g., MMP1 in qPCR) are biologically plausible given their involvement in and partial control of ECM dynamics, cell migration and differentiation niches⁵⁵. Such findings offer a solid basis for future functional readouts or inhibition experiments of, e.g., AKT/mTOR/ERK activation^{25,26}.

Mitochondrial alterations have been described in familial subgroups, however, they are not universal¹³. At the same time, there is evidence for genomic patterns (e.g., CAPSL-regulation in MSL and adipogenesis) as well as for signal activation towards the CK2/AKT/ERK-axis^{25,56}. Therefore, MSL may be viewed as a syndromal rather than a single disease entity, especially from a clinical point of view and its heterogeneous etiology^{5,7}.

Our study has several limitations. A small sample size is inherent to such an orphan disease as MSL and limits statistical power. Thus, no individual protein or gene passed an FDR of 0.05 after correcting for multiple hypothesis testing. Our molecular findings are, therefore, exploratory and hypothesis-generating. Additionally, there is still a risk of confounding due to clinical similarity to other lipomatous entities. For example, Dercum's disease remains a possible source of diagnostic uncertainty. On the other hand, clinical diagnosis was carried out by one senior physician (L.P.) in order to reduce interobserver variability. Another relevant limitation is the use of only one functional readout for metabolic reduction activity with one wt-hASCs cell-line, so that future studies should include orthogonal proliferation assays such as EdU/BrdU incorporation. Finally, only GAPDH was utilized as a reference gene given the standard protocols of our facility. According to the MIQE-guidelines, such constellations require a validation of its expression stability, which was not performed in our study⁵⁷. Additionally, several authors report alternative reference genes alongside GAPDH that can be utilized in qPCR of human ASCs – e.g., eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)^{58,59}. Notwithstanding these limitations, our study still represents the first and largest intraindividual secretome analysis in MSL performed to date. Larger multicenter studies will be required to validate our exploratory results, which suggest a primarily cell-intrinsic dysregulation of adipogenic stem cells in multiple symmetric lipomatosis. Our functional, proteomic and transcriptional readouts provide new hypotheses regarding activated proliferation-associated signaling and extracellular matrix alterations.

Patients and methods

Ethics and study cohort

The study was approved by the Independent Ethics Committee of the University of Regensburg (No.: 24-3802-101, Approval Date: 26.06.2024) and was conducted in full accordance with the Somerset (South Africa, 1996) amendment of the Declaration of Helsinki (1964). Affected and reference tissue was obtained from 15 independent, pseudoanonymized human donors ($n = 15$) during symptomatic treatment. The patients treated were 9 women and 6 men. The distribution of Madelung types according to Donhauser is: I: 5, II: 7, III: 3^{4,5}. Reference tissue was obtained from the forearm and in one case from the lower leg (not depicted in Fig. 1). All patients were informed verbally and in writing about the nature and scope of the study. Informed consent was obtained prior to the procedure.

Surgery & hASCs isolation

During therapeutic liposuction or lipectomy, subcutaneous fatty tissue was excised from the affected and the reference sample from an unaffected region (Fig. 1). Surgery was performed under local anesthesia using Xylonest[™] (Prilocaine). The samples were placed in a sterile tube and immediately transferred to the laboratory at room temperature in under 10 min. The isolation of hASC was carried out as previously described^{26,60}. Samples were mechanically processed and then digested enzymatically using collagenase (Clostridium histolyticum Sigma Blend Type L (Clostridopeptidase A) in PBS (Sigma Aldrich). Digestion was performed in constant motion at 37 °C for 1 h. Digested tissue was forced through a 100- μ m filter (Merck Millipore, Billerica, MA) and centrifuged at 500 $\times$ g for 5 min. The lipid rich layer was discarded, and the cell pellet washed using unsupplemented medium (α -MEM, Sigma Aldrich, St. Louis, MO, USA). Then centrifugation was repeated. The pellet was harvested and incubated in a T175 cell culture flask. After 24 h, possible contamination was visually ruled out and 10% fetal calf serum (FCS) as well as 100U/ml Penicillin/Streptomycin (Gibco) was added to the medium during the medium change. Further medium changes were performed every 2–3 days. At a confluence density of 70–80% cells were passaged as previously described⁶⁰. 5.000 cells/cm² were seeded in triplicates each for RNA and secretome isolation. They were then incubated in unsupplemented medium (α -MEM, Sigma Aldrich) for exactly 24 h.

Metabolic activity and adipogenesis

To determine the effect of the MSL secretome on the metabolic reduction activity of wt-hASCs, a resazurin functional assay was conducted. Wild type (wt) hASCs in passage 2 were seeded in quadruplicates into 96-well plates (800 cells/well), then allowed to grow, washed twice with PBS. Thereafter they were incubated for 24 h with supernatant of cells isolated from affected and unaffected tissue of MSL patients, respectively, which had been collected from the same patient (Type 1 $n = 3$ pairs, Type 2 $n = 3$ pairs, Type 3 $n = 1$ pair; 450 μ l of supernatant and 50 μ l 10%FCS). Unconditioned α -MEM was used as descriptive control after 24 h. For the protocol of supernatant harvesting, see below. Supernatant was removed and hASCs were incubated in 100 μ l of a 0.07 mM resazurin (Sigma Aldrich) solution in PBS per well (Sigma Aldrich 0.07 mM in PBS) for 2 h. Thereafter, resorufin fluorescence was measured at an excitation and emission wavelength of 560 and 590 nm, respectively (VarioScan, Thermo Scientific, Waltham, MA, Software: SkanIt Software 2.4.5 RE).

For adipogenesis induction, MSL-hASCs were seeded in 6-well plates. They were then treated with adipogenic differentiation medium containing 0.5 mM Isobutylmethylxanthine (IBMX), 200 μ M indomethacin, 1 μ M dexamethasone (Dex), and 10 μ M insulin⁶¹. Oil Red staining was used to detect and visualize the triglycerides formed. Cells were fixed for 10 min with 10% formaldehyde solution (Sigma Aldrich), then washed with PBS and stained for 20 min with 5 mM Oil Red O staining solution. Cell count and morphology were determined and imaged on a Nikon Eclipse TS100).

Secretome extraction and analysis

Samples collected from five patients were included in the present secretome analysis (only samples surviving internal quality control, Supplementary Fig. 1). Cells were cultured up to the 6th passage and processed as previously described^{15,60}. Cells were seeded in 6-well plates at a density of $4.8 \cdot 10^4$ cells/well and allowed to attach for 24 h. After seven days, cells were washed twice with PBS prior to incubation in unsupplemented medium (α -MEM, Sigma Aldrich) for exactly 24 h. The secretome was then harvested and stored at -80 °C until analysis. All samples were prepared in technical triplicates and were pooled for analysis (technical pooling within-donors)¹⁵. Secretome analysis was performed using tandem mass spectrometry (TripleTOF 5600 + TM Q-TOF/AB Sciex, Marlborough, MA, USA). Protein intensities were transformed as $\log_2(\text{Intensity} + 1)$. $\log_2$ fold change ($\log_2\text{FC}$) was calculated as the difference between the mean $\log$ -transformed intensities in affected and unaffected samples. The affected/unaffected fold-change ratio was calculated as $\text{FC} = 2^{\log_2\text{FC}}$. For individual protein descriptions and Supplementary Tables 2 and 3, fold-change magnitude was calculated as $2^{|\log_2\text{FC}|}$, with the direction of change indicated by the sign of $\log_2\text{FC}$. For the descriptive classification of all identified proteins in Fig. 4A, FC thresholds of > 1.5 and < 0.5 were applied. Proteins with FC values between 0.5 and 1.5 were classified as falling within the selected interval. In accordance with the UniProt CC annotation, the proteins were assigned to three classes. Secreted; Membrane-associated, defined by the presence of keywords “Cell membrane”, “Cell surface”, “membrane protein”, “Single-pass”, “Multi-pass”, “Lipid-anchor”, or “GPI-anchor”; and Intracellular, defined by the presence of keywords “Cytoplasm”, “Nucleus”, “Mitochondria”, “Mitochondrion”, “Endoplasmic reticulum”, “Golgi”, “Lysosome”, or “Cytosol”. Missing information was curated internally and excluded if no details were provided. Data are available via ProteomeXchange with identifier PXD081657.

RNA extraction and polymerase chain reaction (qPCR)

The expression of candidate genes identified through secretome analysis or by the review of the literature was validated using real-time reverse transcriptase-polymerase chain reaction (rt-qPCR). As in previous experiments the DyNAmo Color-Flash SYBR Green qPCR Kit (Biozym, Hamburg, Germany) and the Eco Real time PCR System (Illumina, Inc., San Diego, CA) were used following the manufacturer's recommendations¹⁷. Primer design and synthesis was conducted using Eurofins Genomics GmbH tools. Exact primer sequences used can be viewed in the Supplementary Table 5. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, whereas *hGAPDH* served as the housekeeping gene. Ct values > 40 were excluded. Additionally, $\log_2 FC$ was defined as $-\Delta\Delta Ct$. The reported affected/unaffected fold change was calculated as $2^{(\text{mean } \log_2 FC)}$. This was corresponding to the geometric mean of the expression ratios across the included patients. Fold-change magnitude was calculated as $2^{|\text{mean } \log_2 FC|}$. Only samples of the first six passages were used. According to the available RNA material, five or ten patients were examined. A total number of ten for *hGAPDH*, *hIL6*, *hHSPB1*, *hHDGF*, *hCLTA*, *hS100A7*, and five for *hCFB*, *hCLCA*, *hLAMA4*, *hMMP1*, *hPAPPA*, *hPCOLCE2*, *hPPAR γ* , and *hPTEN*. Gene expression was analyzed in triplicates for RNA isolated from cells of affected and unaffected tissues of MSL patients. For triplicates with one missing Ct-value, imputation was performed using the mean of the remaining values. Triplicates with more than one missing value were excluded from analysis. ΔCt values were calculated for all target genes, followed by the computation of $\Delta\Delta Ct$ and $\log_2$ fold changes ($-\Delta\Delta Ct$). Statistical significance was assessed uniformly by the two-sided exact Wilcoxon signed-rank test on $\Delta\Delta Ct$ values against zero for all genes. p-values were adjusted for multiple testing using the Benjamini-Hochberg procedure.

Secretome statistical analysis

All analyses were performed using the statistical software R (Versions 4.3.0 and 4.5.1, <https://www.R-project.org/>). For exploratory proteomic comparisons, paired t-tests were applied to $\log_2(\text{Intensity} + 1)$ values from five matched patient pairs. Given the exploratory nature of this study, an uncorrected p-value threshold of $p < 0.05$ were considered nominally significant, and were included in the paired plots (Fig. 5B). Proteins were highlighted in the volcano plot (Fig. 5A) if they met both - nominal significance criterion and the fold-change threshold of $|\log_2 FC| > \log_2(1.5)$. Benjamini-Hochberg correction was applied but no protein remained significant after correcting for multiple hypothesis testing using an FDR of < 0.05 , consistent with the limited statistical power inherent to $n = 5$. A Venn diagram was generated to illustrate quantity and distribution of mass spectrometry data⁶². Functional assay was performed with technical quadruplicates, whereas proteomic analysis and rt-qPCR were carried out using technical triplicates. Resazurin signals were tested using Exact two-sided Mann-Whitney-U-Test between every sample pair. Benjamini-Hochberg correction followed for the above mentioned 7 comparisons of resazurin signals. Artificial Intelligence tools (ChatGPT Sol 5.6, Astra 6 and Claude Opus 5.5) have been utilized to assist with correcting the human written text and while debugging the R script. All experimental results and outputs as well as statistical analyses were only generated by the authors, who maintain whole responsibility for the data integrity and final content.

Data availability

The processed proteomic dataset reporting (fold changes, nominal and adjusted p-values for all identified secreted proteins identified) is provided in Supplementary Table S4. Raw rt-qPCR datasets are not publicly deposited: the study was conducted under an ethics approval and a patient consent framework for molecular-genetic characterization that permits data sharing only in pseudonymized form and restricts transfer to third countries. These data and the R code are available from the corresponding author upon reasonable request, subject to the applicable consent and data-protection provisions. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via PRIDE partner repository with the dataset identifier PXD081657 (<https://ebi.ac.uk/pride/>).

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Declarations

Competing interests

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