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White adipose tissue and adipose-derived stem cells (ADSCs) for the treatment of carpometacarpal osteoarthritis of the thumb

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Content

1. Abbreviations

ASDCs	Adipose-derived stem cells
ADAMTs	A disintegrin and metalloproteinase with thrombospondin motifs
BMP	Bone marrow-derived protein
CCM	Conditioned culture medium
CM	Culture medium
CELA	Cell-enriched lipoaspirate
CMC-1 OA	Osteoarthritis of the 1st metacarpal joint
DASH	Disabilities of the Arm, Shoulder and Hand Questionnaire
HSCs	Hematopoietic stem cells
JSN	Joint space narrowing
IL	Interleukin
iPS cells	Induced pluripotent stem cells
MHQ	Michigan Hand Questionnaire
MMP	Matrix-Metalloprotease
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal stem cells
OA	Osteoarthritis
ROM	Range of motion
SVF	Stromal vascular fraction
TGF-ß	Tissue growth factor beta
TNF-α	Tumor necrosis factor alpha
VAS	Visual analogue scale

2. Introduction

2.1. Osteoarthritis

Osteoarthritis (OA) is not only one of the most common chronic diseases in the elderly population, but also the most common joint disorder in the USA. The lifetime prevalence of OA in Germany is 27.1% for women and 17.9% for men [23]. Before the age of 45, this degenerative joint disease affects men more frequently, then women. After the age of 50, women are more often affected. Osteoarthritis frequently affects the hands, especially the fingers, lower back and neck. Furthermore, it effects weight-bearing joints, such as the knees and hips [21]. The number of symptomatic patients is steadily increasing due to the aging population and the constant rise of obesity worldwide. OA causes pain, stiffness and subsequently weakness, which ultimately result in loss of function of the affected joints. Thus far, OA is a massive and steadily growing factor regarding healthcare costs.

Currently, no known treatment for OA is available and the damage caused by OA is irreversible. OA is an irreversible disease with no curative treatment options. During the course of the disease, loss of the articular cartilage surface occurs; which plays a fundamental role. This is followed by joint space narrowing (JSN) and, subsequently, pathologic changes within the underlying bone occurs. Patients suffer from pain, swelling, joint tenderness and consequently loss of mobility. Ultimately, surgery including salvage procedures or even total joint replacements might be required; to restore some of the joint function [24]. There are multiple causes in the development and progression of OA. These risk factors differ depending on; the affected joint, gender, genetic background, age, and occupation. Most frequently, multiple factors combined, lead to the degenerative processes; which subsequently cause OA [16].

OA is a chronic disease that develops gradually over many years. It is diagnosed based on clinical and radiological examinations. Typical radiological features are JSN, osteophytes and bone cysts in more severe cases. Almost half of the patients with OA, show only radiological features; without any clinical symptoms [21], [25]. Clinically OA can be diagnosed by typical symptoms including; pain, stiffness, reduced movement, swelling and crepitus. Joint pain is the limiting symptom [25]. However, the origin of the pain is yet to be fully understood. Be-cause the articular cartilage is not innervated; it is difficult to determine if the pain is directly involved. Biopsychosocial factors, could play a role [26]. Other structures of the joint; the subchondral bone, periosteum, synovium and joint capsule are all innervated. Therefore, they play a role in the development of pain during OA [25].

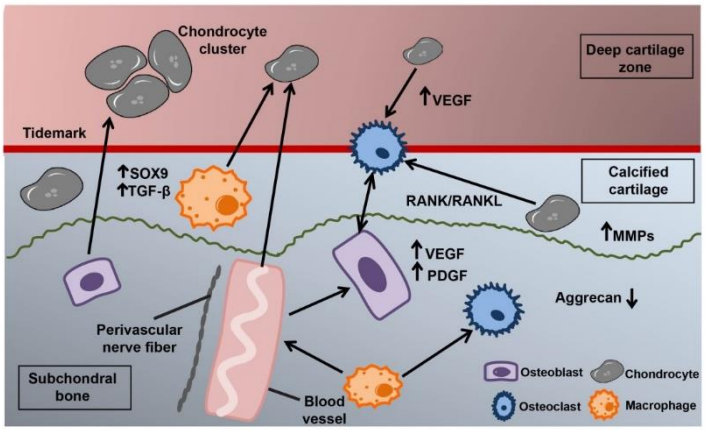

Figure 1: The molecular cross-talk is altered in osteoarthritic joints. The changed interaction in osteoarthritic joints depends on different cytokines and growth factors. These different factors lead to a changed bone and cartilage metabolism. By destruction of the tidemark, the subchondral tissue is prone to increased inflammatory signaling. Articular chondrocytes and the synovium affect the subchondral tissue via different factors, such as vascular endothelial growth factor (VEGF). This leads to differentiation processes an often ends in chondrocyte hypertrophy and enchondral ossification. Adapted and modified after [25], [26].

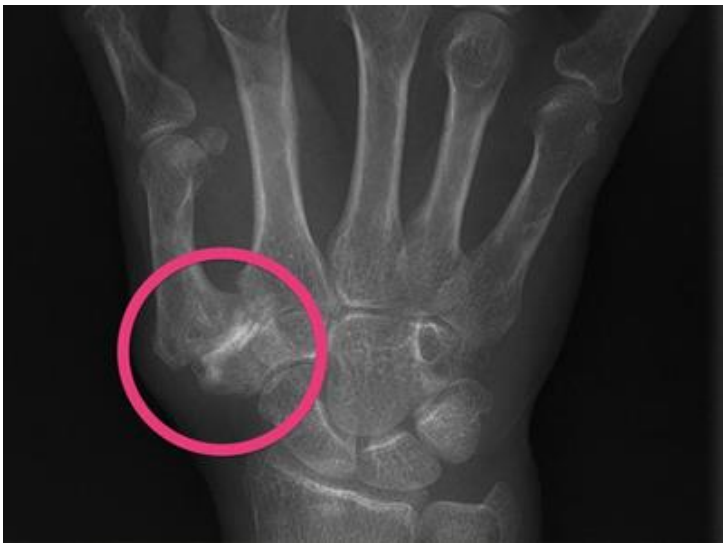

Figure 2: Severe CMC-1 joint OA. The X-ray image shows typical signs of osteoarthritis such as joint narrowing sclerosis, cyst formation and the development of osteophytes (red circle). Modified Eaton and Littler staging system after [35].

To date, there is no curative treatment of OA; mainly because the hyaline cartilage cannot be restored. Therefore, symptomatic treatment is the mainstay of therapy. This includes temporary immobilization, NSAR therapy, occupational therapy, Cryotherapy, direct intraarticular injections, corticoids or buffer substances such as hyaluronic acid, or ultimately, surgery. The main problem with the treatment of OA is, that patients often present late; when the disease has progressed, and significant amounts of cartilage has been destroyed. Early modulation to the progression of the disease, maybe beneficial in order to maintain the hyaline cartilage; and thus increasing the lifespan of the affected joint. One possibility to detect early-stage OA might be the discovery of valid prognostic molecular biomarkers. Early detection of the disease would allow for early treatment. Leading to either a deceleration, or even a halt in the progression of OA. It is therefore, pivotal to gain a deeper understanding of the biology of the entire joint in general and the osteoarthritic cartilage in particular, in response to intrinsic and extrinsic stimuli [27].

2.1.1. Carpometacarpal osteoarthritis of the thumb- the price of an opposable thumb

The CMC-1 joint is a unique joint in the human body representing the only saddle joint. It is formed in a biconcave-convex shape. The joint consists of the trapezium bone of the wrist and the first metacarpal bone of the thumb. Its stabilization is ensured by a specialized ligamentous

apparatus, consisting of 11 different ligaments [32]. The unique shape of the articulating surfaces allows for six different types of movements. These include flexion-extension, abduction-adduction and rotational movements, which when combined, enable the thumb to perform the opposition maneuver, i.e., opposing the thumb's pulp to that of the other fingers [31], [32]. Evolutionarily, the opposition of the thumb distinguished humans from primates. Enabling them to grasp objects, to use tools and to perform many other tasks with their hands; ultimately resulting in an increase of brain volume. Unfortunately, there is a high price for this increased range of motion of the human thumb, namely early onset osteoarthritis. Due to the high degree of mobility along with an extensive utilization and relative overuse of the CMC-1 joint during a lifecycle, it is highly predisposed to age-related degenerative changes. High mobility of the thumb combined with the age-related ligament instability, traumatic ruptures, joint inflammation and hormonal changes collectively contribute to the development of CMC-1 OA [31]–[34].

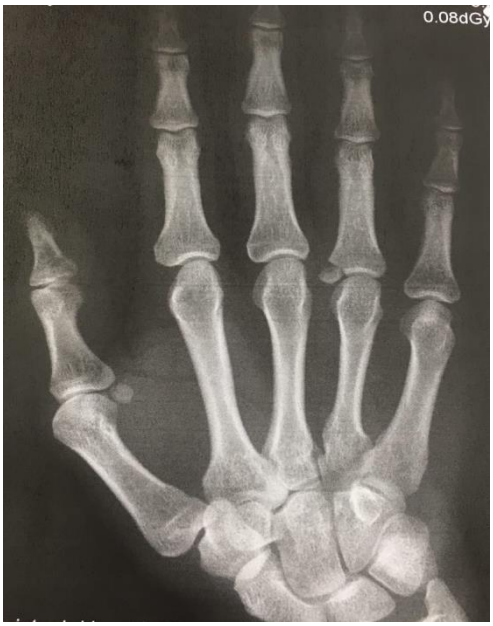

Figure 3: CMC-1 joint. Classification and thereby the diagnosis of CMC-1 OA can be achieved using different radiographic views of the thumb and/or arthroscopy [31] Eaton et al. described 1984 radiographic stages I to IV of CMC-1OA [35].

Arthritis of the distal interphalangeal and CMC-1 OA are the most common degenerative joint diseases of the hand [28]. The largest longitudinal cohort study was conducted in Finland between 1978 and 1980, when 3595 hand radiographs were analyzed from patients aged 30 years and older. The age-adjusted prevalence of CMC-1 OA with a Kellgran and Lawrence stage 2, 3 or 4 was 7% for men and 15% for women [29]. A smaller British study assessed 143 post-menopausal women, aged 45-70 years. It identified a 25% prevalence of radiographically diagnosed CMC-1 OA with an Eaten and Littler stage 2,3 or 4 [30]. Both studies confirmed an age-related occurrence of CMC-1 OA, with its peak in the fifth to sixth decade of life. Furthermore, women were shown to develop Thumb-OA more often, due to hormonal predisposition [29], [30]. Like with almost all other types of OA, patients with CMC-1 OA often present with late-stage form of OA; as pain symptoms often occur in later stages of the disease, when synovitis and inflammation appear [31]. Furthermore, the diagnosis is often delayed because patients try to adjust their hand activity to the circumstances. By using splints and ultimately limiting their hand movement. Like is the case for all other forms of osteoarthritis, there is no curative treatment option for CMC-1 OA available. Hence, treatment is symptomatic and often only temporarily efficient [28], [31].

2.1.2. Diagnosis and treatment options

Classically, the diagnosis can be made clinically. The clinical history along with the clinical examination usually result in a high degree of suspicion. Classification and diagnosis of CMC-1 OA can be achieved using different radiographic views and arthroscopy of the thumb. With progression of the disease patients experience more severe symptoms, including swelling, subluxation and tenderness of the joint, which can be clinically examined [37], [38]. A CT scan is not necessary for diagnosis but reveals details about the localization and extent of the degeneration, as well as the existence of loose intra-articular bodies. MRI scans help to quantify the cartilage damage. They also unveil the amount of inflammatory process resulting from the arthritic changes. In recent years, arthroscopy has become a tool to treat and classify CMC-I-arthritis. This technique has not yet been established as a standard procedure [31]. In 1984, Eaton et al. described four radiographic stages of CMC-1OA (table 1) [32].

Table 1. Stages of CMC1- osteoarthritis

Stage I	Possible widening of the joint space
Stage II	Narrowing of the joint space, osteophytes, loose joint bodies (<2mm)
Stage III	Severe joint space narrowing, osteophytes, loose joint bodies (>2mm)
Stage IV	CMC-1 osteoarthritis combined with scaphotrapezial osteoarthritis

Modified Eaton and Littler staging system after [35].

More importantly however, the radiological stages do not correlate with the clinical severity of the disease [21], [25]. Badia et al. described arthroscopic stages I to III for CMC-1 OA, based on the intra-articular findings. Obviously, arthroscopic evaluation might detect earlier degenerative changes than found in x-rays and may thus change treatment modalities [36].

Taken together, the diagnosis should not only be made by radiographical examination, but should also include the patient's history, clinical assessment, and pain evaluation [31]. For the clinical examination, it is important to measure the range of motion (ROM), as well as the pinch and strength grip. To ensure an objective investigation, measurement devices such as the dynamometer and pinch gauges should be used [31]. In order to evaluate the pain and the associated changes in quality-of-life; there are several questionnaires available. The most frequently used are the Disabilities of the Arm and Shoulder (DASH) and Michigan Hand Questionnaire (MHQ) [31].

The treatment options for patients suffering from CMC-1 OA are very limited. Initially, patients with pain secondary to CMC-1 OA should always be managed conservatively. Conservative treatment options include immobilization with a splint, pain relief with non-steroidal anti-inflammatory drugs (NSAIDs) and physiotherapy [20], [28], [37]. As the disease progresses, intra-articular injection of corticosteroids and hyaluronic acid, combined with splinting (3 weeks), has been shown to give 80% of the treated patients temporary pain relief. However, when the steroids subside, a higher degree of pain may result due to overuse during the pain-free period [39]– [41]. Multiple cortisone injections may be done with caution, as this may induce weakening of the stabilizing joint ligaments [39]. In addition, their effect tapers off with repetitive use. Hyaluronic acid injections may also be considered, yet studies are scarce, and the cost benefit ratio has to be considered. If used, several sequential injections are warranted, yet the exact amount has yet to be determined [39].

When conservative treatment options have failed, surgical treatment/intervention is recommended. Surgical interventions are primarily used in patients with progressed CMC-1 OA (Eaton II-IV). They include osteotomy, trapezium bone excision, ligament reconstruction with or without tendon interposition and various prosthetic implants with or without the excision of the trapezium bone [20], [31].

Overall, conservative treatment options only temporally hinder the development of symptoms, but in the long term, surgical intervention is usually necessary. Most of the techniques irreversibly destroy the natural anatomy of the wrist. They are associated with a significant degree of postoperative pain and usually require an immobilization of the thumb for 4-8 weeks. Therefore, there is a need for new treatment options that help avoid surgical intervention. In an attempt to do so; regenerative therapies like, autologous fat injection was added to the treatment modalities of CMC-I OA.

2.1.3. Autologous fat injection as a novel treatment for CMC-1 OA

Autologous fat injection has recently been investigated as an alternative treatment for CMC-1 OA. It is hoped to be a less invasive alternative to surgery, with the regenerative potential. In a prospective, non-randomized pilot study, we previously compared the clinical outcome of patients, who received a radiologically guided injection of 1-1.5 ml autologous lipoaspirate as a treatment for CMC-1 OA to a classical cortisone injection [42]. In this study, 24 patients aged 47-75 years with early stage OA (Eaton I-II) received a one-time injection of autologous adipose tissue, which was harvested from the abdomen with the Lipivage 200-5 liposuction system. The harvested fat tissue was homogenized using two Luer-Lock Syringes, but not centrifuged, before injection into the patient's CMC-1 joint. After the treatment, the thumb was immobilized with a splint for one week. Patients in the control group received a one-time injection of 10 mg triamcinolon without subsequent immobilization with a splint. The clinical outcome of patients in both groups was measured by evaluating the pinch and grip strength, the pain (VAS), as well as the DASH and MHQ questionnaires, preoperatively as well as two, six and twelve weeks postoperatively. Patients in both groups showed a significant reduction of stress pain after 2 weeks. Neither method had a significant effect on the hand force. The DASH and

MHQ showed an initial improvement of life quality among patients in the control group, which lasted for 6 weeks only, whereas patients in the fat injection group experienced a continuous and prolonged improvement [42]. Bohr et al. used an even more innovative approach for the treatment of CMC-1 OA called cell-enriched lipoaspirate arthroplasty (CE-LA) [4]. For the preparation of a cell-enriched lipoaspirate the SVF of adipose tissue, which is a rich source of pre-adipocytes, endothelial cells, immune cells and MSCs, later called ADSCs is added in different ratios to the lipoaspirate before transplantation. This method is being used to improve autologous fat transplantations through the potential regenerative properties of ADSCs [6]. In the case-report a 62-year-old male with CMC-1 OA at stage II after Eaton and Littler received a one-time CELA. For the CELA, abdominal liposuction with a standard liposuction technique was performed to obtain autologous lipoaspirate. To increase the amount of the SVF the obtained lipoaspirate was centrifuged. Subsequently, 1 ml of the enriched lipoaspirate was injected in the CMC-1 joint, followed by a 10-day immobilization with a splint. According to the authors, this procedure led to freedom from pain after a period of 5 weeks. For outcome analysis the DASH score was evaluated pre- and post-CELA, showing a score of 46/100 pre-CELA compared with a score of 22/100 post-CELA, which indicates an improvement of the patient's life quality after CELA treatment [4].

Both treatment options; the injection of lipoaspirate into the CMC-1 joint and the CELA - may result in pain reduction and improvement of the life quality. However, both studies have limitations, including low patient number and the fact that no objective examination was per-formed to evaluate the OA stage of the patients pre- and post-treatment. The comparison of X-Ray imaging or magnetic resonance imaging (MRI) before and after the treatment, could have shown objective evidence of potential changes in the joint after the different treatments. A more accurate but also more invasive examination would be the analysis of different inflammation markers in the synovial fluid, obtained through arthroscopy, before and after the treatments. It is evident that many questions about the potential effects of these treatments remain unanswered. Most importantly, we need to study, the effects of fat tissue on the osteoarthritic chondrocytes and how adipose tissue can potentially inhibit or modify CMC-1 OA.

2.2. ADSCs for treatment of osteoarthritis - experimental studies

ADSCs are under investigation in several clinical applications for; autoimmune, degenerative and inflammatory diseases, including OA. Articular cartilage destruction during OA causes considerable therapeutic challenges, due to its inability to self-repair. The utilization of ADSCs in the field of articular cartilage regeneration is promised to repair damaged cartilage. Because the application of ADSCs is a not yet clinically approved for any treatment in humans, most of the studies, which explore the effects of these cells on OA focus on *in vitro* and animal model experiments.

In the year 2001, Zuk et al. first described the possibility of ADSCs to differentiate into different mesenchymal cell lineages, including the chondrogenic lineage [43]. In order to differentiate chondrogenically *in vitro*, ADSCs need to be cultured under hypoxic conditions in a three-dimensional culture environment, formed into a pellet or micro-mass culture with specific

growth factors (TGFβ1, TGFβ2, TGFβ3, BMP2, BMP6, BMP7), which promote the chondrogenic differentiation [44]– [46]. Additionally, it has been shown that ADSCs are also able to form cartilaginous tissue in a mouse model [47].

Later, Goldring et al. introduced co-culturing experiments in the field of regenerative cartilage research to study the cross talk between different cells in the joint [17], [48], a model that has since been used excessively by other groups [49].
In different co-culture experiments, using osteoarthritic chondrocytes and ADSCs an increased cartilage formation was detected. This condition might be explained by differentiation of ADSCs into potent chondrocytes or chondrocytes stimulated through ADSCs [49]. Recent xenogenic co-culture studies explored the individual contributions of chondrocytes and MSCs to matrix formation of either cell types [50]. These studies suggested that chondrogenic differentiation of MSCs does not contribute to cartilage formation. The increased matrix production was shown to be the result of MSCs activating chondrocytes through their secretome [49].
At the beginning, it was believed that MSCs and ADSCs have the ability to influence surrounding cells to release bioactive factors which can then lead to tissue regeneration [49], [51]. After different experiments in this field, it is now believed that MSCs and ADSCs are capable of secreting paracrine factors themselves that enhance cell viability, proliferation, and matrix production leading to tissue regeneration [49].
Several studies aimed to mimic the OA microenvironment in joints, by inducing inflammatory processes in chondrocytes via cytokines, including IL1β and TNFα (Figure 1). Platas et al. showed that conditioned culture medium (CCM) from ADSCs enhanced the expression of collagen type II in chondrocytes treated with IL1β while decreasing MMP13 expression [11]. Lai et al. evaluated interactions between ADSCs and osteoarthritic chondrocytes through three different culture models: CCM, bi-layered culture and mixed co-culture in different ratios [10]. It could be shown that CM and bi-layered co-culture had negligible effects on cartilage formation, while a mixed co-culture of chondrocytes and ADSCs at a ratio of 1:3 enhanced cartilage matrix growth and reduced catabolic processes [10]. Furthermore, *in vivo* animal models showed a regenerative effect of ADSCs on OA cartilage. In an experimentally-induced OA mouse model, Huurne et al. showed that injected ADSCs inhibit synovial lining thickening and even prolongate joint destruction by decreasing inflammation in the joint [12]. All together, these studies provide evidence, that ADSCs have a potentially regenerative effect on OA. However, there is no evidence yet that the described regenerative effects on osteoarthritic chondrocytes also apply to the CMC-1 joint.

The pilot study from Ceteno et al. may provide first information of treatment with MSCs for CMC-1 OA [19]. Four male and two female patients (mean age 59.5 years) with symptomatic CMC-1 OA received an intraarticular injection of autologous culture expanded MSCs. The clinical outcome has been compared with the outcome of patients in the control group, who received no treatment except analgesics. The authors claim to observe positive effects in the treatment group regarding the reduction of typical osteoarthritis symptoms compared to the untreated control group [19].

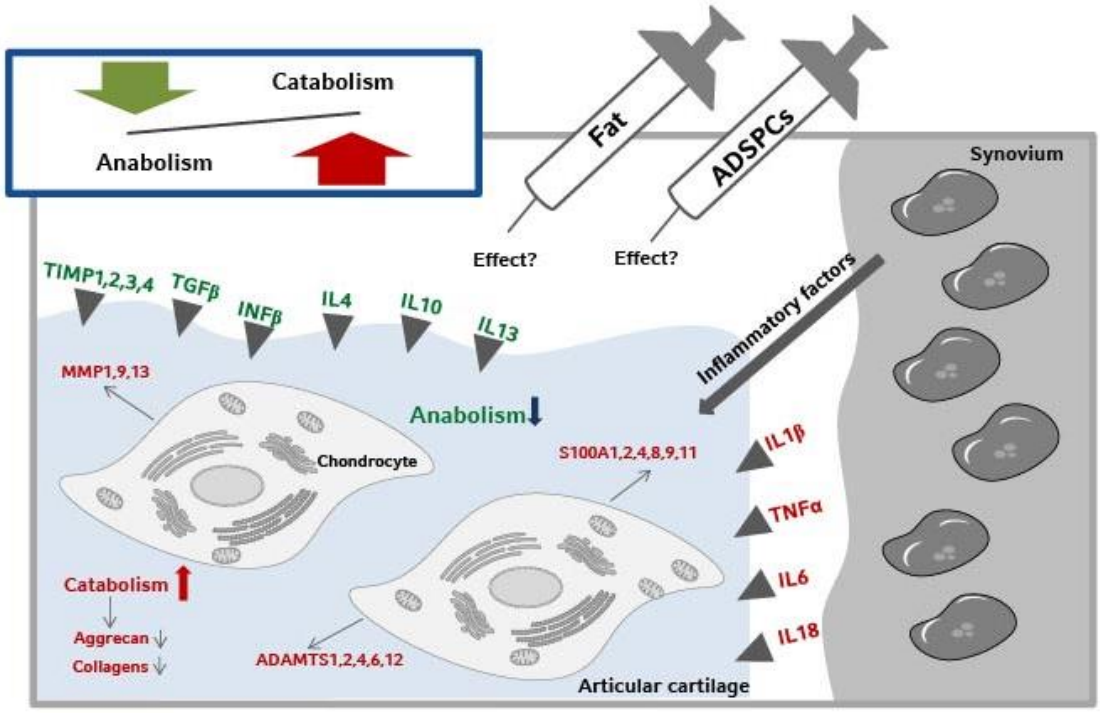

Figure 4: In the osteoarthritic joint anabolism and catabolism is misbalanced. Different cell types in the joint, including cells from the synovium, immune cells and chondrocytes secrete a variety of pro-inflammatory factors. These pro-inflammatory factors, which include for example IL1β, TNFα, IL6 and IL18, promote catabolic processes in the cartilage ECM through activation of degradative factors like for example MMPs and ADAMTs. The expression of ant- inflammatory factors such as INFβ, IL4, IL10 and IL13 is reduced. It is believed, that ADSCs can modulate the inflammatory micro milieu in osteoarthritic joints. Adapted and modified after [52], [53].

2.3. Adipose tissue

2.3.1. Composition and function

Adipose tissue is composed of adipocytes, loose connective tissue matrix and the SVF. The SVF consists of preadipocytes, capillary endothelial cells, infiltrated monocytes/macrophages, and a population of multipotent stem/progenitor cells. Adipose tissue is a richly vascularized and innervated tissue. These different components function as complex metabolic and endocrine organ that fulfills important metabolic and physiologic functions. There are two types of adipocytes: white adipocytes and brown adipocytes. Brown adipocytes, which consist of multiple small lipid droplets, that are responsible for dissipating energy for thermogenesis [7], [54]. Brown adipocytes are almost only found in newborns and rarely in adults; they will not be further discussed here. White adipocytes consist of triglycerides in a fat droplet [54], [55]. Adipose tissue acts as energy storage, regulator of endocrine and metabolic processes, as well as thermal regulator. Furthermore, adipose tissue protects inner organs from mechanical dam-age [55], [56].

As an endocrine organ, adipose tissue secretes different proteins with metabolic and endocrine functions. Leptin is an adipocyte-derived protein and acts as a metabolic signal for energy storage, thereby the leptin levels decrease with caloric restriction and weight loss [54], [57]. Additionally, leptin has several peripheral endocrine functions, including promotion of proliferation

and differentiation of hematopoietic cells, alteration of cytokine production by immune cells, stimulation of endothelial cell growth and angiogenesis, and acceleration wound healing [54], [58]. Furthermore, adipocytes and cells from the SVF secrete the cytokines TNFα and IL6. In the adipose tissue, these cytokines influence the development of obesity and diabetes [54], [59], [60]. In the periphery, these cytokines play a role in inflammatory process-es [61], [62]. Another secreted protein of adipocytes is Adiponectin, whose most important functions include anti-diabetic, anti-inflammatory, and anti-atherogenic effects [54], [63]. In addition to their function to secrete proteins with endocrine functions, adipocytes express many enzymes, which play a role in the activation and inactivation of steroid hormones [54].

2.4. Adipose-derived stem cells

2.4.1. Isolation and characterization of ADSCs

In the past, only two categories of stem cells were known. The embryonic stem cells are derived from the blastocysts and the adult stem cells are derived from postnatal tissues. The latter, include bone marrow (MSCs) and blood (hematopoietic derived stem cells, HSCs) [64], [65]. Within the first couple of cell divisions, embryonic cells are considered totipotent cells, meaning they can form any kind of cell type within the human body. At a later stage they are considered pluripotent, meaning they can differentiate into cells of the mesenchymal, endodermal and ectodermal linage. Multipotent cells such as MSCs, HSCs and ADSCs can differentiate into the different cell types within the mesodermal linage- adipogenic, osteogenic and chondrogenic [65]. Collectively, multipotent stem/progenitor cells show self-renewal capacity and the potential to multilineage differentiation [66].

Adipose tissue is an easily obtainable connective tissue, which contains abundant ADSCs. These can be isolated by enzymatic digestion of the SVF. Therefore, adipose tissue is an easily accessible source for ADSCs. This is an advantage over MSCs, which are traditionally harvested from the bone marrow. Besides the fact that ADSCs can isolated easier, the approach is safer and larger amounts of ADSCs can be harvested as compared to an MSC harvest from the bone marrow. However, there are several differences in functional and biological proper-ties of ADSCs and MSCs, including differences in their phenotype, differentiation potential, transcriptome, proteome, and immunomodulatory activity [67], [68]. Nonetheless, ADSCs fulfill all characteristics of multilineage stem/progenitor cells, including differentiation capacity, proliferation capacity and clonogenicity [66].

2.4.2. Current state of knowledge in the use in regenerative medicine

The field of regenerative medicine using ADSCs and other potent cell types such as MSCs is constantly growing. Due to their mesodermal origin, the application of multipotent stem cells seems obvious in the field of bone and cartilage repair [69]– [72]. Additionally, areas of interest are cardiovascular tissue regeneration, nerve repair, tendon regeneration and the immunomodulatory effect of ADSCs [73] – [78]. It seems that there are no limitations within the potential therapeutically applications of ADSCs. Several research groups have studied the potential of ADSCs in the field of organ tissue engineering, including liver repair, pancreas substitution and urinary tract organ reconstruction [46], [79]– [81]. Furthermore, skin repair reveals to be a big research area using ADSCs [81]. Even diseases such as multiple sclerosis, diabetes and Huntington´s disease have been studied in the field of ADSCs regenerative medicine [82]– [84].

Interestingly, studies have shown that ADSCs might also show the potential of being reprogrammed in so called “induced pluripotent stem cells” (iPS cells), which could open doors to even more advanced regenerative therapies [85].

2.5. Liposuction

Several different methods for harvesting adipose tissue are available. These are ranging from the classical fat resection to the conventional suction-assisted liposuction over to newer techniques; such as the water-jet-assisted, nutational infrasonic and ultrasound-assisted liposuction [13], [14]. Regarding the variety of different liposuction techniques, there are differences in the viability and quality in ADSCs obtained through different techniques. An example for a conventional suction-assisted liposuction method is the so-called Lipivage200-5 system. The Lipivage200-5 is a single-use, disposable system, which can be used for the extraction of adipose tissue up to 200 ml. This technique is easy and timesaving, since fat harvesting and subsequent washing steps are carried out in one step. The harvested tissue can be immediately reinjected due to the system concentrating the fat cells. It is being used primarily for lipofilling in the field of aesthetic surgery as well as for plastic and reconstructive surgery [8], [86]. The Body-Jet system is an example for a water-assisted liposuction method. Hence, this liposuction system consists of a negative-pressure pump linked to a water pump, which creates a fan-shaped water jet during liposuction [87]. This technique allows a gentle cell detachment from tissue resulting in a reduced mechanical injury. In addition, this technique is known to reduce cardiovascular side effects, since the fluid injected into the patient is much lower than during other liposuction methods [88].

3. Zielsetzung und Eigenanteil

Das erste Ziel dieser Arbeit war die Evaluation von zwei im klinischen Alltag gängigen Liposuktionsverfahren, um zu zeigen welches Verfahren sich zur Fettgewinnung besser eignet, um adipose derived stem cells (ADSCs) aus dem gewonnen Lipoaspirat zu isolieren. Die aus Fettgewebe gewonnen ADSCs sind eine leicht zugängliche und reichhaltige Quelle zur Gewinnung von multipotenten Vorläuferzellen. Sie besitzen die Fähigkeit sich in verschiedene Zelllinien zu differenzieren und weisen immunmodulatorische Eigenschaften auf. Diese Vorläuferzellen stellen eine vielversprechende Quelle für zellbasierte Therapie im Bereich der regenerativen Medizin dar. Aus unseren Versuchen geht hervor, dass beide Liposuktionsverfahren im Hinblick auf die Qualität der isolierten ADSCs geeignete Isolationsverfahren sind. Somit sind beide Liposuktionsverfahren als gleichwertige Methoden zur Fettgewinnung anzusehen

Im nächsten Schritt war es unser Ziel die Unterschiede des immunmodulatorischen Potentials von Fettgewebe und ADSCs darzustellen. In der Pathogenese vieler chronisch entzündlicher Erkrankungen wie z.B. Arthrose, Rheumatoider Arthritis und Morbus Crohn verlagert sich das Gleichgewicht von pro-und anti-entzündlichen Zytokinen zu Gunsten der proentzündlichen Faktoren. Humanes Fettgewebe, und viel mehr die darin enthaltenen Fettgewebsstamm-zellen, zeigten im Rahmen experimenteller Studien immunmodulatorisches Potential. In unseren Versuchen wurden Fettgewebe sowie ADSCs erfolgreich mit TNFα behandelt um eine entzündliche Reaktion zu triggern. Hierbei konnten wir eine starke inflammatorische Antwort von Fettgewebe und ADSCs nach Behandlung mit TNFα nachweisen und das veränderte Genexpressionsprofil sowie die veränderten Pathways darstellen. Für klinische Zwecke wurden ADSCs bereits in experimentellen Studien als antiinflammatorische „Bioreaktoren“ für die Therapie

von chronisch entzündlichen Erkrankungen wie beispielsweise Arthrose, Morbus Crohn, Diabetes mellitus und chronische Wundheilungsstörung erprobt. Die Ergebnisse der zweiten Publikation sollen zum Verständnis der immunmodulatorischen Wirkung von Fettgewebe sowie ADSCs dienen und den Weg für zukünftige anti-inflammatorische Therapien weisen.

Beschreibung des Eigenanteils Publikation 1: Ausführliche Literaturrecherche, Mitgestaltung des Versuchsaufbaus, Aquirierung von Fettgewebsproben und Isolierung von ADSCs, Durchführung aller Experimente zur Charakterisierung der ADSCs, Gestaltung der Abbildungen, Verfassen des Manuskripts, Bearbeitung der Revision.

Beschreibung des Eigenanteils Publikation 2: Ausführliche Literaturrecherche, Mitgestaltung des Versuchsaufbaus, Durchführung aller Experimente, gemeinsame Analyse und Interpretation der Versuchsergebnisse mit den Ko-Autoren der Studie, Verfassung des Manuskripts, Bearbeitung der Revision.

4. Abstract

Background: CMC-1 OA is a common degenerative disease of the hand. It causes symptoms such as pain, stiffness and weakness of the CMC-1 joint, which eventually results in the loss of function [1], [2]. Current conservative CMC1 OA treatments are symptomatic and short-lasting. Often surgical therapy, mostly including the removal of the trapezium bone, is the therapy of choice for late stage CMC-I arthritis [2], [3].
In order to overcome this limitation in treatment options, a regenerative approach with autologous fat injections is discussed by different authors [4] – [6]. Adipose tissue is a loose connective tissue consisting of adipocytes, and the stromal vascular fraction (SVF). The SVF is mainly composed of different immune cells, fibroblasts, vascular endothelial cells and adipose-derived stem cells (ADSCs) [7]. The therapeutical benefit of autologous fat injections might be provided by ADSCs, as these cells show impressive capabilities in the area of regenerative medicine and tissue engineering [8], [9]. Interestingly, ADSCs were shown to influence the degenerative inflammatory microenvironment in osteoarthritic joints through their paracrine actions [10] – [12].

Objective: Until now there are only limited analysis of characteristics and functional properties of ADSCs that yield upon different liposuction methods. Regarding the variety of different liposuction techniques, there may be differences in the viability and quality in ADSCs obtained through different techniques. These are ranging from the classical resection, the conventional suction-assisted liposuction over to newer techniques, such as the water-jet-assisted, nutational infrasonic and ultrasound-assisted liposuction [13], [14]. The Lipivage200-5 system can serve as a conventional suction-assisted liposuction method. Whereas the Body-Jet system is a water-assisted liposuction method. In order to the extended field of different fat harvesting methods their clinical properties, such as operative time, harvesting volume, blood loss, skin reactions and usage costs have been investigated and compared [15]. However, there is a serious research gap with regard to the comparison of different liposuction techniques in terms of preservation of the cellular contents. Thus, we compared both methods by examining the clonogenicity, the

proliferation capacity and the metabolic activity. Furthermore, we examined the differentiation potential of ADSCs to differentiate into the adipogenic, osteogenic and chondrogenic lineage. Furthermore, we aimed to reveal paracrine differences of autologous fat and ADSCs that might modulate the biological response of osteoarthritic chondrocytes with a focus of donor variability.

Methods: The proliferation potential of ADSCs, was examined by different experiments including cumulative population doublings (cumPD), population doubling time (PDT), colony forming units (CFU) and cell metabolism assays. In order to show the multipotency, ADSCs were differentiated into adipogenic, osteogenic and chondrogenic lineages.
Furthermore, fat tissue- and ADSC-conditioned medium were produced by culturing both groups for 48 hours with TNFα (tumor necrosis factor alpha) to trigger the inflammatory response in vitro. Normal cell culture medium served as a control. To validate an effective induction of inflammation, stimulated and unstimulated samples were analyzed for upregulated inflammatory markers by Next-Generation-Sequencing.

Results: Our study show that ADSCs isolated from adipose tissue after liposuction with different liposuction systems show similar cell characteristics and functional properties. Technical differences such as different cannula sizes used for the different systems might contribute to the slightly different cell characteristics. Still both methods should be equally considered for clinical use. The second part of our study shows that fat tissue and ADSCS treated with TNFα responded very heterogeneously to TNFα treatment. Our analysis reveals that ADSCs are potentially more reliable and predictable when used therapeutically. Furthermore, our study shows potential changes in biological processes, such as immune system response, inflammatory response, and cell activation in treated ADSCs.

5. Zusammenfassung

Einleitung: Rhizarthrose ist eine vorwiegend im höheren Alter vorkommende Erkrankung, die bis zu 80% der älteren Bevölkerung betrifft [19]. Vor allem Menschen die beruflich viel Handarbeit leisten und/oder Sportarten ausüben, haben ein höheres Risiko an Rhizarthrose zu erkranken. Bis heute gibt es keine Heilungsmöglichkeiten, sondern lediglich krankheitsverzögernde und schmerzlindernde Therapiemaßnahmen im konservativen und chirurgischen Bereich. Im Rahmen der konservativen Behandlung werden Physiotherapie, Ruhigstellen des Gelenks mittels einer Schiene, Analgetika und Injektionen (Kortison- und Hyaloronsäure) angewandt [20]. Nach Ausschöpfung der konservativen Therapieoptionen, besteht die Indikation zu operativen Therapiemaßnahmen. Zu den operativen Maßnahmen zählen die isolierte Trapezektomie, die Suspensionsarthroplastik, die Suspensionsinterpositionsarthroplastik sowie die Arthrodese [20].
Oftmals bieten diese Therapieoptionen den betroffenen Patienten keine zufriedenstellende Besserung ihrer Beschwerden. Somit besteht zunehmend die Notwendigkeit neuer Therapiemöglichkeiten, vor allem in Hinblick auf die Regeneration des Knorpelgewebes. In den letzten Jahren wurde die Anwendung von mesenchymalen Stammzellen (MSCs) zur Therapie von Osteoarthrose häufig diskutiert. Aus dem menschlichen Fettgewebe lassen sich einfach und in gro-

ßer Zahl die sogenannten adipose-derived stem cells (ADSCs), isolieren. In verschiedenen Studien konnte bereits gezeigt werden, dass ADSCs, sowohl immunsuppressive, als auch anti-inflammatorische Effekte aufweisen können [12]. In den letzten Jahren wurde die Anwendung von mesenchymalen Stammzellen zur Therapie von Arthrose häufig diskutiert. Es ist anzunehmen, dass ADSCs durch ihre anti-inflammatorischen und immunsuppressiven Eigenschaften den therapeutisch bedeutendsten Zellanteil im Rahmen autologer Fettinjektionen ausmachen. Daher besteht ein großes Interesse daran zu zeigen, welche Liposuktionsverfahren sich zur Fettgewinnung eignen.

Methoden: Um Patienten eine bestmögliche Therapie mit Eigenfettinjektionen zu ermöglichen, gilt es die beste Methode zur Fettgewinnung, sowie Isolierung von ADSCs festzumachen. Infolgedessen wurden zwei gängige Liposuktionsverfahren Lipivage200 und HumanMed Bodyjetmiteinander verglichen. Dazu wurden Patientenproben hinsichtlich des Liposuktionsverfahrens in zwei Gruppen unterteilt. Um den mesenchymalen Stammzellcharakter und die Qualität der isolierten Zellen aus den zwei Verfahren zu untersuchen, wurde die kumulative Populationsverdopplung zur Wachstumsanalyse, die Fähigkeit zur Kolonienbildung sowie die metabolische Aktivität bestimmt. Des Weiteren wurde das chondrogene, adipogene und osteogene Differenzierungspotential analysiert.

Ergebnisse: Aus unseren Versuchen geht hervor, dass es zwischen beiden Liposuktionsverfahren keinen Unterschied im Hinblick auf die Qualität der isolierten ADSCs gibt. Bezogen auf die Zellqualität sind beide Liposuktionsverfahren zur Gewinnung von Lipoaspirat für zukünftige Therapien mit ADSCs als gleichwertig anzunehmen.
Um zukünftig Therapieerfolge mit einer Eigenfettinjektion und in weiterer Folge auch mit isolierten ADSCs verzeichnen zu können, ist es wichtig zu zeigen, welche immunomodulatorischen Unterschiede Fettgewebe und die daraus isolierten Vorläuferzellen aufweisen. Arthrose ist mit einer Entzündungsreaktion assoziiert. Es kommt im Verlauf der Pathogenese zur Freisetzung einer Vielzahl von pro-inflammatorischen Zytokinen. Diese Zytokine führen in der Folge zu einer Rekrutierung sowie Aktivierung von weiteren pro- und anti-inflammatorischen Zellen. In unserer Studie wurden Fettgewebe und ADSCs inflammatorisch behandelt und die unterschiedliche Reaktion untersucht.
So wurden Fettgewebe sowie ADSCs erfolgreich mit TNFα behandelt um eine entzündliche Reaktion zu triggern. Dazu bestätigte die erste Tiefensequenzierung der mRNA den „proof-of-concept“. Hierbei konnten wir einerseits eine starke inflammatorische Antwort von Fettgewebe und ADSCs nach Behandlung mit TNFα nachweisen und das veränderte Genexpressionsprofil sowie die veränderten Pathways darstellen. Anderseits konnten wir zeigen, dass das gewonnene Fettgewebe erhebliche interindividuelle Unterschiede beim Genexpressionslevel aufwies, jedoch die daraus isolierten ADSCs ein äußerst homogenes Expressionsprofil hatten.

6. First publication

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Adipose-derived stem/progenitor cells from lipoaspirates: A comparison between the Lipivage200-5 liposuction system and the Body-Jet liposuction system

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KEYWORDS
Adipose-derived stem/progenitor cells;
Suction assisted liposuction;
Water-jet-assisted liposuction;
Lipivage200-5;
Body-Jet;
Stem cell characterization

Summary *Background:* Adipose-derived stem/progenitor cells (ADSPCs) are under investigation in many clinical applications for their regenerative potential in a variety of autoimmune, degenerative, and inflammatory diseases. Adipose tissue, which is mainly harvested by manual liposuction, is the main source of these ADSPCs.
Objective: In the past years, a variety of different liposuction devices have been commercialized. To ensure a high quality of obtained ADSPCs, it is crucial to show the advantages and disadvantages of frequently used liposuction methods.
For this reason, the objective of this study was to compare ADSPCs harvested by either the suction-assisted LipiVage200-5 or the water-assisted Body-Jet system.
Methods: The proliferation potential of ADSPCs, harvested from 20 patients, was assessed by cumulative population doublings (cumPD), population doubling time (PDT), colony-forming units (CFU), and cell metabolism assays. To prove the multipotency of the primary isolated cells, ADSPCs were induced to differentiate into adipogenic, osteogenic, and chondrogenic lineages.

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Results: Our data show a significantly higher cumPD, as well as a significantly lower PDT for cells obtained by the Body-Jet system. No significant differences were found regarding the CFU efficiency and the cell metabolism. Furthermore, we showed that the adipogenic, osteogenic, and chondrogenic potential of ADSPCs is similar in both groups.
Discussion/conclusion: In our study, we provide evidence that the cell characteristics and the functional properties of ADSPCs isolated after liposuction with different techniques are largely similar. However, we observed a significantly higher cumPD and a slightly higher adipogenic potential in cells isolated after liposuction with the Body-Jet system. Different cannula sizes and sheer stresses in the used methods might play a role here.

Introduction

Adipose tissue can be used in a wide range of clinical applications, including autologous fat grafting and lipofilling. Liposuction has become an important method to obtain fat tissue for further therapeutic purposes. Adipose tissue is a complex and highly active metabolic and endocrine organ composed of adipocytes, loose connective tissue matrix, and the stromal vascular fraction (SVF). The SVF consists of preadipocytes, capillary endothelial cells, infiltrated monocytes/macrophages, and a population of multipotent adipose-derived stem/progenitor cells (ADSPCs)[1,2]. It can be isolated by enzymatic digestion of adipose tissue, and some clinicians already use the SVF, for example, in cell-enriched lipotransfers[3]. Zuk et al. have previously described a stem/progenitor cell population in human adipose tissue that displays self-renewal potential and multilineage potency[4]. Thus, ADSPCs fulfill all characteristics of multilineage stem/progenitor cells, including differentiation and proliferation capacity, as well as clonogenicity[5]. For clinical purposes, ADSPCs seem to be the most promising cells of the SVF for the purpose of regenerative medicine. Since the discovery of these cells, the number of fields for their application in tissue engineering and regenerative medicine has grown immensely. Because of their mesodermal origin, the clinical utilization of these cells seems obvious in the field of bone, cartilage, and tendon repair[6–10]. Other areas of interest range from cardiovascular tissue regeneration and nerve repair to immunomodulatory effects of ADSPCs[11–15]. Furthermore, ADSPCs display several advantages, when compared to bone marrow-derived stem cells (MSCs). In contrast to MSCs, ADSPCs can be more easily and more safely obtained through liposuction. The latter procedure is also associated with a higher harvest[16].

Currently, several methods are available for harvesting adipose tissue. They range from classical resection, conventional suction-assisted liposuction to newer techniques such as the water-jet-assisted, nutational infrasonic, and ultrasound-assisted liposuction[17,18]. These methods are developing continuously, and newer methods will be introduced in the future. Specifically, the Body-Jet and LipiVage200-5 liposuction devices are reported to provide favorable clinical outcomes. These two frequently used methods show great differences with regard to the fat-harvesting procedure. The suction-assisted LipiVage200-5 system is a single-use, disposable system that allows to obtain 200 ml of lipoaspirate. This technique provides efficient handling, as fat harvesting and subsequent washing steps are carried out in one step. The harvested tissue can be re-injected in the same treatment. After removing fluid and oil by means of filtration, it is used primarily for lipofilling in the field of esthetic surgery, as well as in plastic and reconstructive surgery[19,20]. In contrast, the Body-Jet system is a water-jet-assisted liposuction method. Hence, the liposuction tube, which is connected to a negative-pressure pump and a water pump, enables the ejection of fan-shaped water in specified frequencies during liposuction[21]. This technique is known to reduce cardiovascular side effects, as the fluid injected into the patient is much lower than that during other liposuction methods[22].

To maintain a high clinical standard, it is essential to analyze and compare the most frequently used liposuction techniques. To date, there are several studies that compare either the LipiVage or the Body-Jet system to classic manual methods. Ferguson et al. compared the viability of cells isolated from adipose tissue harvested with the LipiVage system and classic manual liposuction[19]. Bony et al. have recently published a study comparing cell properties of ADSPCs isolated after manual and water-jet-assisted liposuction[22]. Furthermore, studies have compared novel liposuction techniques that have not yet found widespread application. Recently, Bajek et al. evaluated biological properties of ADSPCs isolated after liposuction with the newer laser-assisted technique, as well as after power-assisted liposuction and surgical resection[23]. To the best of our knowledge, there is no study specifically investigating the two general most widespread liposuction approaches. For this reason, we compared the influence of LipiVage and Body-Jet liposuction methods for ADSCPs properties by examining their clonogenic potential, proliferation capacity, metabolic activity, and differentiation potential into the adipogenic, osteogenic, and chondrogenic lineages.

Methods

Ethics statement and sample acquisition

Human lipoaspirates were obtained from 20 patients undergoing liposuction after written informed consent was signed by them. Patients received liposuction either as an esthetical procedure or for the treatment of carpometacarpal joint osteoarthritis using unprocessed autologous fat tissue for injection into the joint capsule. The study was approved by the Ethics Committee of Ludwig-Maximilians-

Table 1 Patient information.

Patient overview	Female donors total	Male donors total
Number	10	10
Average age	51.2 years (range 27-69 years)	46.5 years (range 20-69 years)
Harvesting site	Abdomen and thighs	Abdomen and breast
Average fat sample weight	8.7 g (range 4.6-10 g)	9.3 g (range 6.2-10.2 g)

University, Munich (275-16). Patient information is listed in Table 1.

All lipoaspirates were harvested either through water-jet-assisted liposuction with the Body-Jet system (Human Med AG, Germany) or through suction-assisted liposuction performed with the LipiVage system (Bondimed, Austria) by a surgeon according to common surgical standards. The Body-Jet liposuction technique was used for patients receiving esthetic liposuction, whereas the LipiVage technique was used for obtaining adipose tissue for injection of lipoaspirate into the carpometacarpal joint capsule. Tumescent fluid containing saline, lidocaine, and epinephrine was used for liposuction with the Body-Jet system but not for liposuction with the LipiVage technique. To obtain comparable samples of adipose tissue, samples (300 g) harvested with the Body-Jet device were centrifuged for 5 minutes and the mid-layer, consisting of tumescent fluid, was extracted before further use of the samples for experiments.

All patients were tested negative for HIV (human immunodeficiency virus), HCV (hepatitis C virus), and HBV (hepatitis B virus). The average patient age was 50.15 years. All patients were in a generally healthy condition.

Cell isolation and culture condition

ADSPCs were enzymatically isolated from approximately 10 g lipoaspirate with a semi-automated centrifuge system (ARC™-Processing Unit, InGeneron, USA) following the manufacturer's protocol using their enzyme blend (Matrase™) and 37 °C warm lactated Ringer's solution (Fresenius Kabi, Germany). The obtained SVF was resuspended and cultured in standard culture medium consisting of DMEM-high glucose (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, USA). During cell expansion and experiments, the medium was changed twice per week. Cells were cultured in a humidified incubator to approximately 80% confluency, detached with 0.5% Trypsin (Merck, Germany), and frozen down in passage 1 for further experiments.

Cell characterization

To examine the clonogenic potential, colony-forming unit (CFU) efficiency (CFU% = (number of colonies/initial cell number) × 100) was determined following the protocol described previously[24]. ADSPCs were seeded at the concentration of 10 cells/cm² in 10 cm culture dishes, cultured for 10 days, and the formed colonies were visualized by staining using a 0.5% solution of crystal violet in methanol.

Proliferation capacity was quantified by calculating the cumulative population doublings (cumPD) and the population doubling time (PDT) during 30 days, corresponding to 10 consecutive passages, following the protocol described in[25].

Metabolic activity was examined with a WST-1 (water-soluble tetrazolium) assay after a protocol detailed in[24]. Optical density (OD) was measured with an ELISA-reader (MultiSkan FC, Thermo Fisher Scientific, USA) at a wavelength of 450 nm. A 620 nm filter was used as the reference wavelength.

Adipogenic differentiation

Adipogenic differentiation was induced for 21 days according to the protocol described earlier[25]. Briefly, 5000 cells/cm² were seeded in 6-well plates and expanded to total confluence. After 5 days, differentiation was initialized by changing normal culture medium to induction medium, consisting of standard medium supplemented with 1 µM dexamethasone, 0.2 mM indomethacin, 0.01 mg/ml insulin, and 1 mM 3-isobutyl-1-methyl-xanthin (all from Sigma-Aldrich) for five days. The induction phase was followed by a two-day conservation phase using standard medium with 4 mM L-glutamine and 0.01 mg/ml insulin (all from Sigma-Aldrich). Cells cultured in standard culture medium served as controls.

Lipid vacuole staining

After 21 days, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA). Subsequently, cells were stained with 5 mM Bodipy 493/503 (Thermo Fisher Scientific, USA) solution. Newly formed lipid droplets were quantified with the open-source program ImageJ by calculating the fraction of the area occupied by the droplets relative to the total area[26].

Osteogenic differentiation

For osteogenic differentiation, a standardized protocol was followed[25]. Briefly, 5000 cells/cm² were plated in 6-well plates and expanded to total confluence. Differentiation was initialized by exchanging standard culture medium with induction medium consisting of standard culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerolphosphate and 50 µM ascorbic acid (all from Sigma-Aldrich). The medium was changed twice a week. Cells in standard medium served as a control.

Alizarin red staining

After 21 days, cells were stained after fixation with a 40 mM Alizarin Red solution (pH 4.1, adjusted with ammonium hydroxide). Afterwards, stained cells were imaged with a Zeiss Axiovert 40 CFL microscope. For quantification, the bound

dye was dissolved, followed by multiple centrifugation steps and the collection of the supernatant. Finally, samples were measured at a wavelength of 405 nm with an ELISA reader (Thermo Fisher Scientific, USA) and compared to a standard curve.

Chondrogenic differentiation

Chondrogenic differentiation was performed in pellet culture as described before[27]. Briefly, ADSPCs were preconditioned during monolayer expansion in a hypoxia incubator (2% O_2, 5% CO_2, 37 °C) (Sanyo, Japan) for 4 days. For pellet formation, 2.5×10^5 cells were centrifuged at 400 g for 5 minutes in 96-well, v-bottom polypropylene microplates (Corning, USA). Basic chondrogenic medium consisted of DMEM-high glucose (Thermo Fisher Scientific, USA) with 10 µM dexamethasone, 1 mM sodium pyruvate, 0.195 mM ascorbic acid 2-phosphate, 1% ITS+3 (insulin, transferrin, and selenite, all from Sigma-Aldrich), and 1% Penicillin-Streptomycin. This basic chondrogenic medium was used for controls. Additionally, BMP6 (100 ng/ml) and TGFB3 were added to the differentiation medium (20 ng/ml, both R&D systems). Pellets were cultured under hypoxic conditions, and the medium was changed every two days.

Safranin-orange and aggrecan staining

After 28 days, cell pellets were fixed, washed, and incubated overnight in a 30% sucrose solution in PBS. The next day, pellets were embedded with Tissue-Tek O.C.T (Sakura, USA), compound, and frozen at −80 °C. Embedded pellets were cut with a cryotome, and sections were stained with 0.1% safranin-orange (Sigma-Aldrich, USA) in dH_2O to evaluate the glycosaminoglycan content within the pellets. Furthermore, immunocytochemistry on pellet sections for aggrecan was performed. The anti-aggrecan primary antibody (Chemicon AB1031, USA) was diluted 1:600 in a blocking solution consisting of 1% bovine albumin serum (Life Technologies, USA) in PBS and incubated overnight at 4 °C. The following day, sections were washed in PBS and then the secondary antirabbit antibody (Vectastain, USA) was 1:400 diluted and added into blocking solution for 1 h. Afterwards, sections were washed in PBS and stained with 3,3-diaminobenzidine (DAB) (Sigma-Aldrich, USA) for 7 min, followed by another washing step in tap water. Subsequently, pellet sections were mounted with Roti-Mount-Aqua (Roth, Germany). In parallel, negative control stainings were performed by omitting the primary antibody. Finally, pictures were taken on an AxioObserver Z1 microscope using AxioCam ICc3 color camera (Carl Zeiss, Germany). Safranin-Orange- and aggrecan-positive areas were quantified with the open-source program ImageJ and compared to the total pellet area.

Statistical analysis

For all experiments, 10 donors per liposuction method were evaluated. All cell culture experiments were performed in triplicates except for the cumPD and CFU-assay. A Mann-Whitney-*U* test was used for statistical analysis, and a *p*-value of ≤ 0.05 was considered significant. All values were plotted as box and whisker plots (median, quartiles, and minimum/maximum).

Results

Cells isolated with the Body-Jet system show a significantly higher proliferation rate than those isolated with the Lipivage200-5 system

The extent of self-renewal capacity, including the proliferation potential, is one of the main characteristics known to reflect the regenerative potential of ADSPCs. Therefore, we examined the cumPD (Figure 1(A)). During a period of 30 days, ADSPCs isolated with the BodyJet system showed a significantly higher cumPD of 15.92 ± 1.86 than the ADSPCs that were isolated with the LipiVage system (cumPD 13.44 ± 1.90). The mean population doubling time (PDT) was 47.12 ± 6.50 h for the Body-Jet and 56.76 ± 8.31 h for the LipiVage system (Figure 1(B)). By monitoring the cell morphology by phase-contrast microscopy during *in vitro* cultivation, we could prove a spindle-like shape typical for mesenchymal fibroblasts during the first weeks of culture (Figure 1(C)). However, most ADSPCs isolated with both methods adopted a flattened shape after 30 days in culture (Figure 1(C)).

To determine the clonogenic potential of the isolated cells, we performed a CFU assay. In both groups, ADSPCs were able to form colonies after a period of 10 days (Figure 2(A/B)). CFU efficiency was comparable between LipiVage (18.53 ± 6.48%) and Body-Jet isolated ADSPCs (18.23 ± 8.29%) (Figure 2(C)).

Furthermore, metabolic activity, measured by a WST-1 assay, resulted in a non-significant difference in mean OD of 0.21 ± 0.16 and 0.19 ± 0.15 in the LipiVage and Body-Jet group, respectively (Figure 3).

ADSPCs isolated from both liposuction methods showed a comparable adipogenic, osteogenic, and chondrogenic potential

To validate stem cell properties, the isolated cells were induced to differentiate toward the adipogenic, osteogenic, and chondrogenic lineages. Cells obtained by LipiVage (Figure 4(A/A')) and water-jet liposuction (Figure 4(B/B')) showed prominent formation of lipid vacuoles after 21 days when stimulated with adipogenic supplements. The control groups from both liposuction methods showed no formation of lipid vacuoles (Figure 4(A/A') and (B/B'), inserts). Quantification of the amount of accumulated intracellular lipid vacuoles revealed a slight but insignificant (p=0.1377) tendency toward a stronger adipogenic differentiation potential in the Body-Jet group (12.45 ± 7.47%) than in the LipiVage system (8.17 ± 4.47%; Figure 4(C)).

ADSPCs derived from both methods showed strong mineral depositions (Figure 5(A/B)), which were absent in control cells (Figure 5(A/B), inserts). Quantification of the mineral nodule formation by Alizarin Red staining revealed a minor tendency toward a stronger osteogenic potential of

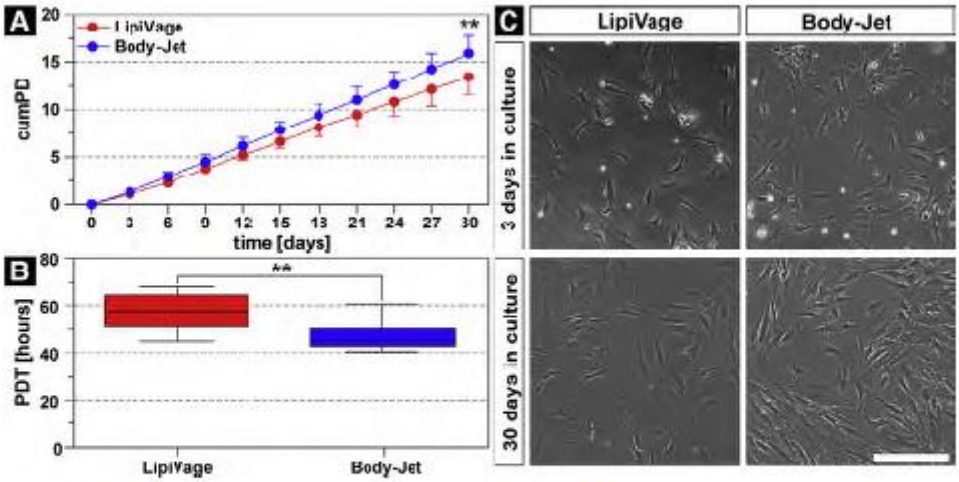

Figure 1 ADSPCs isolated with the Body-Jet system showed a significantly higher cumulative population doubling. Statistical analyses of the mean cumPD (A) after 30 days (corresponding to 10 consecutive passages) revealed a significantly higher rate for cells isolated with the Body-Jet system. The PDT showed a significantly lower rate for cells isolated with the Body-Jet system (B). Phase-contrast microscopy of cells cultivated for 3 days shows a spindle-like cell shape (C). Along with passaging, cells became flattened, resembling the shape of senescence cells (C). Scale bar: 100 μm.

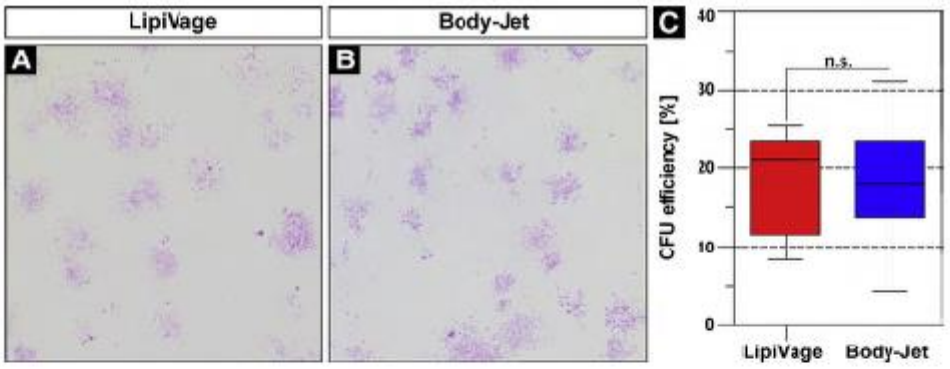

Figure 2 Number of colony forming units did not reveal a significant difference. Formed colonies were visualized with crystal violet after 10 days (A/B). Both groups showed a comparable number of colonies formed, no significant (n.s.) difference could be found (C).

cells derived from Body-Jet samples (1.83 ± 0.96 mM) in contrast to ADSPCs in the LipiVage group (1.65 ± 1.42 mM; Figure 5(C)).

ADSPCs isolated with both liposuction methods were able to differentiate into proteoglycan-producing chondrocytes in pellet culture potential (Figure 6(A/A') and (B/B')), whereas cells from both control groups did not (Figure 6(A/B)). The quantification of the Safranin-Orange-positive area on cryosections of the pellets did not show any significant difference ($p = 0.7394$) in the chondrogenic differentiation potential between cells obtained with the LipiVage (45.06 ± 11.96%) and the Body-Jet (43.75 ± 10.18%; Figure 6(C)). In addition, the quantification of the aggrecan-positive area by immunohistochemical staining did not indicate any significant difference in the chondrogenic differentiation potential between the LipiVage (46.26 ± 9.71%) and Body-Jet (49.70 ± 4.19%; $p = 0.4318$; Figure 6(D)).

Taken together, ADPSCs isolated with the two systems have a high proliferation capacity and can differentiate into the adipogenic, osteogenic, and chondrogenic lineages. However, cells derived from the Body-Jet system proliferate significantly faster and exhibit a slight tendency to

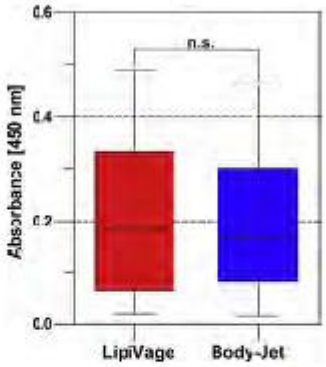

Figure 3 Metabolic activity of isolated ADSPCs is comparable between the LipiVage and Body-Jet isolation methods. The WST-1 measurement was performed with ADSPCs isolated by the two liposuction techniques. There was no significant difference among the different liposuction techniques.

accumulate more lipid vacuoles than ADSPCs isolated with the LipiVage system.

Discussion

In recent years, the regenerative potential of ADSPCs has become an interesting area in the field of regenerative medicine and tissue engineering. Because of legal restrictions, the clinical application of ADSPCs is still in its infancy. However, legal restrictions alone do not constitute the only barrier to a more widespread clinical use of ADSCPs. The incomplete understanding of the effects of different harvesting methods on the properties of these cells presents an additional limitation. Numerous fat-harvesting methods have been compared with regard to their clinical properties such as operative time, harvesting volume, blood loss, skin reactions, and usage costs[23]. However, there is a serious gap regarding the comparison of different liposuction techniques in terms of the preservation of cellular properties. The key question is whether the cellular functions of ADSPCS, derived from adipose tissue harvested with different liposuction techniques, are comparable or differ significantly.

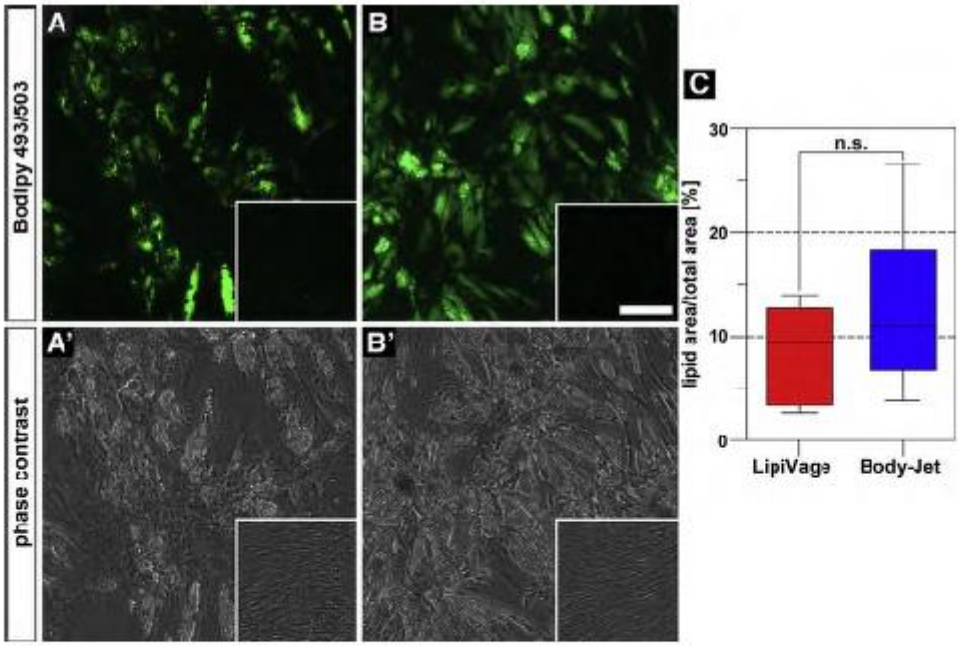

Figure 4 ADSPCs isolated after liposuction with the Body-Jet system exhibit a tendency of stronger adipogenic potential. Accumulated lipid vacuoles (green) were detected by Bodipy staining after 21 days of stimulation with adipogenic supplements (A/B). The phase-contrast microscopy pictures show the same donors at day 21 after adipogenic stimulation (A'/B'). Unstimulated negative controls showed no formation of lipid vacuoles (Fig.4A/A' and B/B', inserts). Quantitative analysis of the accumulated intracellular lipid vacuoles revealed a stronger, but statistically not significant adipogenic differentiation potential in the Body-Jet group compared with the LipiVage (C). Scale bar: 50 μm.

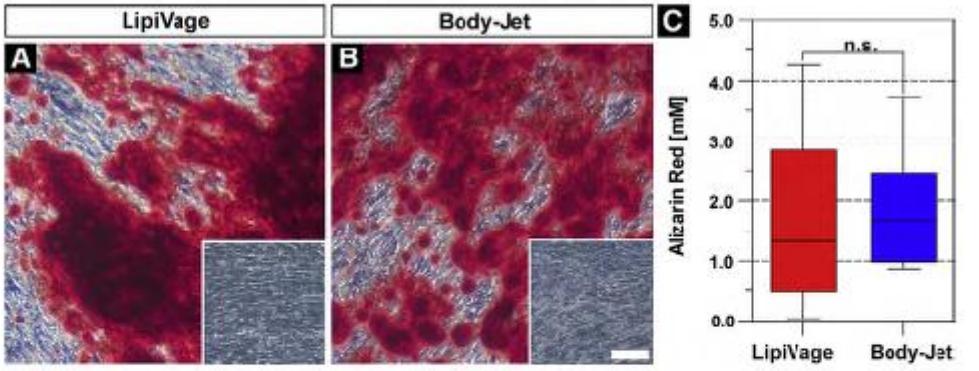

Figure 5 Quantification of extracellular mineralization demonstrates comparable osteogenic differentiation of ADSPCs isolated by LipiVage and Body-Jet. Deposited calcified matrix was revealed by Alizarin Red staining after 21 days (A/B). Unstimulated negative controls showed no formation of extracellular mineralization (A/B, inserts). Quantitative analysis of the Alizarin Red accumulation (stained red) revealed a moderate tendency towards a stronger osteogenic differentiation potential in the Body-Jet group when compared to the LipiVage group (C). However, the difference is not significant. Scale bar: 400 μm.

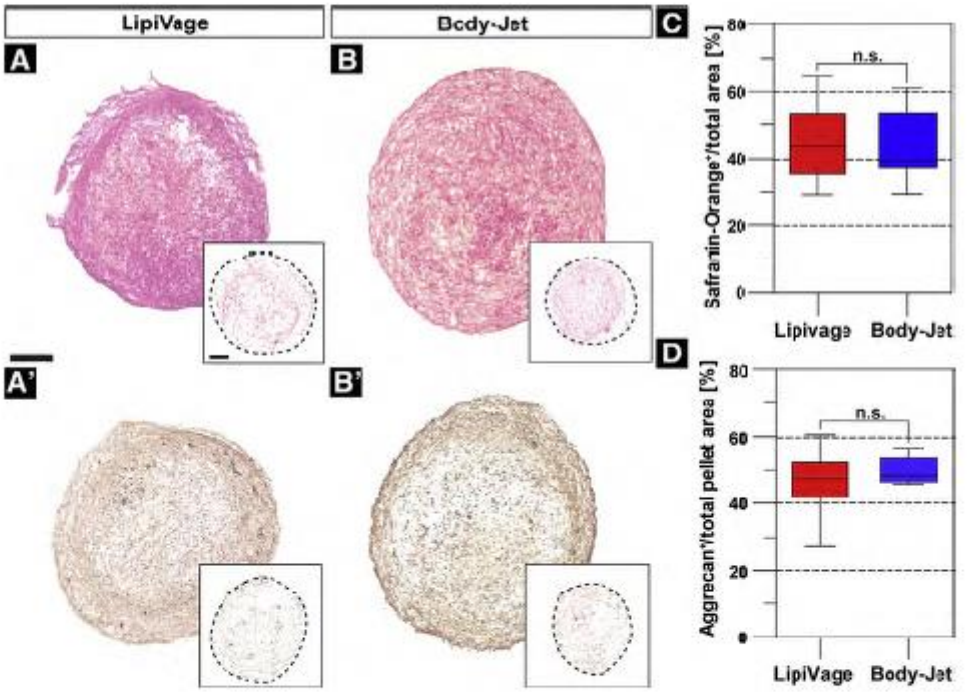

Figure 6 Deposition of proteoglycans in pellet culture indicates comparable chondrogenic differentiation of LipiVage- and Body-Jet-isolated ADSPCs. Extracellular matrix sulfated proteoglycan deposition was revealed by Safranin- Orange (A/B) and aggrecan staining (A'/B') after 28 days in pellet culture. Unstimulated negative controls did not show deposition of proteoglycans (Fig.4A/A' and B/B', inserts). Quantitative analysis of the Safranin-Orange and aggrecan accumulation did not reveal a significant difference in the chondrogenic differentiation potential between the Body-Jet and LipiVage200-5 groups (C/D). Scale bar: 400 μm.

Thus, we studied the differences between two techniques. The selected methods Body-Jet and LipiVage200-5 show great differences with regard to the fat-harvesting procedure. Nonetheless, both systems are used in everyday clinical practice for different indications[19,21].

Within the scope of our study, we evaluated cell proliferation by calculating the cumulative population doubling and the population doubling time during a period of 30 days. In addition, we assessed the number of colonies formed with the CFU assay and the cell metabolism activity with the WST-1 assay. We could show that ADSPCs isolated from fat tissue with either method displayed similar results for metabolic activity. However, ADSPCs from the Body-Jet group showed a significantly greater population doubling rate than ADSPCs in the LipiVage group.

Further studies regarding currently commercially available medical devices have shown similar results to ours[21,28]. A study by Bony et al. showed that ADSPCs derived from fat tissue, harvested with the Body-Jet system, display similar cell characteristics as those of ADSPCs derived from fat obtained through manual liposuction[22]. To the best of our knowledge, there currently is no comparable study that assesses cell properties of ADSPCs isolated with the LipiVage liposuction system. Our results provide evidence that ADSPCs from fat tissue, obtained with the Body-Jet and LipiVage system, feature a comparable metabolic capacity but a significantly different proliferation capacity. Regarding the functional properties of ADSPCs, we analyzed their potential to differentiate toward the adipogenic, osteogenic, and chondrogenic lineages. Our results could not show any significant differences between the two liposuction techniques. Previous studies from Yin et al. and from Bony et al. have shown an equivalent adipogenic and osteogenic differentiation potential for ADSPCs isolated from adipose tissue harvested with a water-jet-assisted liposuction system compared to that with the manual liposuction[21,22]. However, these studies did not examine the chondrogenic potential of isolated ADSPCs. Our findings verify a strong chondrogenic potential for ADSPCs obtained with both liposuction techniques. Altogether, the present study shows equivalent cell characteristics and functional properties for ADSPCs isolated with the two procedures, confirming that both liposuction techniques may be used to isolate functional ADSPCs. Furthermore, our findings are well in line with the studies available[3,21,22].

However, we emphasize that our data revealed a significantly higher cumPD and a tendency of higher adipogenic potential for cells isolated with the Body-Jet system. These findings might be explained through differently composed SVFs, containing heterogeneous subpopulations of stem and more committed progenitor cells, which were isolated through the two different liposuction techniques. A study by Bajek et al. demonstrated that ADSPCs obtained during manual and ultrasound-assisted liposuction show differences in antigen expression in 52 surface markers from the 242 studied[23]. We hypothesize that these different subpopulations of stem/progenitor cells might display different cell functions leading to the differences in cumPD rate and adipogenic differentiation potential.

Özsoy et al. revealed that the cannula diameter of liposuction devices affects the number of viable adipocytes. The study showed that the usage of a 4-mm-diameter aspiration cannula results in a greater number of viable adipocytes than utilization of a 2.5-mm-diameter cannula[29]. However, considering the following facts, greater cell viability might not necessarily lead to greater cell proliferation rates. During adipogenesis, pre-adipocytes undergo cell hyperplasia and hypertrophy to differentiate into adipocytes[30,31]. Due to the use of bigger cannulas, more committed progenitor cells might be isolated in higher amounts. These more committed progenitor cells in the isolated SVF might lead to a lower proliferation rate and a weaker adipogenic differentiation potential than the potentially more effective early-stage progenitor/stem cells. For liposuction with the LipiVage system aspiration cannulas with a diameter of 3-5 mm are used. In contrast, liposuctions with the Body-Jet system are performed with cannulas with a diameter of 3.5 or 3.8 mm. In practice, however, the diameter is smaller, as the cannula contains a water-jet tube, resulting in a diameter of approximately 2 mm. Furthermore, Hasanzadeh et al. showed in their study that chemical and mechanical stress plays a major role in the differentiation of ADSPCs toward other cell linages[32]. Kim et al. demonstrated in their study that shear stress leads to higher proliferation rates and stronger actin structures of ADSPCs[33]. These findings also might support the hypothesis that different cannula sizes have an impact on the characteristics of ADSPCs, as the different cannula diameters potentially lead to various shear stresses. Taken together, a bigger cannula diameter with less shear stress on cells might result in a greater number of viable cells. However, these cells might be in a greater number hypertrophic, more committed progenitor cells with a low proliferation potential. In contrast, shear stress in cannulas with a smaller diameter might lead to a lower number of viable hypertrophic cells but a higher number of smaller more, potent progenitor cells. Nevertheless, these assumptions should be studied in a follow-up study, in which for each liposuction technique, different cannula sizes are used and compared.

From a clinical point of view, it has been shown that the Body-Jet technique is associated with lower pain levels and ecchymosis, indicating less trauma to nerves, tissue and blood vessels than those used in manual liposuction. Further advantages of the Body-Jet system over manual liposuction are the rapidity and large quantity of fat tissue collection[34]. These findings indicate that the Body-Jet system can ideally be used for extensive liposuctions, where a high quantity of adipose tissue needs to be harvested. The LipiVage is a closed system that combines tissue removal and filtering, as well as its transfer back to the patient in one setting. As the cannula may hold up to 200 ml of tissue at a time, this system is ideal for smaller procedures, which require a smooth detachment.

In addition, the financial aspect should be taken into consideration, as the LipiVage system represents a more cost-effective method than the Body-Jet system. The single use of the LipiVage system costs 328 Euro, whereas the costs for the Body-Jet are 371 Euro in Germany. However, the Body-Jet liposuction device itself has to be purchased for several thousands of Euros, whereas the LipiVage device is a disposable system and does not require any additional costs. This difference in the usage costs can lead to a more frequent use of the LipiVage system, even though the Body-Jet system might be the slightly better technique.

In summary, our comparative analysis shows that the tested liposuction techniques LipiVage and Body-Jet can be seen as equally viable methods for the isolation of ADSPCs for further use in regenerative research and medicine. However, donor heterogeneity with regard to age and fat ratio may constitute the limitation of this study.

Conclusion

To date, the properties of ADSPCs harvested with different liposuction techniques have not been investigated sufficiently. The lack of knowledge may be one of the factors limiting the use of ADSPCs in clinical approaches. We show convincing evidence that the cell characteristics and the functional properties of ADSPCs isolated by different fat-harvesting methods are similar. Consequently, ADSPCs obtained with the Body-Jet and LipiVage liposuction technique should be equally considered for clinical use.

Declaration of Competing Interest

All authors declare no competing or financial interests.

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7. Second publication

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Article

Differences in the Inflammatory Response of White Adipose Tissue and Adipose-Derived Stem Cells

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Abstract: The application of liposuctioned white adipose tissue (L-WAT) and adipose-derived stem cells (ADSCs) as a novel immunomodulatory treatment option is the currently subject of various clinical trials. Because it is crucial to understand the underlying therapeutic mechanisms, the latest studies focused on the immunomodulatory functions of L-WAT or ADSCs. However, studies that examine the specific transcriptional adaptation of these treatment options to an extrinsic inflammatory stimulus in an unbiased manner are scarce. The aim of this study was to compare the gene expression profile of L-WAT and ADSCs, when subjected to tumor necrosis factor alpha (TNFα), and to identify key factors that might be therapeutically relevant when using L-WAT or ADSCs as an immuno-modulator. Fat tissue was harvested by liposuction from five human donors. ADSCs were isolated from the same donors and shortly subjected to expansion culture. L-WAT and ADSCs were treated with human recombinant TNFα, to trigger a strong inflammatory response. Subsequently, an mRNA deep next-generation sequencing was performed to evaluate the different inflammatory responses of L-WAT and ADSCs. We found significant gene expression changes in both experimental groups after TNFα incubation. However, ADSCs showed a more homogenous gene expression profile by predominantly expressing genes involved in immunomodulatory processes such as *CCL19*, *CCL5*, *TNFSF15* and *IL1b* when compared to L-WAT, which reacted rather heterogeneously. As RNA sequencing between L-WAT and ADSCS treated with TNFα revealed that L-WAT responded very heterogeneously to TNFα treatment, we therefore conclude that ADSCs are more reliable and predictable when used therapeutically. Our study furthermore yields insight into potential biological processes regarding immune system response, inflammatory response, and cell activation. Our results can help to better understand the different immunomodulatory effects of L-WAT and ADSCs.

Keywords: white fat tissue; adipose-derived stem cells; immunomodulation; inflammation; TNFalpha

1. Introduction

Inflammation is a complex, multifaceted state for many chronic conditions. The ability to regulate an adequate inflammatory response is pivotal to prevent the development and progression of any disease. Inflammatory processes are characterized by an interplay between pro- and anti-inflammatory cytokines. Cytokines, such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and gamma-interferon (IFN-γ), are classified as pro-inflammatory, whereas IL-4, IL-10 and IL-14 are classified as anti-inflammatory cytokines [1–3]. However, this classification is far too simplistic, since most cytokines may act as a pro- as well as an anti-inflammatory cytokine [1,4]. In many chronic diseases, such as osteoarthritis, rheumatoid arthritis and Crohn's disease, the balance of pro- and anti-inflammatory cytokines shifts toward pro-inflammatory factors and ultimately requires treatment [5]. Thus, novel treatment options targeting cytokine imbalance in inflammatory conditions are under investigation.

Human white fat cells and stem cells, derived of white fat tissue (ADSCs), have been shown to exert immunomodulatory effects both in vitro and in vivo [6–8]. White adipose tissue (WAT) is a highly complex organ. Rather than functioning as a mere energy storage, it also plays a potent role in metabolic and endocrine balance. It is composed of adipocytes, loose connective tissue matrix and the stromal vascular fraction (SVF). The SVF consists of preadipocytes, capillary endothelial cells, infiltrated monocytes/macrophages and a small subpopulation of multipotent ADSCs. The cellular composition, cell size and cell activity are highly variable and dependent on the donor and the tissue source [9–11]. Similar therapeutic effects are described for both WAT and ADSCs, yet there is a trend in regenerative and immunomodulatory medicine toward the utilization of the supposedly more versatile ADSCs. They are thought to be the most promising cells of the SVF regarding medical benefits [12–17], and their molecular features make them promising candidate cells, not only in the field of regenerative medicine, but also for the treatment of inflammatory-related disorders [7,18–24]. ADSCs can exert immunomodulation through direct contact with immune cells or by secretion of paracrine factors [5,7,8]. Dependent on their microenvironment, ADSCs exhibit pro-inflammatory and anti-inflammatory properties [6]. ADSCs have the ability to interact with many components of the innate immune system, including soluble complement, macrophages, dendritic cells, neutrophils, mast cells and natural killer cells [6,25,26]. Furthermore, ADSCs have the capacity to interfere with the adaptive immune system [27–29]. For clinical purposes, ADSCs have been used as anti-inflammatory "bioreactors" in the case of inflammatory bowel diseases, osteoarthritis, diabetes mellitus, chronic wounds and scar treatment [13–15,19,30–32]. Nevertheless, there is still some ambiguity regarding the utilization of ADSCs as a cellular treatment option. Potentially harmful short- or long-term effects may still be discovered, and as the exact working mechanisms are yet to be unraveled, the application as a treatment option is legally restricted in most countries.

In contrast, the use of simple WAT for clinical purposes is not subject to such strict legal restrictions in most countries, as long as it is used as an unpurified autologous lipo-transfer. Current clinical applications include scar treatment, breast reconstruction after breast cancer surgery, aesthetic rejuvenation, cleft-lip repair and liposculpture for body deformities [30,33–36]. The treatment of bone defects, osteomyelitis or chronic wounds is being debated [13,30,35–39]. Although having a higher immunomodulatory and regenerative potential, the clinical use of purified ADSCs is, as mentioned above, even more restricted. Given the huge potential of treating chronic inflammatory processes with the more appealing ADSCs, the mechanisms of action need to be further explored in order to facilitate legalization of future clinical applications.

For this purpose, we aimed to generate an unbiased dataset of transcriptional changes after an extrinsic inflammatory stimulus. Therefore, liposuctioned white fat tissue (L-WAT) and ADSCs from the same donor were treated with tumor necrosis factor alpha (TNFα) in vitro. Subsequently, a bioinformatic analysis of obtained RNA sequencing was performed to evaluate the different inflammatory responses of L-WAT and ADSCs.

2. Results

2.1. Gene Expression after TNFα Treatment of ADSCs Is More Homogenous in Comparison to L-WAT

To assess the immunomodulatory response of L-WAT and ADSPC of the same donor after TNFα exposure, we performed a deep RNA-sequencing (Figure 1).

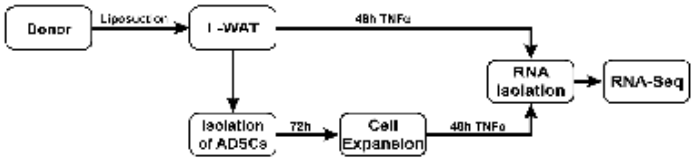

Figure 1. Experimental timeline and setup. Human L-WAT was harvested from five different donors through water-jet-assisted liposuction. Fresh fat samples were incubated with TNFα for 48 h, to mimic an inflammatory milieu that triggers a strong immune response. Simultaneously, ADSCs were isolated from the same donors' fat samples harvested during earlier liposuction. After an expansion period of 72 h, ADSCs were also treated with TNFα. Subsequently the incubation with TNFα, RNA from fat and cell samples was isolated and sequenced. A portion of the same sample without TNFα was implemented in parallel and used as reference control.

TNFα induced significant changes in gene expression in L-WAT and ADSCs, when compared to controls. After normalization and correction for multiple testing, differential gene expression analysis revealed 83 genes that were significantly upregulated and 29 genes that were significantly downregulated in L-WAT treated with TNFα, when compared to untreated L-WAT. Interestingly, ADSCs from different donors showed a considerably more homogenous transcriptional response, which results in 1404 and 1109 genes that were significantly upregulated and downregulated, respectively, in ADSCs treated with TNFα, when compared to ADSCs in normal culture conditions. Common to both groups, 68 genes were significantly upregulated, and 11 genes were significantly downregulated. While Table 1 shows the five most TNF-dependent upregulated and downregulated genes, when comparing ADSCs to ADSCs, and TNFα or L-WAT to L-WAT and TNFα (Table 1), Supplementary Table S1 (Table S1) includes all significantly changed genes of ADSCs and L-WAT after TNFα treatment.

Table 1. Five most TNFα-dependent upregulated and downregulated in ADSCs and L-WAT; logFC: logarithmic fold-change.

ADSCs vs. ADSCs and TNFα		
Gene symbol	**Description**	**logFC**
CXCL10	C-X-C motif chemokine ligand 10	10.08
CXCL11	C-X-C motif chemokine ligand 11	9.67
CCL5	C-C motif chemokine ligand 5	9.60
CXCL8	C-X-C motif chemokine ligand 8	9.38
LINC01539	long intergenic non-protein coding RNA 1539	8.49
PLA2G2A	phospholipase A2 group IIA	−6.22
WISP2	WNT1 inducible signaling pathway protein 2	−6.47
TNNT3	troponin T3, fast skeletal type	−6.48

ASPN	Asporin	−7.06
H19	H19, imprinted maternally expressed transcript	−8.17

L-WAT vs. L-WAT and TNFα

Gene symbol	Description	logFC
CCL22	C-C motif chemokine ligand 22	5.68
ANO9	anoctamin 9	5.30
MMP9	matrix metallopeptidase 9	5.17
EBI3	Epstein–Barr virus induced 3	4.94
CCL5	C-C motif chemokine ligand 5	4.68
ECSCR	endothelial cell surface expressed chemotaxis and apoptosis regulator	−2.97
AC091939.1	novel transcript	−3.13
MNDA	myeloid cell nuclear differentiation antigen	−3.57
CA4	carbonic anhydrase 4	−4.08
AC002546.1	novel transcript	−4.13

2.2. Gene Expression Regulation in Fat and ADSCs Treated with TNFα

To further highlight the different gene expression response of L-WAT and ADSCs to TNFα treatment, we analyzed the significantly upregulated and downregulated genes in the L-WAT vs. ADSCs, as well as in the experimental group L-WAT and TNFα vs. ADSCs and TNFα. We found 3388 genes significantly higher expressed in ADSCs, in contrast to L-WAT. Furthermore, our evaluation showed 2397 significantly higher expressed genes in ADSCs and TNFα, when compared to L-WAT and TNFα. Interestingly, 699 genes out of the 2397 significantly upregulated genes in ADSCs were TNFα-dependent (Figure 2A). In comparison, 3129 genes were significantly lower expressed in ADSCs, when compared to L-WAT. After incubation with TNFα, 3047 genes were significantly downregulated in ADSCs, when compared to L-WAT. Hereof, 1160 genes were significantly downregulated in ADSCs and TNFα, when compared to L-WAT and TNFα, due to the effect of TNFα. The remaining 1887 significantly downregulated genes in ADSCs and TNFα, when compared to L-WAT and TNFα were not related to the effect of TNFα (Figure 2B). The whole list of significantly changed genes in ADSCs and TNFα vs. L-WAT and TNFα is provided in Supplementary Table S2 (Table S2).

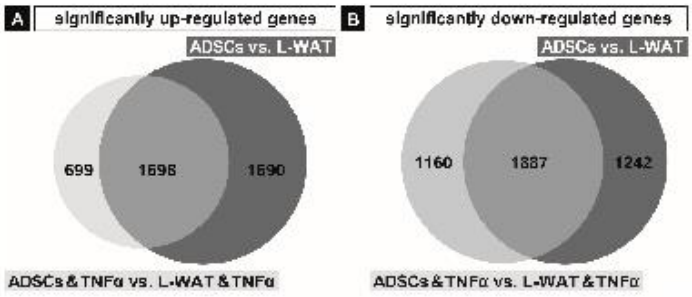

Figure 2. Significantly differentially expressed genes in L-WAT and ADSCs. Significantly upregulated genes in ADSCs compared to L-WAT and in ADSCs and TNFα compared to L-WAT and TNFα (**A**). Significantly, downregulated genes in ADSCs compared to L-WAT and in ADSCs and TNFα compared to L-WAT and TNFα (**B**).

While a hierarchical cluster analysis of the principal component 1 (PC1), revealed a clear separation of ADSCs and L-WAT, PC4 clearly shows that ADSCs have a substantial higher homogenous gene expression profile, when compared to L-WAT (Figure 3). Moreover, ADSCs showed greater transcriptome changes after incubation with TNFα, when compared to L-WAT (Figure 3, PC4).

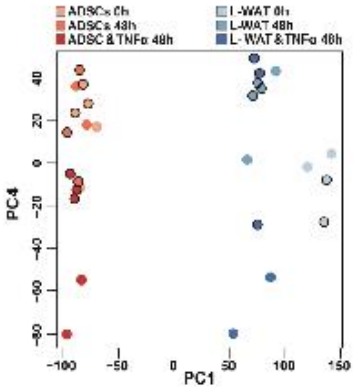

Figure 3. Principal Component Analysis (PCA) of L-WAT and ADSCs. The PCA revealed a clear separation of L-WAT (blue dots) and ADSCs (red dots) along the main component PC1. In addition, TNFα treatment showed a separation along PC4, with a more homogeneous response of ADSCs (dark-red dots), when compared to L-WAT (dark-blue dots). There was no clear clustering of samples from the abdomen (black border) or the thighs (no border).

The 30 most to PC1 contributing genes are fat-related marker genes, including *LEP*, *FABP4* and *ADIPOQ*. Furthermore, it revealed six genes that are higher expressed in ADSCs; these include *GREM1*, known to be involved in limb development [40], *CNN1*, which plays a role in smooth-muscle function [41], and *ALPK2*, which is important for cardiac muscle cell development [42] (Figure 4A).

Interestingly, the analysis of the fourth cluster (PC4) revealed the separation among genes associated with immunomodulatory processes such as *CX3CL1*, *IL-4I1*, *IL-31* and *CCL5*. (Figure 4B). However, this separation was strongly visible in ADSCs, whereas, in L-WAT, the inflammatory effect was not as strong. The top five prominent genes in this separation are as follows: BIRC3, which is important for the inhibition of apoptosis [43]; *MEOX1*, which plays a role in sclerotome development [44]; *CX3CL1*, which is pivotal for chemotaxis and cell adhesion [45]; *CCL19*, which plays a crucial role in different inflammatory processes [46,47]; and *ANO9*, which might play a role in different types of cancer [48].

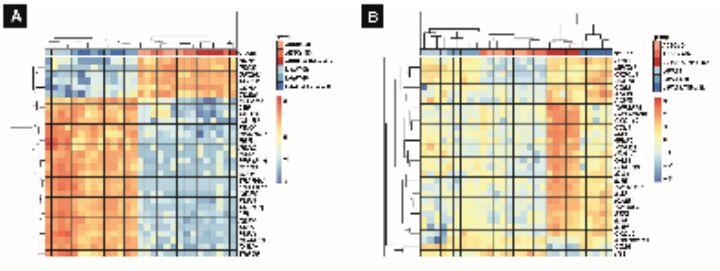

Figure 4. Hierarchical cluster analysis of the 30 most contributing genes of PC1 and PC4. The 30 most to PC1 contributing genes are fat-related marker genes including *LEP*, *FABP4* and *ADIPOQ* (**A**). The analysis of the PC4 reveals the separation among genes associated with immunomodulatory processes (**B**).

2.3. Biological Pathways that Are Regulated Upon TNFα Exposure in L-WAT and ADSCs

As TNFα is a pleiotropic cytokine with important functions, such as homeostasis, inflammation, pathogenesis, apoptosis or necroptosis [49], different biological processes were significantly changed in the experimental groups after exposure to TNFα. We carried out pathway and functional analysis, using Gene Ontology (GO), including all genes that were differentially regulated upon L-WAT and ADSCS treated with TNFα (ADSCs and TNFα vs. L-WAT and TNFα). The evaluation with GO of all significantly higher regulated genes in ADSCs, when compared to L-WAT treated with TNFα, revealed a plentitude of different biological functions, like immune system processes, extracellular matrix organization and response to an inflammatory stimulus (Figure 5A). Furthermore, the analysis with GO of significantly lower-expressed genes in ADSCs, when compared to L-WAT treated with TNFα, showed alterations in different biological pathways, including developmental processes, biological adhesion and leukocyte migration (Figure 5B). The whole list of significantly different biological processes is provided in Supplementary Table S3 (Table S3).

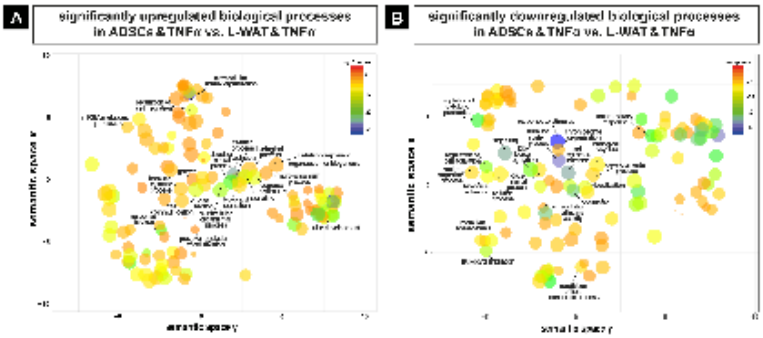

Figure 5. Significantly changed biological pathways in ADSCs and L-WAT after incubation with TNFα. Differential gene expression analysis revealed hundreds of significant upregulated (**A**) and downregulated (**B**) biological pathways in ADSCs treated with TNFα, when compared to L-WAT treated with TNFα.

To get a better understanding of the differentially regulated pathways in ADSCs and L-WAT upon exposure to TNFα, we performed a gene set enrichment analysis (GSEA). Interestingly, ADSCs and TNFα showed a significant increase of inflammation-related gene hallmarks, as well as gene hallmarks, like epithelial to mesenchymal transition and apical junctions, when compared to L-WAT and TNFα (Figure 6). Intriguingly, while TNFα exposure of ADSCs led to interferon alpha-, as well as gamma-related response, L-WAT showed a mild response in the interferon gamma pathway and nearly no gene-set enrichment in the interferon alpha hallmark (Figure 6, blue arrowheads). Additionally, the most significantly underrepresented gene sets of ADSCs and TNFα mainly consist of fat-related metabolism and development sets, when compared to L-WAT and TNFα (Figure 6).

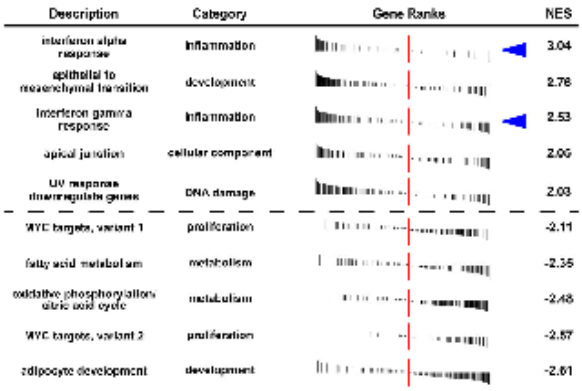

Figure 6. Visualization of the five most positively (NES > 0) or negatively (NES < 0) TNFα-dependent hallmark gene sets in ADSCs, when compared to L-WAT. Blue arrowheads indicate the difference of interferon alpha and gamma response of ADSCs and L-WAT upon TNFα treatment. NES: normalized enrichment score. Adjusted *p*-value < 0.05.

3. Discussion

L-WAT and ADSCs are seen as a promising therapy tools in the field of regenerative medicine. While the use of simple fat tissue is straightforward, the therapeutic use of isolated stem cells is controversial and, to date, not clinically approved in most countries. Because of the inflammatory component, which characterize several clinical conditions, it is of upmost importance to discover novel immunomodulatory treatment options and to understand their mechanisms of action. To date, there are several studies that investigated the immunomodulatory function of (L-)WAT and ADSCs, but to our knowledge, there is none specifically investigating the differences in both in an unbiased manner [20,50–52]. In the early 2000s, Zuk et al. described the in vitro potential of human ADSCs to differentiate under specific culture conditions into different mesenchymal cell linages [16,53]. Later, mesenchymal stem cells and ADSCs were introduced as trophic mediators for tissue repair, and it was proposed that they secrete factors that stimulate the release of functional bioactive factors from surrounding cells [54,55]. This view has been evolved, and MSCs and ADSCs are now believed to secrete paracrine factors themselves that promote cell viability, proliferation and matrix production in the surrounding environment [55]. Different studies show that the secretome of ADSCs, exerted through extracellular vesicles, is a promising source of new cell-free therapies in the field of regenerative medicine [24,56–61]. The identification of the exact overall immunomodulatory response of L-WAT and ADSCs is crucial for clinical approaches, in order to introduce targeting therapies. Therefore, our aim was to investigate the differences in the inflammatory response of L-WAT and ADSCs.

In our study, we identified genes in L-WAT and ADSCs, as well as pathways induced or repressed in inflammation that are modulated by TNFα exposure and may represent candidates for targeting treatment in inflammatory conditions. As expected, the analysis detected genes specifically involved in TNFα-induced inflammatory processes. After correction for multiple testing, 5444 genes showed significant differential gene expression in ADSCs treated with TNFα, when compared to L-WAT treated with TNFα. Our data provide evidence that ADSCs display greater transcriptional changes after TNFα treatment, when compared to L-WAT (Figure 3). The PCA clearly shows that ADSCs display a more homogenous gene expression between cells isolated from different individuals, when compared to a strong heterogeneous gene expression profile in L-WAT. This fact might indicate that L-WAT is much more susceptible to inter-individual factors and thus might influence its therapeutic effect. This appears to be even more interesting, when considering the circumstance that isolated ADSCs and fat tissue were harvested from the same donors.

WAT is a whole tissue with its intrinsic and complex cellular and biochemical components, which makes its clinical use difficult. In addition, inter-individual donor factors, such as age, sex, ancestry and medical conditions, have a hardly predictable and yet unknown influence on the therapeutical outcome. Furthermore, WAT contains not only progenitor cells, but also adipocytes, blood cells, immune cells and soluble factors that can influence the gene expression profile. On the other hand, since WAT is composed of different components, it might be concluded that when used for therapeutic purposes, these components can synergistically exert their positive effects. Different studies showed that ADSCs cannot exert their claimed therapeutic effects solitary but need different "co-factors" [50]. Furthermore, ADSCs injected into osteoarthritic joints are not detectable anymore after a few days [62,63]. Therefore, it is assumed that ASPCs "imprint" their anti-inflammatory effects on cells of the immune system, which then give a prolonged ameliorating effect [15,62,63]. These findings indicate that a composition of progenitor cells and different cells of the immune system, as found in L-WAT, might have a stronger positive therapeutic effect than its individual injected factors. On the other hand, isolated ADSCs are referred to be the most promising and potent component of L-WAT, because these cells behave in a more predictable manner [13,17,20,64]. Different studies have shown that an inflammatory environment, as found in many chronic diseases, extensively enhances the immunosuppressive effects of ADSCs [65]. However, it remains uncertain if there is a special "threshold" that needs to be reached to activate the immunomodulatory effects of L-WAT and

ADSCs. Therefore, searching for the strategies that can activate the trophic functions of L-WAT and ADSCs is fundamental for their application in regenerative medicine.

While an inflammatory response to TNFα treatment was shown in all donors, the effect of TNFα seemed to be stronger in ADSCs. This might be mostly due to the highly heterogeneous gene expression profile and the high proportion of fat-related gene sets in L-WAT treated with TNFα, which may alleviate its inflammatory effect. Another limitation of the presented study is the utilization of ADSCs and L-WAT from different anatomical regions and the in vitro expansion of ADSCs, as the complexity of the immunomodulatory action of ADSCs and WAT cannot be resolved by pure in vitro experiments [66]. Nonetheless, our GSE analysis revealed that gene sets for interferon alpha and gamma were differently regulated between ADSCs and L-WAT upon TNFα treatment. This novel result can be utilized in future experiments, by confining genes that are involved in the immunomodulatory properties of ADSCs. Future studies should focus on the direct transcriptional analysis of WAT in different inflammatory conditions (IFNα/β, IFNγ and/or TNFα), on a single-cell level, to obtain biologically relevant data. This experimental approach will help to unravel the interactions between the various cell types of WAT, including ADSCs.

4. Materials and Methods

4.1. Ethics Statement and Sample Acquisition

After obtaining written informed consent, human L-WAT was obtained from five patients without systemic diseases (mean age: 47.4 years), undergoing water-jet-assisted liposuction with the Body-Jet system (human med AG, Germany) from subcutaneous regions, for aesthetic reasons. Liposuctions with the Body-Jet system were performed with 3.5 and/or 3.8 mm cannulas and a pressure of approximately 550 bar. This study was conducted in accordance with the declaration of Helsinki and approved by the ethics committee of Ludwig-Maximilians-University, Munich (275-16). All lipoaspirates were harvested from the abdomen or thighs, through liposuction by a surgeon following common surgical standards. All patients were previously screened and tested negative for HIV (human immunodeficiency virus), HCV (hepatitis C virus) and HBV (hepatitis B virus). Patients' information is summarized in Supplementary Table S4.

4.2. Preparation of White Adipose Tissue, Cell Isolation and Culture Conditions

For all samples, a portion of 1.5 g L-WAT was washed twice with phosphate-buffered saline (PBS), to remove residual blood, and afterward they were directly incubated in standard culture medium, consisting of DMEM-high glucose (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA), 100 U/mL of Penicillin and 100 µg/mL of Streptomycin (Life Technology, USA), in a humidified incubator (21% O_2, 5% CO_2 and 37 °C), supplemented with 50 ng/mL of recombinant TNFα (Merck, Germany), for 48 h, to stimulate the secretion of inflammatory paracrine factors. ADSCs were isolated from approximately 10 g of the same L-WAT with a semi-automated centrifuge system (ARC™-Processing Unit, InGeneron, USA), following the manufacturer's protocol and using its enzyme blend (Matrase™) and 37 °C warm lactated Ringer's solution (Fresenius Kabi, Germany). Stem cell properties were proven by differentiating a proportion of isolated cells into the osteogenic, adipogenic and chondrogenic lineage, as previously published [67]. For RNA-Seq experiments, freshly isolated cells were expanded for 3 days in standard culture medium, as described above. After cell expansion for 72 h, 150,000 ADSCs in passage 1 were cultured for an additional 48 h in standard culture medium, supplemented with 50 ng/mL recombinant TNFα. ADSCs and L-WAT in standard culture medium in the absence of TNFα served as a control. ASCs after 72 h (ASC 0 h) and fresh L-WAT after washing (L-WAT 0 h) served as baseline controls. After cultivation, ADSCs and L-WAT were lysed in Trizol (Invitrogen, USA) and stored at −80 °C, until RNA sequencing.

4.3. Deep RNA-Sequencing and Bioinformatics

Total RNA was isolated by following a standardized protocol. RNA quality and quantity were measured with a BioAnalyzer (Agilent, USA), and libraries for sequencing were prepared with a SENSE mRNA-Seq Library Prep Kit V2 (Lexogen, Austria). All libraries were sequenced on a HiSeq1500 device (Illumina, USA) with a read length of 50 bp and a sequencing depth of approximately 20 million reads per sample. After demultiplexing, reads were aligned to the human reference genome (version GRCH38.85) with STAR (version 2.5.3a) [68]. Expression values (TPM) were calculated with RSEM (version 1.3.0) [69]. Genes detected in less than five samples were removed from further analysis. Differential gene expression analysis was performed, using the "voom" function in edgeR (version 3.26.5) [70] with a linear model encompassing biological condition and sequencing lane as fixed and random effect, respectively. An adjusted p-value (FDR) of less than 0.05 was set to determine significantly changed genes.

5. Conclusions

Autologous fat grafting has been carried out for decades as a standard procedure in many fields of plastic and reconstructive surgery, whereas the use of isolated ADSCs is highly restricted in most countries. In this study, we show that both L-WAT and ADSCs exhibit a strong response when exposed to an inflammatory environment. However, the inflammatory effect of TNFα on transcriptome regulation is more pronounced and predictable in ADSCs, when compared to L-WAT, which displays a very heterogeneous gene expression profile.

Supplementary Materials: The total list of significant differentially expressed genes (DEGs) of ADSCs and L-WAT after TNF treatment, as well as DEGs of ADSCs and TNFα compared to L-WAT and TNFα and corresponding changed gene ontologies, are provided in Supplementary Tables S1, S2 and S3. Patients' information is provided in Supplementary Table S4. All supplementary tables can be found at www.mdpi.com/xxx/s1. All sequencing raw data is available upon request and approval of the local ethics committee.

Author Contributions: Conceptualization, S.T., E.V., P.A., A.A. and M.M.S.; data curation, T.S. and M.M.S.; formal analysis, S.T., T.S. and D.D.-R.; funding acquisition, E.H. and M.M.S.; investigation, S.T., P.A. and M.M.S.; project administration, P.A. and A.A.; resources, R.G.; software, D.D.-R.; supervision, E.V. and M.M.S.; validation, M.M.S.; visualization, S.T. and T.S.; writing—original draft, S.T., E.V., P.A. and M.M.S.; writing—review and editing, S.T., E.V., E.H., P.A., T.S., A.A., R.G. and M.M.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors have declared that no competing interests exist.

Abbreviations

ADSCs	adipose-derived stem cells
GO	gene ontology
GSEA	gene set enrichment analysis
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
IFN	Interferon
IL	Interleukin
L-WAT	liposuctioned white adipose tissue
PCA	principal component analysis
SVF	stromal vascular fraction
TNFα	tumor necrosis factor alpha
WAT	white adipose tissue

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9. Appendix

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