Adipose tissue: from obesity to tissue regeneration

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Abstract
Nowadays, adipose tissue is considered as a multi-function organ and more recently a source of regenerative cells. Therefore, adipose tissue has gained significant attention. How to reduce it when it is in excess? How to take advantage of it? And even how to increase it when needed? In this thesis, we will demonstrate a) how adipose reduction by plastic surgery can improve quality of life and weight loss, b) how to improve fat grafting outcome by adding platelet-rich plasma (PRP), c) how to have a safer and more effective technique for in vitro adipose-derived stem cells expansion by using autologous PRP instead of actual cell culture media, and e) finally, how to increase soft tissue by an innovative technique composed of microspheres loaded with lipids, which could increase the adipose tissue volume in situ.

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ADIPOSE TISSUE: from obesity to tissue regeneration

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1 Abstract

Adipose tissue represents an important body weight in mammalian. Although often decried and fought against it in case of obesity, it is an essential multi-function organ. Adipose tissue was regarded for a long time as a relatively passive site of energy storage. Besides to store fat, adipose tissue participates significantly in energy and thermal homeostasis. Nowadays, adipose tissue is also considered as an endocrine organ and more recently a source of regenerative cells. Therefore, adipose tissue has gained significant attention. How to reduce it when it is in excess? How to take advantage of it as a multifunctional organ? And even how to increase it when needed?

In this thesis, the physio-pathology of adipose tissue is overviewed at first. We will then demonstrate how plastic surgery after bariatric surgery can improve quality of life and weight loss. These data are important for insurance companies to consider body contouring as a reconstructive surgery and reimburse it.

On the other hand, the comprehension of adipose tissue physiology offers the possibilities to develop innovative techniques to even increase adipose tissue volume when needed. Autologous fat grafting is nowadays used to increase soft tissue volume. Despite having various advantages, the inefficient fat graft survival rate is an important issue. Therefore, to improve fat grafting survival, we propose to add platelet-rich plasma (PRP) to fat graft. PRP is autologous plasma with a high platelets concentration. Platelets secrete orchestrated growth factors known to increase cell proliferation and differentiation. Taking advantage of this regenerative capacity, in order to have a safer and more effective technique for tissue engineering and cell therapy, we demonstrate that PRP could substitute actual cell culture media for adipose-derived stem cells expansion.

For the future, as stem cells are known to play a crucial role during wound repair, we aim to evaluate the effectiveness of adipose-derived stem cells for the promotion of wound repair and investigate the homing capacity of these cells as they migrate to the injured site.

And finally, in order to propose a bedside product that can increase soft tissue cost-effectively, we are developing an innovative technique composed of microspheres loaded with lipids, which could increase the adipose tissue volume in situ.
In conclusion, excess of adipose tissue is harmful and should be prevented and treated. But increasing knowledge about this multifunction and abundant tissue, will allow us to modulate and take advantage of it.
2 Introduction

Adipose tissue is one the largest organs of human body; it can reach 45kg or even more in obese. Traditionally the image of adiposity is associated with obesity and related disease. In parallel to this negative vision, we have to take on consideration that adipose tissue is a precious multifunction organ. Besides participating in essential way in energy hemostasis by storing fat, adipose tissue plays key metabolic and hormonal roles.

Adipose tissue is used since last decades in plastic surgery to reconstruct volumes following various deformations. Even recently, identification of regenerative cells in adipose tissue has drawn the attention of many investigators. Given the practical and ethical advantages of its use, the adipose tissue will become the main source for cell therapy, tissue engineering and regenerative medicine.

In this thesis, after an up-to-date overview of adipose tissue characteristics, we are going to demonstrate the role of plastic surgery in the treatment of obesity. Finally we develop how we can take benefit of adipose tissue for tissue regeneration.

2.1 Adipose tissue: anatomy and physiology

2.1.1 Different types of adipose tissue and their distribution

There are two main types of adipose tissue: the brown adipose tissue, whose major role is to release the heat, and the white adipose tissue which establishes the biggest energy reserve. In contrary to the brown adipose tissue, which declines after the birth, the white adipose tissue develops mainly in postnatal.

2.1.1.1 Brown adipose tissue

The brown adipose tissue is present at almost all the newborn mammals, hibernating mammals or adult rodents. It produces heat from triglycerides during reheating of the newborn children or at the end of the period of hibernation of the hibernating animals.

In most of the species, brown adipose tissue declines so strongly after the birth as it becomes almost undetectable. The brown fat cells are smaller than the white adipocytes, contain numerous lipid inclusions which occupy only a small part of the cellular volume, contain very
numerous mitochondria, and have a central nucleus. The brown adipose tissue, thanks to decoupling protein UCP1 present in mitochondria, quickly warms the newborn children or the hibernating if necessary. UCP1 uncouples the respiratory chain, allowing for fast substrate oxidation with a low rate of ATP production and high heat generation\(^6\). Besides these thermoregulatory functions, the brown adipose tissue could have a regulating role of the energy balance during the food taking. It was demonstrated that the overfed rats present a brown adipose tissue hypertrophied and very reactive as well as a greater thermogenesis during the post-prandial period.

In human adults the role of brown adipose tissue is not very well known. In 1980s it was generally accepted that there is no brown adipose tissue in adult man. Later, it has been demonstrated that in adult human, brown adipocytes are often present in peri-renal region\(^7\) and neck muscles.\(^8\) Since many researches claim that brown adipose tissue may contribute to body temperature regulation, fatty acid oxidation and body fat content control. Our knowledge concerning the brown adipose tissue is its infancy, but brown adipose tissue regulation could be helpful for metabolic disorders treatment.

### 2.1.1.2 White adipose tissue

The white adipose tissue is present as well in adults as in newborn. It manages the energy fluctuations by storing it in the form of triglycerides, or by releasing it in the form of fatty acids, when needed. This type of storage is very advantageous, because as adipose tissue are anhydride (low water concentration), it is of very low volume with regard to its stored energy.

The white adipose tissue represents at least 15 % of the total body mass in a non-obese man. The body distribution of white fat tissue is variable from one person to another, depending on different factors such as sex and age. The main deposits of white adipose tissue are in the subcutaneous and intra-abdominal area. In an adult man with a normal weight, the subcutaneous locations represent 50 % (eg femoral, abdominal) of all fat body mass. Intrabdominal part is 15 % (eg omentum, peri-renal region), and the rest is distributed in perigonadic region and muscles. The metabolism of adipocytes varies according to the anatomical site and can condition the privileged extension of certain deposits. For example because of difference of adrenergic receptors in woman, the femoral subcutaneous adipose tissue, more
collectively called “culotte de cheval”, is much more developed and resistant in weight lose than its abdominal equivalent. Of more the distribution of the fat mass establishes a secondary sexual character: the android profile is characterized by a more important fat deposit in the abdominal region; and in the gynoide profile adipose tissue development is more considerable in the lower parts of the body.

2.1.2 Development of adipose tissue (adipogenesis)
The adipose tissue develops from the second third of gestation from the embryonic mesoderma. During the childhood the number of fat cells increases (hyperplasia). At the adult, on the contrary, the number modifies little, only the cellular volume varies (hypertrophy) according to the nutritional and environmental changes.

Adipogenesis is the process by which precursor cells differentiate into mature cells, termed adipocytes, and accumulate triglycerides. This process is controlled by an intricate network of signaling cascades and transcription factors.

As demonstrated by Spalding et al., a human adipocyte can live for about 10 years, and that during that period of time, its triglycerides are renewed six times. Moreover, neither obesity nor variation of cell size changes the adipocyte half-life.

In general, from childhood to adult, the adipogenesis takes place in 2 phases (see Figure 1):
1st phase consists of production of pro-generative fat cells (pre-adipocytes) from multipotent mesenchymal stem cells (MSC). During the 2nd phase, these pre-adipocytes differentiate into functional mature fat cells (adipocytes).

According to the in vitro studies, during this phase after an exponential proliferation of “fibroblastic” cells derived from MSC, the cells express specific markers in particular the lipoprotein lipase (LPL). This allows the cells to begin the fat storage and to enter the late phase of differentiation after one or two additional mitoses. Then after, enzymes allowing the synthesis of fatty acids appear. The lipogenic function becomes refined then, and the first droplets of lipids appear inside cells. By expressing other proteins, in particular the perilipine, the fat cells become...
mature. The adipocyte maturation is characterized by the spherical shape of the cells which contain a unique and voluminous vesicle, resulting from the fusion of the small lipid vesicles. This process of differentiation is under the influence of diverse hormones. Among the most important are the triiodothyronine, the insulin and the growth hormone which stimulate this differentiation. Other agents can inhibit, even same to invert the process of differentiation (e.g. FGF, TGFβ, TNFα, retinoic acid). One the key regulator of adipogenesis is the ligand activated transcription factor peroxisome proliferator-activated receptor gamma (PPARγ).

During physiological weight gain adipocytes become bigger (hypertrophy) and accumulate lipids. Indeed, the adipocytes present the highest capacity of volume variation among cells: their size can vary from 15 to 200 µm. The increased size of adipocytes comes along with an increased expression of certain proteins, such as the anti-lipolytic receptors (eg. PYY, α2-adrenergic) that provokes an augmentation of fat storage capacity. However adipocytes cannot indefinitely accumulate lipids, and once hypertrophy is maximal they will recruit adipocyte precursors to form new mature adipocytes. Even if the number of adipocytes is considered to remain mostly constant during adulthood, adipogenesis is essential for adipocyte turnover and maintenance of adipose tissue. A phenomenon depending on the renewal capacity of pre-adipocytes.

2.1.3 Mechanism of fat storage in adipocytes (lipogenesis)

In adipose tissue, lipid is stored as triglycerides. In a lean young adult human the weight of stored triglycerides represents about 10-20 kg, corresponding to 90000-180000 kcal. This energy can be released as fatty acids (lipolysis) in case of energy shortage. Fat storage allows to survive up to 60-70 days of starvation in humans, even extended to 90-100 days in case of obesity.

The origin of lipids is for an important part the diet. Lipids represent a heterogeneous group consisted of: 1) fatty acids, where some are essential because the body cannot synthesize them (eg linoleic acide), 2) phospholipides, 3) steroids and 4) triglycerides which are the main fatty component of foods.
The pathways from the fat ingested in the diet to the lipids stored in the adipocytes are complex and multiple. (see Fig. 2) Ingested lipids are digested by different enzymes (e.g. lipases, biliary acids) and absorbed at the level of the duodenum and jejunum. However, as triglycerides are highly hydrophobic, they cannot circulate in the blood. Therefore they are delivered as chylomicrons into the circulation after intestinal hydrolysis. Chylomicrons are composed of lipoproteins (e.g. VLDL, HDL) which are spheric macromolecules with a hydrophilic envelop made with apoproteins and a hydrophobic nucleus containing triglycerides. They transport absorbed lipids (exogenous) or those produced by the body (endogenous) in the plasma.

As chylomicrons are too large to cross the endothelium and to enter into adipocytes, the triglycerides contained in chylomicrons are hydrolyzed into fatty acids. This hydroxylation is done by lipoprotein lipase (LPL), an enzyme synthesized by the adipocytes and exported to the luminal side of the capillary endothelium. LPL activity is increased in the prandial states, essentially by the influence of insulin and decreased during fasting, due to decreased insulin and increased catecholamine. Once liberated from chylomicrons, fatty acids must enter into adipocytes. Although fatty acids are lipophilic and could theoretically passively diffuse through the plasma membrane, this process is too slow due to insufficient free fatty acids gradient.

**Figure. 2** Long and complex pathways from ingested fat to stored lipid in adipocytes through 8 steps (Lehninger & al., Principles of biochemistry, 2nd edition, chap. 16)
Therefore a number of proteins have been demonstrated to be involved in this process, including fatty acid transport proteins (FATP)\textsuperscript{14}, receptor CD36 \textsuperscript{15} and plasma membrane fatty acid binding proteins (FABPpm).\textsuperscript{16} Parton et al. have even suggested that extracellular lipids can enter directly to the lipid droplets through specific 50-100nm caveolae invaginations in the cell membrane.\textsuperscript{17} Interestingly, several receptors involved in fatty acids uptake (i.e. CD36, FATP) are enriched in these caveolar membranes.\textsuperscript{3}

Once inside the adipocytes, fatty acids are transported in the cytoplasm to various compartments by specific proteins: cytoplasmic fatty acid-binding proteins (FABPs). In order to be metabolized for triglyceride synthesis, fatty acids should be transformed intracellularly into acyl-CoA. In addition to FATP, acyl-CoA synthetase (ACS) are the key element for this transformation.\textsuperscript{18} Once fatty acids transformed intracellularly to acyl-CoA, lipids can be stored in the form of triglyceride within lipid droplet. Many pathways exist, but the main one starts with glycerol-3-phosphate backbone to which acyl-CoA are added. This process is controlled by different enzymes (i.e GPAT, AGPAT) which are activated by insulin.\textsuperscript{19}

Another source of lipids stored in adipocytes is the glucose. (see Fig. 3) The excessed glucose is transformed to fatty acids which are then esterified in triglycerides and stored in adipocytes. This lipogenic activity is strongly dependent on insulin and GLUT4. Glucose is transported through the adipocyte membrane in a facilitative manner, by Glut protein family that comprises 14 members.\textsuperscript{20} This transport is highly stimulated by insulin. This control by insulin is perturbed in fasted condition, obesity and type II diabetes. Adipocytes are hyperresponsive to insulin for glucose transport following refeeding after fasting and the beginning of obesity development. Numerous natural and chemical compounds are also able to induce glucose transport, by acting on the insulin signaling pathway (i.e. adenosine\textsuperscript{21}, orexin A\textsuperscript{22}) or by inducing directly another glucose transport non-insulin dependent (i.e. acetylsalicylic acid, S-nitrosylation\textsuperscript{23}). There is also some counter-regulatory
hormones such as glucagon, adrenaline or cortisol that inhibit glucose transport into adipocytes during hypoglycemia or stress conditions.

During fasting and effort, energy is released from adipose tissue in the form of non-esterified fatty acids, a procedure named lipolysis. (see Fig. 4) These fatty acids are transported to different organs (e.g. muscle, brain) and to give them energy that is needed. Catecholamines, natriuretic peptides and growth hormones are the major lipolysis activator, while insulin has an antilypolitic effect.\textsuperscript{24}

\textbf{2.1.4 Angiogenesis during adipogenesis}

There is a close relationship between adipogenesis and angiogenesis. Vascularization determines the accessibility of the energy substrata and the hormones to the tissue. Besides adipogenesis requiring angiogenesis, the adipose tissue variation needs constant vessel growth, regression and remodeling.\textsuperscript{25}

The adipose tissue has a low metabolic rate and requires 30-50-fold lower blood flow in comparison to liver, heart or brain. The blood flow of the adipose tissue in a rested man is 2-3mL/min for 100g of tissue. This debit is more important in fat deposits presenting small cells than in those with hypertrophic cells. In response to feeding, the adipose tissue blood flow\textsuperscript{26} and recruitment of capillaries increase.\textsuperscript{27} Such a process increases the exposure surface of lipoprotein lipase, located at the luminal side of capillaries, to the circulating lipids, promoting therefore lipid uptake and storage in adipose tissue.

Angiogenesis is a complex process that requires extracellular matrix, endothelial cell proliferation and several biological factors: VEGF, FGF, HGF, MMP, angiopoeitin. Most of these pro-angiogenic factors have been described to be up-regulated during adipogenesis. Several of those are produced in adipose tissue and have been identified in the pre-adipocytes’ microvesicles.\textsuperscript{28}

The most known condition linked to increased angiogenic factors production is hypoxia. In hypoxic culture, preadipocytes and adipocytes up-regulate the expression of VEGF, leptin and
MMP\textsuperscript{29}. Therefore it could be suggested that hypoxic condition increases angiogenesis, and then adipogenesis.\textsuperscript{30} Interestingly treatments with angiogenesis inhibitor prevented obesity in mice models.\textsuperscript{31} Sympathetic activation has also been demonstrated to stimulate angiogenesis in murine adipose tissue via VEGF up-regulation.\textsuperscript{32} The innervation containing noradrenaline or the neuropeptide Y is rather distributed at the vascular level than adipocytes. The importance of this innervation varies according to the localization of the fat deposits.

### 2.1.5 Histology of adipose tissue

Adipose tissue is a loose connective tissue composed essentially of fat cells (adipocytes), separated by a thin layer of extracellular matrix containing collagen fibers and vessels. Mature white adipocytes are characterized by its spherical aspect, containing a wide and unique lipid vesicle made up of 95% of triglycerides. The volume of this vesicle can be very variable according to the quantity of lipid which it contains.

Lipid vesicle is the most prominent intracellular organelle, occupying a central cell position, and filling almost the cytoplasm. The lipid vesicle, surrounded with a network of vimentin is bounded by a specific monolayer phospholipid where diverse proteins including perilipin are inserted.\textsuperscript{33} Perilipin proteins operate as shells on lipid vesicles, protecting the neutral lipid core from hydrolysis by cytoplasmic lipases.\textsuperscript{34}

The cytoplasm containing the nucleus, small mitochondria, some elements of reticulum endoplasmic and a significant number of micro-vesicles is pushed away in periphery by the lipid vesicle. The cyto-skeleton of adipocytes, composed essentially by actin microfilaments, intermediate filaments and microtubules, is characterized by its big plasticity. This characteristic is essential for these cells which have to undergo drastic morphological modifications. On one hand during their differentiation, they have to change from fibroblastic shape into a round shape. On the other hand, they have to adapt their size to lipid vesicle representing more than 90% of their volume and susceptible to change size rather quickly.

The extracellular matrix consists mainly of collagens, elastin and fibronectins which are imbricated in a hydrated gel of glycosaminoglycan. It allows the cohesion of the tissue and plays
an essential role in the regulation of the development and the metabolism of adipocytes by letting the diffusion metabolites, nutriments and hormones. It could also play a role in the gene expression regulation involved in the proliferation and the differentiation of adipocytes.\textsuperscript{35} For example, if cells are cultured on a collagen matrix the adipocyte differentiation is normal, but it decreases on a fibronectin matrix.\textsuperscript{36}

### 2.1.6 Fonctions of adipose tissue

Adipose tissue is frequently considered only as a harmful tissue that should be eliminated. Scientific experts recommend to reduce adipose tissue, because of medical comorbidities related to overweight. Men and women fight daily against fat to get rid of adipose tissue, and to be in “good shape”.

In reality adipose tissue is a multi-function organ that plays key physiological roles for the body survival:

a) The main function of adipose tissue is energy homeostasis: energy storage (lipogenesis) and energy liberation (lipolysis). The first one takes place essentially during post-prandial period. The abundance of nutriment is managed by the insulin which stimulates numerous mechanisms to put in reserve the energy. The second appears when the body needs energy (i.e. urgent physical effort, fasting). (see Fig. 5) The energy concentrated in adipocytes is released in form of fatty acids that can be used by other organs such as the brain.

b) Thermogenesis is another key function of adipose tissue. Homothermous species, particularly aquatic and hairless animals, and humans possess subcutaneous fat panniculus which is an efficient thermic isolation. Moreover, brown adipose tissue, produces an important
heat quantity when required. Although rich in energy substrata and mitochondria, brown adipocytes synthesizes very few ATP but consumes many O₂ because of UCP protein present in mitochondria. It results then an important heat production.

c) The adipose tissue is a real endo-and paracrine gland. It releases lipids including fatty acids, glycerin and some enzymes, collectively referred to as adipokines. They regulate the metabolism; but they are thought to be also involved in the metabolic risk associated with obesity (i.e. IL-6, TNFα).37

d) The adipose tissue constitutes a privileged lipid reservoir. In addition to their metabolic and energetic roles, lipids have a structural role in the composition of cell membranes including nerves, a transporter role for lipophilic vitamins (e.g. Vit A, D, E, K) and a hormonal role as major steroids are made from cholesterol; and finally

e) The adipose tissue has a “volumator” function. On one side, the loose and expandable adipose tissue protects against mechanic choc that the body have to sustain in everyday life. For example the foot fat pad or the gluteal fat avoids skin ulcers that can be produced against the bone structure in standing or seating position. Patients with fat atrophy present more risk for pressure ulcers. On the other hand, the 3D “aesthetic” shape of the body is thanks to the harmonious volume that the adipose tissue offers. With age, subcutaneous fat tissue reduces, and provokes wrinkles and hallows in the face. In some diseases such as lipodystrophy, related or not to HIV, some part of the body such as face and lower limbs present lipo-atrophy, and some other as abdomen and cervical region lipo-hypertrophy, that makes unattractive.
2.2 Obesity

Adipocyte could be considered as an “Axis of Evil”. An excess in adiposity is clearly associated with numerous comorbidities, including metabolic and mechanical complications. It is recognized as a major cause of morbidity, handicaps, and mortalities, as well as a bad quality of life. There is a direct relation between the level of the obesity and the mortality associated with the cardiovascular diseases and the diabetes. Sex, age, BMI and central adipose tissue distribution are clearly the main parameters of these complications. However, new concepts have emerged during the last decade, as limited adipose tissue expandability, adipose tissue dysfunction, abnormal free fatty acid trafficking, specific role for visceral adipose tissue, ectopic fat accumulation, chronic inflammation or lipotoxicity.

Figure 6 Adiposopathy: simplified relationship between pathogenic adipose tissue and cardiovascular disease. (Bays HE. Journal of the American College of Cardiology 2011;57:2461-73)
Therefore, metabolic abnormalities could be viewed as the result of impaired adipose tissue function, a phenomenon described by Bays et al. as “adiposopathy”. Targeting not only the caloric imbalance but also adipose tissue dysfunction may represent a novel strategy to prevent obesity-related diseases or to treat obesity.

2.2.1 Definition
The obesity, of the Latin Ob-esium "eaten quantity ", is due to an imbalance between the individual energy needs and energy taken. In 4th century BC, Hippocrates already noted that obesity is linked to “greater risk of sudden death”. In 2000, WHO defined obesity as “a condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that the health may be impaired”. Body fat deposition and its relation to ill health are therefore central to the definition.

2.2.2 Body mass index
The weight cannot be directly correlated with the fat mass. The “ideal weight” would correspond to index where morbidity and mortality are minimal. The concept of “ideal weight” appeared only in the early of 19st century, when Adolphe Quetelet, a Belgian scientist by analyzing the anthropometric characteristics of military conscripts, defined the famous Body Mass Index (BMI). This formula gained popularity in 1950-60s, when insurance companies, defined different BMI thresholds to fix new premiums according to obesity related complications risk. Obesity is considered when the weight is >15-20% more than the “ideal weight” or the BMI > 30kg/m².

2.2.3 Development of obesity
Human obesity evolves 3 main phases: constitution (a dynamic phase which gives evidence of a positive energy balance), maintenance (static phase where a new energy balance and storage capacities are stored) and resistance to weight loss phase. During the constitution phase, under positive energy balance, adipose tissue volume increases. Lipids, important constituents of the diet, are the main source of energy: up to 40 % of the daily energy furniture. A diet rich in lipids and sugars, associated with the absence of physical
exercise, will favor triglyceride storage. Although the food excess favors the weight gain, the calorie intake is not the only factor. The genetic and environmental components play also an important role.

During development and childhood adipose tissue augmentation is principally due to adipocytes hyperplasia. In adults, the development of the fat mass is mainly due to the increase of adipocytes size (hypertrophy). The number of fat cells (approximately 20 billions) is not the main characteristic of the level of fattening. In severe obese, the number of fat cells is increased only 3 - 4 times.

Under chronic positive energy balance, the adipocyte hypertrophy reaches its limit. A limited expansion capacity of differentiated adipocytes loaded with triglycerides results on recruitment of new adipocytes either by multiplication of stem cells or by release of the differentiation processes of latent pre-adipocytes. Therefore, more adipocytes can be produced, leading to hyperplasia. In case of positive energy intake, the number of adipocytes continues to increase.

2.2.4 “Lipotoxicity”

The mechanism of adipose tissue augmentation has a limited capacity. When the storage capacity of adipose tissue by hypertrophy and hyperplasia is exceeded, lipids are released into the circulation. The unoxidized free fatty acids are deposited in other fat depots (i.e. visceral, epicardical adipose tissue), and even in non-adipose tissue organs such as liver, skeletal muscle, heart muscle and kidney. Moreover, as insulin is adipogenic by activating the entrance of triglycerides to the adipocytes, insulin resistance is directly associated with a high free fatty acid concentration in the

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(Carruthers M & al., Cardiovascular diabetology 2008;7:30)
blood circulation and its deposition in ectopic organs. The primary signal leading to adipose tissue dysfunction could be adipocyte hypertrophy, since enlarged adipocytes are more insulin resistant and produce more pro-inflammatory adipokines.\(^40\) (see Fig. 7)

Therefore, when subcutaneous white adipose tissue storage capacity is overcome, due to limited expandability, ectopic fat deposition particularly in liver and skeletal muscle is favored.\(^52\) This ectopic fat deposition has been shown to be the fundamental mechanism leading to the metabolic syndrome.\(^43,53,54,55\) in obese subjects, and not only the amount of total body fat mass by itself. The visceral adipose tissue which is significantly linked to increased cardiometabolic risk, predicts the best ectopic fat accumulation.\(^56\) Therefore, as hypothesized by Desprès et al., increased visceral fat mass may represent a marker of the inability of a patient to store fat in subcutaneous depots, and the visceral adipose tissue could be a surrogate marker and not a casual risk factor.\(^53\)

The expandability of the adipose tissues stores differs globally among individuals.\(^39\) Some individuals even with BMI >50, may be able to undergo almost unlimited expansion of their adipose tissue, and hence remain protected from the metabolic syndrome. Those with large hip and thigh would belong to this category, because the adipose tissue on this region is more expandable than intra-abdominal fat depots. The ability to expand adipose tissue has a strong sexually dimorphic component. Women at any BMI, have a greater fat depots in gluteo-femoral region, and they are more protected against insulin resistance and lipotoxicity phenomenon than men with the same BMI.\(^39\) (see Fig. 8)

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**Figure 8** Ectopic fat and metabolic disorders. (Courtesy of Mayo Clinic)
2.2.5 Histopathological changes in obesity

During obesity, there are some histopathological changes in adipose tissue. Healthy adipose tissue is characterized by a large number of small fat cells, low level of inflammation, good vascularization and minimal fibrosis. It also shows preserved insulin sensitivity and mitochondrial function. In obese, Cancello et al. demonstrated, that the concentration of macrophages is increased, reaching 15-30 per 100 adipocytes, specifically in visceral adipose tissue; and it is partially reversible by weight loss induced by gastric bypass. Moreover, enlarged adipocytes which can reach impressive sizes (>100um), exceeds the maximum oxygen diffusion distance and creates areas of hypoxia. This hypoxia could explain the death of enlarged adipocytes and infiltration of inflammatory cells, particularly macrophages, creating a chronic inflammation status.

Obesity is also associated with extracellular matrix deregulation, where the fibrotic tissue is increased. Divoux et al. demonstrated that weight loss is associated with a decrease fibrosis, but on the other hand the excess fibrosis may alter the remodeling of adipose tissue and limit the fat loss.

In resume cell types and signals involved in adipose tissue in obese are multiple and complex. They can create a local micro-environment that promotes inflammation and fibrosis. (see Fig.9) This pro-inflammatory condition could play a role in metabolic complications and cardiovascular disease.

Figure 9 Pro-inflammatory status of obese adipose tissue leading to metabolic disorders. (Wellen KE et al. J Clin Invest 2003;112:1785-8)
2.2.6 Epidemiology of obesity

Obesity was initially marginalized in the beginning of 20th century because of its low prevalence and for a long time the problem was confined to the United States. The lack of medication and physopathological knowledge also contributed to a certain lack of medical interest. From the 1980s, epidemiology changed radically and prevalence increase reached Europe and emerging countries (i.e. China, Brazil) in 1990-2000s. The WHO estimates the prevalence of obesity in 7% of the world’s population, and could reach 12% in 2020 if current evolution trends continue.59 (see Fig. 10)

In Switzerland, according to last survey in 2012, nearly one person out of three was in overweight (BMI 25-29.9 kg/m²) and one on ten was obese (BMI>30 kg/m²). The prevalence has more than doubled during past 20 years. The overweight and obesity and their comorbidity engendered a total cost of 8 billion CHF including direct health cost close to 4.7 billion CHF in 2012.60 (see Fig. 11)


Figure 11 Prevalence of overweight and obesity in Switzerland according to age. Source: National Swiss Survey 2012. Office fédérale de statistique (OFS). www.bfs.admin.ch
2.2.7 Treatment of obesity

It is difficult to determine the minimal weight quantity necessary to lose in overweighted patients, in order to reach a measurable profit for the health. A weight loss of only 5-10% was demonstrated to be sufficient to decrease the comorbidity. Because of strong prevalence of comorbidities, even small decreases of their prevalence by the weight loss, will have a significant impact on the public health. In obese a weight loss of 10kg was associated with a decrease of 20% of comorbidities including 30% of diabetes. Besides the improvement of the joint and dorsal pains, the sleep apnea was improved. Samsa and al. demonstrated furthermore that this moderate weight loss also allowed an improvement of patients quality of life. Indeed, WHO declared a weight loss of even 5% was clinically significant.

For weight reduction, different approach such as behavioral or nutritional could be intended. However, as adipose tissue resists to diminish, particularly on obese individuals, an important weight loss that stays stable over time is difficult to obtain in obese.

In normal weight individual as well in overweight persons, after the constitution phase of adipose tissue development, the fat mass is spontaneously maintained stable (maintenance phase). This phenomenon is due to the fact that once differentiated, adipocytes do not return to the precursor stage; and hyperplasia seems not or lightly reversible. Weight loss is linked to a reduction in the size of adipocytes (hypotrophy), not the number which remains stable. Moreover, cell size cannot be maintained below a certain value; the minimum level of fat mass which can be reached is limited by the number of adipocytes. Leptin, secreted by the adipose tissue, would also be involved in this autoregulation of the fat mass during the maintenance phase. This adipose tissue resistance could explain partially, the resistances to weight loss, fast return to the initial weight after a weigh-reducing diet, or the difficulty of former obese to keep a stable weight. It would explain that only sustainable modifications permit a decrease of the long-term weight. And unfortunately most of the medical treatments based on diets, physical exercise, modifications of food behavior or drugs are often insufficient, particularly to the morbid obese individuals.
2.2.7.1 Bariatric surgery

Bariatric surgery (from Greek baros, weight; and iatrikos, being a part of the medicine) could be proposed to obese individuals that cannot lose weight with non-surgical approaches. The surgical treatment of the obesity is only justified if the morbidity and the mortality caused by the obesity are more important than those associated with the operation and its aftereffects. Therefore, patient’s and procedure selection is primordial.

The surgical treatment of the obesity began in 1956 with Payne and De Wind and since then, numerous techniques were developed. They are grouped in three categories: those reducing only the length of intestine absorption called "malabsorptives" (e.g. Scopinaro, jejuno-ileal bypass), those purely "restrictive" creating a small gastric pocket (e.g. vertical gastroplasty, adjustable gastric loop), and those who combine both principles and called "mixed" (e.g. Roux-en-Y gastric bypass (RYGBP). These operations permit a massive weight loss not only because of reduced estomac volume and intestinal absorption, but also by changing gastro-intestinal hormones and flora. Recently it has been demonstrated that bariatric surgery, influence also appetite and energetic metabolism through entero-hypothalamic pathways. Ghreline rate, considered as main appetite activator is reduced after a bariatric surgery. After RYGBP, Glucagon-like peptide-1 (GLP-1), used for diabetes treatment, and peptide YY (PYY) secretion are increased.

Numerous studies demonstrated that the bariatric surgery solves up to 95 % of the diabetes, 100% of dyslipidemia, 67,8% of blood hypertension, 92.2% of the sleep apnea syndromes and 96% of the metabolic syndromes. These improvements allow an increase of the life expectancy and a decrease of the mortality. Sjöström and al., in an important study including 7925 patients who underwent a bariatric surgery, concluded that the global mortality of the operated patients was decreased 40 % compared with the not operated group. Arterburn et al. in a retrospective cohort study, confirmed that obese patients who underwent a bariatric surgery had a lower all-cause rate of death at 5 up to 10 years after the surgical procedure, compared with obese patients without surgery (13,8% vs. 23,9%).

With a favorable risk-profit balance, the bariatric surgery became the gold standard treatment for morbid obesity. By taking into account the disadvantages of the operation: immediate post-
operative pains, operating stress, food inconveniences, possible complications and a per-operative mortality risk of 0.04-0.16%, the advantages of the intervention are clearly superior.\textsuperscript{63} In fifteen years these interventions have more that quintupled to reach more than 100'000 operations per year in the USA.\textsuperscript{70} According to Swiss Society for the Study of Morbid Obesity and Metabolic disorders (SMOB) in 2013, more than 4000 bariatric interventions were made in Switzerland, in 51 centers (www.smob.ch).

\textit{2.2.7.2 Roux-en-Y gastric bypass}

Numerous studies have already demonstrated the beneficial effect of RYGBP on the comorbidity and mortality, with a long-term efficiency in terms of weight loss.\textsuperscript{63} On average patients lose 66 \% of their excess of body weight (EBW) during 1-3 post-operative years, 60 \% in 5 years and 50 \% between 5 to 10 years.\textsuperscript{63,71} Considering the risk/advantages balance, the RYGBP is nowadays recognized as one of the best solution for morbid obese (BMI>40 kg/m\textsuperscript{2}) or those with BMI>35 kg/m\textsuperscript{2} presenting more than 2 comorbidities.

For many patients the psychosocial improvement is also important, sometimes even more than the effective decrease of their weight. Indeed, the request of the patients for a medical or surgical treatment of their weight excess ensues more often from a decreased quality of life than from fears of medical comorbidities. The psychosocial distress is the main motivation of a surgical treatment for more than two thirds of the patients, against only 10 \% for purely medical reasons.\textsuperscript{72} The purpose of the surgery treatment of obesity for the patients seems to be more the improvement of their physical image, their self-esteem and their quality of life than only the weight loss.\textsuperscript{73,74}

Several studies demonstrated that RYGBP besides comorbidities and mortality reduction, improves patients health-related quality of life (HRQoL).\textsuperscript{75-79} In one of our study, we demonstrated that the quality of life was evaluated as “much better” by 22 \% of patients after RYGBP. This improvement concerned all sub-items of HRQoL: self-esteem, physical activity, social life, work ability and sexual activity.\textsuperscript{80} (Annexe. Paper 1)

However, despite a massive weight loss, HRQoL of these patients who lost a massive weight after a bariatric surgery remains always unsatisfactory in comparison with those of non-
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operated persons with the same weight. The cutaneous excess appeared after a massive weight loss can be considered as a new handicap by the patients. Up to 95.6 % of patients report residual morphology dissatisfaction associated with loose sagging skin.\textsuperscript{81} This side effect of the bariatric surgery can strongly bother patient’s daily activities (e.g. mobility, clothing) and raise hygienic problems (e.g. macerations, intertrigo) requiring medical treatments. It can induce, despite considerable weight loss, severe psychosocial problems because of a lack of self-confidence and a disturbed physical image.\textsuperscript{82} More than two-thirds of patients who have undergone bariatric surgery consider their excess skin as a negative consequence of surgery.\textsuperscript{83} This dissatisfaction motivates 74 % of patients to seek body contouring procedures after bariatric surgery, but only 21 % achieves at least one of them.\textsuperscript{81} Most of them give up the surgery for economic reasons, as often health insurances don’t cover such kind of plastic surgery.

2.2.8 Benefits of plastic surgery

The basic principle of body contouring after massive weight loss is to remove excess skin and tighten the cutaneous tissue to the new body volume and reach a harmonious silhouette. It could therefore eliminate physical or psychological handicap bound to the excess skin. It is thus essentially a functional surgery. According to the American Society of Plastic Surgeons, body contouring procedures concerned 52,603 patients in the USA in 2010.

Body contouring is proposed to patients who reached a stable weight after a massive weight loss, usually at least 12-18 months after bariatric surgery. According to the status and the request of the patient these interventions are mainly made at the abdominal level (abdominoplasty) and breasts (mammoplasty) but also in inner thighs (cruroplasty) or arms (brachioplasty). These interventions are not free of complications, and they create scars which, in certain cases, can be disappointing for the patients. Therefore patients have to be informed about these sequels, even before to undergo bariatric surgery. In collaboration with our “bariatric” multidisciplinary team, we participate actively in preparation programs of patient who will undergo bariatric surgery in our center. We inform them about the skin excess that could appear after bariatric surgery, and its possible treatment by body contouring. Seen the risks and scars, the patients so wonder it's worth it?
2.2.8.1  Effects of body-contouring on quality of life (Paper 1)

In a prospective study we demonstrated that plastic surgery improves significantly HRQoL after RYGBP. 98 consecutive patients who had body contouring after gastric bypass for obesity were included, and with a mean follow-up of 7.5 years their HRQoL was compared to their situation before body contouring and to a matched control group containing 102 patients who had only RYGBP. Of the patients who had body contouring, 57% evaluated their HRQoL “much better” in comparison to only 22% of patients without body contouring (p<0.001).

The improvement was significant in all sub-items of HRQoL: self-esteem, social life, work ability, sexual activity and physical activity (p<0.001), and remained stable over time. (Annexes. Paper 1) This HRQoL improvement by plastic surgery has been confirmed later by other authors.

2.2.8.2  Benefits of body-contouring on weight loss stability (Paper 2)

Even RYGBP offers a fast and massive weight loss within the first 18 months after surgery, as many as 50 percent of patients may unfortunately regain some of the lost weight. The mean weight regain within the first 18 to 36 months after surgery is 5 to 10 percent and it becomes 10 to 15 percent over the course of the next 10 years. This weight regain can be associated with a recurrence of comorbidities, such as hypertension, diabetes, and hyperuricemia. Kalarchian et al. concluded that any interventions that improved the psychosocial functioning of a patient would also strengthen the weight loss maintenance. Considering that body contouring improves HRQoL weight loss, we were interested to assess the effect of body contouring on weight loss and stability after RYGBP.

In a prospective match-control study, we intended to evaluate the benefit of plastic surgery on body weight control after RYGBP. By comparing 98 patients who underwent body-contouring after RYGBP, to 102 matched patient without body-contouring, we demonstrated that plastic surgery improves significantly weight loss stability. RYGBP alone resulted in an initial massive mean weight loss of 45.2 kg. Then, the patients reached a plateau approximately 12 to 18 months after surgery, thereby allowing them to obtain a minimal mean weight of 78.3 kg (range, 65 to 92 kg) and a mean BMI of 29.9 kg/m² (range, 26 to 34 kg/m²) (p < 0.001). Beyond the second year after RYGBP, patients without body-contouring started to regain significant more weight than those with body-contouring (1.78 kg/year vs versus 0.51 kg/year, p = 0.001).
The differences between groups gradually became more significant over time: from the minimum weight achieved by RYGBP, the mean weight regain at 7 years was only 4.8 kg for patients with body-contouring and 20.1 kg for those without (p< 0.001). This resulted in a higher final weight for patients with RYGBP alone as compared with those who had body-contouring (101.2kg/ BMI= 37.2 versus 82.5 kg/BMI=30.6 kg/m2 respectively; p = 0.01).91 (Annexe. Paper 2)

As hypothesized in the beginning, this weight improvement by plastic surgery could be explained by improved HRQoL that these procedures offer. However, the reduction of fat and skin during body-contouring could also participate to this weight stability. During the body-contouring procedure, a mean mass of 2.04 kg (range, 0.45 to 6.3 kg) of adipose tissue was excised from patients included in our study. Most of these tissues were from the abdominal part, the most resistance adipose tissue.

As demonstrated before, it is known that physiologically adipose tissue show some resistance to lose volume and the fat mass is spontaneously maintained stable. (Sect. 2.2.2) This phenomenon is due to the fact that the weight loss, even massive is linked to a reduction in the size of adipocytes and not in the number of cells. As mature adipocytes do not return to the precursor stage; and hyperplasia seems not or lightly reversible, hypotrophic adipose tissue maintains its high capacity to store triglyceride again. This adipose tissue resistance could explain partially the difficulty of obese patients who underwent a massive weight loss, to keep stable their weight over time. Fat tissue discarding by body contouring could explain partially why patients who underwent plastic surgery gained less weight, in comparison to those how had still an adipose-cutaneous excess.

Moreover, obesity creates a local micro-environment in adipose tissue that promotes inflammation and fibrosis. This inflammatory condition could play a role in metabolic complications and cardiovascular disease. As described before (Sect. 2.2.1) weight loss is associated with a decrease fibrosis, but the pathological excess fibrosis created by obesity is not completely resorbed and may alter the remodeling of adipose tissue and limiting the fat loss.58

Body-contouring surgery by reducing a part of this residual “pro-inflammatory and fibrotic adipose tissue” could contribute to weight stability and maybe to reduce metabolic diseases. In
a meta-analysis performed by Boriani et al., and a review by Esposito et al., it has been demonstrated that adipose tissue mass reduction even by liposuction alone, could reduce adipokines and improve metabolic disease, particularly insulin resistance. \(^{37,92}\)

In resume, we concluded that plastic surgery plays an important role in treatment of patients after massive weight loss. We demonstrated that body contouring, despite important scars, significantly improves satisfaction, HRQoL and weight of patients after gastric bypass. Therefore, the treatment of morbid obesity should not be deemed achieved unless plastic surgery has been considered. Data of our clinical studies have to be used as an argument in favor of cost coverage of body contouring by health insurances. These procedures should be considered as a reconstructive surgery of obesity and the treatment of its sequela.

### 2.3 Tissue regeneration

For decades, fat tissue has been considered as a demonic tissue, and different technique and treatment have been developed to reduce the fat body mass. Recently, and particularly in 21\(^{st}\) century, an important interest has been grown among the scientific and medical community, to take benefit from advantages of adipose tissue, a precious organ. This particular attraction comes from the fact that adipose tissue is a unique and multifunctional tissue, mostly abundant and rich in regenerative cells. At first, adipose tissue was used by plastic surgeons to restore soft tissue volume by transferring it from one site to another. Later on, once stem cells richness of adipose tissue was discovered, particular attention grown for its use in tissue regeneration, tissue engineering and even cell therapy.

#### 2.3.1 Autologous fat transfer

The transplant of autologous fat tissue, so called lipomodelage, liposculpture or lipofilling, shows an increase interest since the end of 20\(^{th}\) century. The principle of the technique is to transfer patient’s own fat tissue from a donor site (e.g. abdomen, flanks) to a site where there is a volume deficit.
Despite Neuber’s use of fat transfer in 1893 by transplantation of a lipoma to a breast, the first 
description of the fat transplantation by infiltration dates to 1962 by Miller. Given the little 
satisfactory long-term results, this technique did not have the expected success and other 
unsuccessful methods were described, until that developed by Coleman in 1986.93 His method 
takes into account the fragility of fat cells during manipulation and the importance of oxygen for 
fat grafts. Given better results offered by Coleman’s technique, at present, it is the most used 
method for the fat harvesting, purification and infiltration. Its first indications were for aesthetic 
surgery of the face.94 Nowadays, fat grafting is also used to reconstruct a soft tissue lost due to 
a trauma, a surgery, a congenital disease or lipodystrophy. 95 In addition to a volumizing effect, 
the transferred fat leads to a neoangiogenesis effect.96 As described (Sect. 2.1.3), adipose tissue 
is an angiogenic tissue thanks to several pro-angiogenic factors (e.f. VEGF, FGF) that it 
produces.28 Fat grafting is thus also recommended for antiaging treatment97, wound healing98, 
scar reduction99 and radiodermatitis treatment.100 
Main advantages of fat grafting include a) a long lasting result contrary to the synthetic 
resorbable products such as hyaluronic acid, b) prevention of granuloma and allergic reactions 
which could be provoked by the permanent injectable products, c) a natural consistency and d) 
an improvement of cutaneous and subcutaneous trophicity. On the other hand, disadvantages 
of autologous of fat grafting are a) its complexity, requiring a more important learning curve 
with regard to the prepared products, b) the availability of the donor site, that sometimes could 
not be enough, c) the morbidity of the donor site, and principally d) the unpredictability of the 
remaining volume by partial uncontrolled absorption rate of grafted fat.101,102 
The survival rate and longstanding results depend partially on indications and patients, but 
mostly on surgical technique. However, even with the best surgical technique, the fat grafting 
survival is variable, with a variable resorption rate reported throughout the literature of 10 to 
90%. 
Directly after the transplantation, the grafted fat tissue undergoes a state of acute ischemia, 
which, if it persists, leads to adipocytes necrosis and subsequent oily cysts formation, fibrosis, 
and calcification.103 The fat tissue has to be revascularized at the transplantation site within 48 
hours. During this time, it is fed by diffused material in the plasma. Eto & al. have demonstrated
that after the transfer of fat into the receptor site, 3 zones appear in the fat graft. On the most superficial zone, surviving zone, adipocytes will survive. In the second zone, regenerating zone, most stem cells and pre-adipocytes survive and after one week they are differentiated into adipocytes. And in the most central part, the necrotizing zone, cells die and adipogenesis did not occur and many inflammatory cells are observed after 2 weeks.\(^5\) (see Fig. 12)

This dynamic remodeling after fat grafting depends on the microenvironment, particularly on the vascularity of the receptor zone. Therefore effective neoangiogenesis into the fat graft is one of the most crucial aspects for adipocyte survival\(^5,104\) and adipogenesis (as detailed in section 2.1.3) to give a long-term satisfactory result.

Thus the main obstacles preventing permanent augmentation are ischemia and partial absorption of the transplanted fat tissue, which often necessitate multiple transplantations. Then, the quality of transplanted tissue becomes highly dependent on the healing process, restoration of vascularization and adipocyte differentiation. Different techniques have been proposed to improve the survival rate of fat grafting and its predictability. The most efficient technique proposed until today is to highly enrich the grafted tissue on mesenchymal stem cells.\(^105\) However most of these technique are time consuming and expensive with significant harvested fat loss; nevertheless the results still remain unsatisfactory.\(^106\) Another approach to improve the fat grafting results could be stimulation of transplanted tissue by growth factors. Unfortunately, the exogenous and synthetic growth factors treatment has not provided the desired expected results in clinic. One of the reasons is the protein fragility and instability of growth factors once they have been directly injected. To address the important disadvantage of fat grafting resorption, we propose the addition of autologous platelet rich plasma (PRP).\(^107\)
2.3.2 Fat grafting and PRP (Paper 3)

Platelet-rich plasma (PRP) is blood plasma that has been enriched with platelets. Platelets are enucleated circulating blood particles that derive from the fragmentation of megakaryocytes. They are known as a natural reservoir of growth factors (e.g. PDGF, TGFβ, IGF, VEGF and EGF), that physiologically activates angiogenesis, cell proliferation, cell differentiation and new matrix formation for tissue repair.

PRP could be used safely as an autologous product by a simple bedside technique preparation. There is broad variability in the production of PRP by various equipment and techniques. With any methods, patient's whole blood (8-50ml) is collected in a specific tube containing an anticoagulant. After few minutes (5-20 minutes) of centrifugation, the PRP aliquot is separated from red and white blood cells. In humans, the typical baseline blood platelet count is approximately 200,000 per µL; therapeutic PRP concentrates the platelets by roughly 3-5 fold.\(^\text{108}\)

PRP was first developed in the 1970s but PRP therapy began gaining popularity in the mid-1990s. It has since been applied to many different medical fields. The benefit and safety of PRP is documented in more than 5,000 studies where the authors observed enhancement of bone regeneration\(^\text{109-112}\), wound healing,\(^\text{112-115}\) tendon and cartilage healing\(^\text{116-119}\), corneal healing\(^\text{119,120}\) and skin rejuvenation.\(^\text{110}\)

The efficacy of growth factors within PRP is the theoretical basis for the use of PRP in tissue repair. Taking advantage of angiogenic and regeneration capacities of PRP, we proposed the combination of fat graft with PRP to improve long-term result of fat grafting. We hypothesized that PRP growth factors, by increasing the vascularization of the grafted tissue, and enhancing the adipocytes survival and stem cells proliferation, would augment the “surviving” and “regenerating” zone of fat graft (Sect. 2.3.1). We published this new concept on 2013, and concluded that in clinic, the addition of PRP might improve fat grafting survival and would make fat graft application easier.\(^\text{107}\) (Annexe: Paper 3)
2.3.3 Adipose regenerative cells

Adipose tissue contains a high concentration of regenerative cells, including mesenchymal stem cells (MSCs). MSCs are multipotent cells that have the ability to differentiate to mesodermal lineages such as adipocytes, osteocytes, and chondrocytes. The use of MSCs has gained significant interest in many medical fields particularly for regenerative medicine and tissue engineering. There have been promising results in the therapy of myocardial infarction, liver cirrhosis, cornea damage and other disorders where tissue regeneration is crucial.\textsuperscript{121} For many decades, bone marrow has been used as the main source of MSCs.

2.3.3.1 Adipose tissue-derived mesenchymal stem cells (AT-MSC)

Presently, an increasing interest is devoted to MSC isolated from adipose tissue (AT-MSC).\textsuperscript{122,123} Adipose tissue, as a MSCs source, presents several advantages in comparison to bone marrow: (i) adipose tissue is easier to harvest, (ii) it is widely available, (iii) MSC concentration is \~1000x more and (iv) AT-MSC grows faster in culture.\textsuperscript{123,124} Zuk et al.\textsuperscript{123} were the first to demonstrate that AT-MSCs are similar to bone marrow-derived mesenchymal stem cells (BM-MSCs) but not identical. Each of them has specific genomic and proteomic markers. As BM-MSCs, AT-MSCs are multipotent but more easily oriented toward an adipose phenotype, whereas BM-MSCs into osteoblasts and chondrocytes.\textsuperscript{125}

AT-MSCs are mostly pericytes\textsuperscript{126} that could be in perivascular position and be obtained from the stromal vascular fraction (SVF) of adipose tissue.\textsuperscript{123,127} SVF can be extracted from adipose tissue simply by collagenase and centrifugation. SVF is strongly heterogeneous and contains several cellular subpopulations, including AT-MSCs, endothelial cells, and hematopoietic cells which represent a large portion of the fraction (20-50\%).\textsuperscript{128}

Several clinical studies using SVF or only AT-MSC have been produced in the osteoarticular, cardiovascular, immunomodulation and wound healing field. Most successful results are currently in the area of modulating immune system and inflammation: treatment of autoimmune encephalomyelitis\textsuperscript{129}, arthritis\textsuperscript{130}, perianal fistula\textsuperscript{131} or host-versus-graft disease.\textsuperscript{132}
2.3.3.2 **ROS influences AT-MSC differentiation (Paper 4)**

Pre-adipocytes are differentiated from AT-MSCs. However, factors that regulate their proliferation and differentiation are not very well known. Factors as FGF2\(^{133}\), TGF\(\beta\)\(^{134}\), and miARN \(^{135}\) have been demonstrated to play a key role. Through a literature review, we demonstrated that reactive oxygen species (ROS), an oxygen derived molecule, influence tightly MSCs differentiation: it increases adipogenesis while MSCs osteogenesis is blunted by ROS.\(^{136}\) (Annexe: Paper 4) In the future, ROS modulators could be used to bring under control MSC differentiation for tissue engineering use or cell therapy.

2.3.3.3 **PRP as an ideal culture media for AT-MSC expansion (Paper 5)**

The possibility to obtain the SVF quickly and simply from adipose tissue on patient’s bedside presents a considerable benefit. Another advantage comes from the synergistic effects existing between different cellular populations in SVF. These advantages are offset, by the heterogeneity of this fraction since the cellular composition varies considerably depending on the patient, the difficulty to establish quality controls due to brief time allocated and the limited number of cells. Only cell culture would permit to have a more homogenous product and more cell numbers with a strong quality control. Moreover, although adipose tissue is highly concentrated on AT-MSCS, for most tissue engineering or cell therapy uses, the number of MSCs is not enough if collected directly from adipose tissue.

Ex vivo cell culture is therefore mandatory for most clinical applications of AT-MSCs. Cell expansion requires a basal medium supplemented with proteins, growth factors, and enzymes. Classical protocols use culture media supplemented with xenogeneic additives (e.g., fetal calf serum or fetal bovine serum (FBS)). These non-autologous products present a potential risk of infection and immunological reaction. Furthermore, they are inefficient as MSCs culture is slow. Therefore, a safe and effective culture supplement is urgently needed to comply at best with national and international regulatory agencies’ requirements for clinical applications of MSCs. The ideal cell culture media could be an autologous product.

To define an autologous system for AT-MSC proliferation, we proposed the use of autologous PRP, a plasma riche on platelets known to secrete orchestrated growth factors. (Sect. 1.3.2) In a study, we assessed in vitro the efficiency of autologous PRP on AT-MSC proliferation in
comparison to the classical FBS-supplemented medium. We demonstrated that the culture media supplemented with PRP showed dose-dependent higher AT-MSC proliferation than did FBS. Twenty percent of PRP was the most effective concentration to promote cell proliferation. This condition increased 13.9 times AT-MSC number in comparison to culture with FBS, without changing the AT-MSC phenotype, differentiation capacity, and chromosome status. We concluded that autologous PRP is a safe, efficient, and cost-effective supplement for AT-MSC expansion, and it should substitute current non-autologous culture media, even for other cells than AT-MSC.¹³⁷ (Annexe: Paper 5)

Then after we aimed to assess the mechanism of action of PRP on AT-MCSs proliferation and elucidate the extra- and intra-cellular pathways involved in that. We submitted recently a paper, in which we concluded that PRP regulates AT-MSCs proliferation mainly through secreted growth factors (PDGF-AB, FGF, TGFβ1, VEGF and MIF). And after binding to their specific receptors, they mainly activate AKT and Smad2 signaling pathways.
3 Perspectives

3.1 Fat grafting and PRP: in vivo experiments

Since our publication of combined use of fat grafting with PRP, this technique has gained an important interest, and it is actually widely used by plastic surgeons. Several studies have confirmed this positive effect of PRP on fat graft. However the type of PRP to be used, its concentration, and its mechanism of action is not yet elucidated. We believe that growth factors, particularly angiogenic factors, present in PRP are key elements for its positive effect on fat graft. PRP could increase the surviving zone described by Eto & al. Furthermore, as we demonstrated that PRP increases AT-MSC proliferation, it could also augment the regenerative zone in the fat graft, and decreasing therefore the necrotic zone, resulting in a better fat graft survival. (see fig. 13)

To confirm and assess more in detail the effect of PRP on fat grafting survival, we actually realize animal experiments. We aim to investigate the efficacy of PRP in enhancing fat graft survival that has been injected in mice. Up to 90 days, the volume of fat graft will be measured by micro-CT-scanning and viability of adipocytes in fat grafts will be assed with immunohistochemistry assay. These data will be compared to control animals that received fat graft without PRP. A better understanding of the efficacy of PRP in enhancing fat graft survival could improve current technologies.

Moreover PRP is actually a very heterogeneous product: on one hand different companies propose different techniques preparation, and on the other hand, PRP as an autologous product has a high inter-individual variability. Therefore, a standardization of PRP products is needed. To achieve this goal, first of all, studies have to be undertaken to define the best PRP characteristic (e.g. platelet concentration, plasma purity) that have to be used for each indication. Then after, new methods have to be developed to measure PRP characteristics simply, fast and cost-effectively.
3.2 Adipose derived mesenchymal stem cells for wound healing enhancement (SNF application)

MSCs have been demonstrated to play a critical role in tissue repair and regeneration.\textsuperscript{123} MSCs are believed to migrate to a specific destination of damaged tissue in a process called homing. When the skin is injured, MSCs from the surrounding tissue or those mobilized from bone marrow or adipose tissue are attracted to the wound site.\textsuperscript{138} Once MSCs arrive at the wound site, they respond and modulate their function and differentiation as they are exposed to biochemical factors characteristic of an injured environment.\textsuperscript{139} In response to inflammatory molecules, MSCs produce crucial growth factors promoting wound repair by increasing fibroblast, epithelial and endothelial cell division as well as neo-vascularization.\textsuperscript{140} MSCs could also play a role in the wound’s ability to progress beyond the inflammatory phase and to avoid a progress to a chronic inflammatory phase by decreasing secretion of pro-inflammatory cytokines and increasing the production of anti-inflammatory cytokines.\textsuperscript{141} Furthermore, MSCs seem to play a primordial role in wound neo-vascularization as they promote proliferation, migration, and differentiation of endothelial cells.\textsuperscript{142} Further research should focus on the fate of MSCs in order to establish the role of MSC differentiation during wound repair.

Several studies described the potential use of MSCs for the treatment of chronic wounds\textsuperscript{143}, diabetic foot ulcers\textsuperscript{144, 140, 141}, limb ischemia\textsuperscript{145}, radiodermitis\textsuperscript{146} and even pressure ulcers.\textsuperscript{147} The majority of these studies have used bone marrow-derived MSCs, and various methods of administration have been used, from topical application with or without carriers to intramuscular injections. The engraftment of MSCs into damaged tissues is crucial for their efficacy in enhancing tissue repair and regeneration. The optimal method of delivering MSCs in wound therapy is still a matter of controversy. Local administration will require an appropriate carrier to be used with the MSCs. Their viability, migratory capacity as well as the diffusion of their growth factors into the wound is also questionable. Systemically administered MSCs are an attractive option for regenerative medicine as they have been shown to have the ability to home to sites of acute inflammation. For instance, it has been reported that systemically injected MSC preferentially home to ischemic myocardium.\textsuperscript{148} Systemic administration mimics the physiological route that MSCs would take to the target destination. However, the main
disadvantage is side uptake of MSCs in non-target organs such as the lung. Efforts to enhance MSC homing to sites of injury and inflammation are hampered by a lack of understanding of the mechanisms of MSC recruitment and migration. Improved understanding of how AT-MSCs are recruited and how they exert their therapeutic effects to the injured site is a crucial process that needs to be understood to achieve clinical efficiency in wound repair.

On the other hand, although most studies show promising results of AT-MSCs use for wound healing and met safety endpoints, efficacy has not yet been clearly established. So, further studies are still needed to investigate a) the benefit of AT-MSCs, in comparison to bone-marrow derived MSCs, b) the optimal administration method, c) timing and frequency of AT-MSCs administration, and d) the number of cells to administer.

To answer to these questions we developed a project that has been submitted for Swiss National Fond (SNF) in 2015, and started preliminary experiments from January 2015. The main aim of this project is to determine whether systemically administered AT-MSCs promote wound healing. To achieve this goal, first of all we need to understand the homing capacity of AT-MSCs in vitro and in vivo. We will investigate the homing capacity of ASCs in vitro by considering: (1) the interaction between ASCs and endothelial cells by assessing the expression of cell surface markers believed to be important for homing and correlating this at the level of gene expression; (2) the role of adhesion molecules, chemokines and cytokines in ASC migration; and (3) the transmigration of ASCs through or integration within the endothelium under conditions of shear flow.

To study the homing capacity of systemically administered AT-MSCs and to evaluate their therapeutic effect on wound repair, we are going to use the animal model of chronic wounds that we have developed previously in our lab. Each rat with a ischemic or not-ischemic wound on the limb will receive systemic AT-MSCs isolated from isogenic rat inguinal fat pad and cultured in isogenic PRP as a media supplement. The homing capacity of AT-MSCs will be assessed by tracing techniques. Planimetry and digital photography of the wound on each time point will allow us to follow the progression of wound closure in comparison to rats without AT-MSCs treatment. We expect to gain knowledge regarding the distribution of AT-MSCs in response to inflammation and tissue damage as well as the effect of AT-MSCs to improve wound repair, to translate this innovative regenerative cell therapy to clinic.
3.3 *In situ* fat tissue augmentation (CTI project)

Addressing the soft tissue volume loss is an important issue in plastic, reconstructive and aesthetic surgery. Soft tissue augmentation could be needed after a trauma or surgery as well as in ageing. Although in some cases a reconstruction with tissue flaps, a complex procedure, is mandatory, often just filling the subcutaneous tissue with a “volumazaitor” is enough. Nowadays, besides implants for breast augmentation, the most used techniques for soft tissue augmentation are either autologous fat grafting or injection of synthetic product (e.g. hyaluronic acid, collagen). Even there is an increase demand for such kind of procedures, none of them are ideal. Main disadvantages of actual synthetic products are their complete resorption after ~6 months for resorbable products such as hyaluronic acid or the high risk of granuloma for “permanent” products like calcium hydroxyapatite. Fat grafting, although its numerous advantages, has a resorption rate of 30 to 80% which is a major issue requiring often multiple surgical procedures. Moreover, some patients do not have enough fat depots to be harvested.

Recently, there is tempting to pharmacologically target one or several steps of triglyceride synthesis to modulate the adipocytes volume and to improve autologous fat grafting survival. However, since lipids are ubiquitous, a systemic action could have dramatic consequences by forwarding fatty acids to other tissues (e.g. liver, vessels) and triggering lipotoxicity. In addition this may affect the cell signaling and cell’s integrity as lipids play a key role in these processes. Therefore, a solution that offers the possibility to “nourish” adipocytes in situ, and only in the desired site would be ideal.

For reminder, an adipocyte is fundamentally composed of triglycerides (up to 80-95% of volume) which generally derive from extracellular sources. Lipids enter adipocytes in the form of fatty acids. This transport and intracellular lipid storage, adipogenesis, are orchestrated by different factors such as insulin as detailed in Sect. 2.1.2. Taking in account all these phenomenon, and the clinical need for *in situ* adipocyte augmentation, in collaboration with a start-up (PB&B) and Ecole Polytechnique de Lausanne (EPFL) we are developing an innovative product.
Innovation resides in the sustained delivery of lipids by a biodegradable microparticles. Injecting lipids alone into fat tissue degrade and diffuse rapidly, leading to two main problems: one being a short lived effect and the second being the risk of inducing side effects in an unintended area of the body. The most promising solution to solve these problems is to have a localized depot of these lipids that can be released in a controlled manner.

In our innovation, we aim to use microspheres encapsulating lipids that will be released slowly over a precise period of time. Furthermore to improve adipogenesis locally, lipid absorption by adipocytes and their storage, bioactive molecules known to be adipogenic could be added to these microspheres and be released over time.

The designed product would be composed of 3 main elements:

1) a selection of lipids that are known to increase efficiently adipocyte’s volume (e.g. oleic acid, linoleic acid),
2) biological factors recognized for their lipogenic effect (e.g insulin, FGF) and
3) a biocompatible slow-degradable microspheres made of polylactic-glycolic acid (PLGA) containing lipids and biological factors that they release over time. (see Fig 14)

The final product would be an injectable liquid that physicians can inject, like hyaluronic acid, to patients to enhance soft tissue volume “permanently”. We expect that the volumator effect will last for a long term. As mentioned (Sect. 2.2.2) once adipose tissue reaches a volume, they tend to conserve their volume by an autoregulation system. Moreover, as Spalding et al. demonstrated, adipocytes have a 10 years live. And interestingly this long live is independent of adipocytes size changing.

We performed preliminary in vivo experiments by injecting our microspheres to mice inguinal fat pad. They suggested that the specific microspheres, encapsulating lipids and biological
factors, enhance significantly the inguinal fat pad volume compared to all controls on day 15 and day 30 after injection. (see Fig. 15)

**Figure 15** Sample image depicting the 3D CT scan reconstruction of mice inguinal fat pad (bleu) 15 days after injection of empty PLGA microspheres (left image) and lipid loaded microspheres (right image). Significantly the loaded microspheres enhance the volume of the inguinal fat pad 15 days compared to empty microspheres.

Based on these encouraging results, we obtained a Commission for Technology and Innovation (CTI) funding. The aim of this CTI project is to conduct the pre-clinical trials necessary to initiate clinical trials and bring to market a product for use in plastic, reconstructive and aesthetic procedures.

The overall goals of this project in collaboration with EPFL are: 1) proof of technology in terms of efficacy and durability of the results, 2) producing safety and pharmacokinetic data, 3) optimizing the formulation and 4) establishing concrete protocols for production and quality control of microspheres to be used for clinical testing and scale up in the future.
4 Conclusion

Adipose tissue, formerly decried as the main responsible for metabolic diseases, has acquired an entirely different status of a useful regenerative cells reservoir.

With increasing prevalence of obesity, particularly in children, prevention of the obesity, specifically at the young child is primordial. As the adipocyte hyperplasia during childhood or adult excessive weight gain seems mostly irreversible, this prevention is significantly more efficient than the overweigh treatment.

New knowledge about the adipose tissue behavior and its related comorbidities opens new therapeutic perspectives for weight loss. Anti-inflammatory or lipolysis moderators could be in future the main approaches for adipose-related comorbidities treatment.

Currently for morbid obese patients, the gold standard treatment is bariatric surgery. Besides its efficiency for weight and comorbidities reduction, it provokes often bothering skin excess that has to be corrected by plastic surgery. Unfortunately, this procedure is considered as an “aesthetic treatment” by health insurance companies that don’t usually reimburse the cost. It is therefore the duty of scientific community, to demonstrate that plastic surgery after massive weight loss is a cost-effective reconstructive surgery. Morbid obesity is a real disease. Therefore, skin excess should be considered as a sequela of obesity treatment. And the cost of body contouring, considered as the treatment of this sequela, should be covered by health insurance.

On the other hand, adipose tissue gained a significant interest as a promising and cost-effective source of autologous stem cells that can be used for tissue engineering, tissue regeneration or cell therapy.

To make the AT-MSCs culture safer and more efficient for bench-to-bed translation, autologous PRP could substitute all non-autologous culture media that are used at present. As a concentrated source of growth factors and cytokines, PRP is nowadays recognized as a new developing area for clinicians and researchers. Autologous PRP could be applied in the future with safety for
expansion of other cell types than AT-MSCs, such as keratinocytes culture for burns or Langerhans pancreatic cells for diabetics. The abundance of regenerative cells in adipose tissue raises the possibility to consider adipose tissue as a physiological cell reservoir that can be recruited in the event of an injury or tissue regeneration. However, the enthusiasm for stem cells therapy has to be moderated by the fact that our current knowledge is limited. Nowadays, we don’t have enough skills to control the multipotency properties of stem cells. The immunosuppressive effects associated with the angiogenic properties of AT-MSCs raise the crucial question of a possible interaction between stem cells and cancer cells. Before to treat any of our patients with “stem cells”, all the scientific steps required to confirm the safety and efficiently of the treatment have to be respected. In vitro and in vivo experiments are essential, and clinical trials primordial.
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Adipose tissue: obesity vs regeneration

Adipose tissue: obesity vs regeneration


Adipose tissue: obesity vs regeneration


6 Annexes

6.1 Paper 1: Plastic surgery after gastric bypass improves long-term quality of life

Plastic Surgery After Gastric Bypass Improves Long-Term Quality of Life

A. Modarressi • N. Balagué • O. Huber • M. Chilcott • B. Pittet-Cuénod

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Abstract

Background Excess skin after massive weight loss impairs patient’s health-related quality of life (HRQoL). Therefore, body-contouring surgeries can be proposed. However, few data exist concerning the effect of body contouring after bariatric surgery on HRQoL, including control group with a long-term follow-up.

Methods In a prospective study, 98 consecutive patients who had body contouring after gastric bypass for obesity (BMI > 40) were included (group A). A matched control group containing 102 patients who had only gastric bypass was selected (group B). HRQoL was measured by Moorehead-Ardelt questionnaire before (group A1) and after (A2) body contouring, and at different time points for group B until 8 years post-gastric bypass. To evaluate the effect of body contouring by two parallel methods, HRQoL was compared between groups A1 and A2, and between A2 and B.

Results We found that body contouring procedures improved significantly patients’ HRQoL, in comparison to those who had only gastric bypass. Of the patients who had body contouring (group A2), 57% evaluated their HRQoL “much better” in comparison to only 22% of patients before body contouring (group A1) or those who never had body contouring (group B) (p<0.001). The improvement was significant in all sub-domains of HRQoL: self-esteem, social life, work ability, sexual activity and physical activity (p<0.001), and remained stable over time.

Conclusions Our study confirms the important role of plastic surgery in treatment of patients after massive weight loss. We demonstrated that body contouring, despite important scars, significantly improves satisfaction and HRQoL of patients after gastric bypass. Therefore, the treatment of morbid obesity should not be deemed achieved unless plastic surgery has been considered.

Keywords Obesity • Plastic surgery • Quality of life • Body contouring surgery • Gastric bypass • Bariatric surgery

Introduction

Obesity carries several co-morbidities (e.g. cardiovascular diseases, diabetes, cancer) that conspire to double mortality [1]. Furthermore, most overweight patients suffer from health-related quality of life (HRQoL) impairments and other psychosocial distresses [2, 3]. For these patients, the main purpose of therapy appears more to be the improvement of their physical image, self-esteem and quality of life rather than the search for weight loss and correction of related medical co-morbidities [4, 5].

Regrettably, most medical treatments associating diet, physical exercise, eating behaviour modifications or drugs are ineffective in most cases for patients presenting a BMI more than 40 kg/m² [6]. With a positive risk–benefit balance, bariatric surgery (from Greek baros, weight, and iatrikos, being a part of the medicine) has become the treatment of choice of morbid obesity [7]. Among surgical options,
Roux-en-Y gastric bypass (RYGBP) is presently considered the gold standard [7–12]. In the last 15 years, these interventions have more than quintupled to reach more than 100,000 operations per year in the USA [13]. This procedure achieves the best weight loss and co-morbidity improvement [6, 14] offering a 40% decrease in mortality [15, 16], with the lowest complication rate at short- and long-term follow-up. Bariatric surgery was shown to improve HRQoL too [3], but after massive surgical weight loss, up to 95.6% of patients report residual morphology dissatisfaction associated with loose sagging skin [17]. This cutaneous excess invalidates the patients in daily life (e.g. mechanical limitation of physical activities, hygienic problems caused by intertrigo or maceration) and can induce, despite considerable weight loss, severe psychosocial problems because of a lack of self-confidence and a disturbed physical image [18]. More than two-thirds of patients who have undergone bariatric surgery consider their excess skin as a negative consequence of surgery [19]. They often mask their deformities by clothing and limit themselves to superficial social relations. Furthermore, the disappointment is stronger if weight loss is massive [19]. The patients who have gained years of life expectancy and have a new vision of life desire a corrected silhouette to fully regain self-esteem and to function normally in society.

This dissatisfaction motivates 74% of patients to seek body contouring (BC) procedures after bariatric surgery, but only 21% achieve at least one of them [17]. According to the American Society of Plastic Surgeons, BC concerned 52,603 patients in the USA in 2010.

The basic principle of all of these operations is to tighten the cutaneous tissue to reach a harmonious silhouette and to eliminate physical or psychological handicap bound to the excess skin. It is thus essentially a functional surgery which is going to improve the silhouette, but at the price of a scar.

Several publications have already demonstrated the cost-effectiveness of RYGBP for morbid obesity treatment and underlined patients’ HRQoL improvement after bariatric surgery [8–10]. With the explosion in the number of plastic surgery interventions after massive weight loss, it seems necessary to estimate its cost-effectiveness as well. Its relative costs have to be compared with its psychological, social and long-term functional results. Some outcome data following body contouring have been reported regarding HRQoL [20–22]; however, few data including control group and regarding long-term effect exist. The purpose of our study is to measure the contribution of BC on HRQoL after RYGBP by two parallel methods: firstly by comparing patients HRQoL before and after BC and secondly by comparing between a group of patients with BC after RYGBP and a control group with RYGBP alone.

Methods and Procedures

Subjects

Two groups were sorted:

Group A (patients with RYGBP and BC): 98 consecutive patients (89.8% females, mean age 42.6 [34–55 years]) who had BC procedures after RYGBP were included. All had been submitted to RYGBP for morbid obesity (BMI > 40) at least 18 months before plastic surgery, with stable body weight during the last 6 months.

Group B (patients with RYGBP only): for each patient from group A, a matched patient was randomly selected from a total of 538 patients with RYGBP alone. These patients without BC had either no demand for plastic surgery or did not undergo BC because health insurance did not cover the cost. Patients included in this group (102; 81.4% females, mean age 38.6 [31–48 years]) were each matched to a patient in group A by decreasing order of criteria importance of pre- and post-RYGBP BMI, excess body weight loss (EBWL), age and gender. It has to be noted that four patients in group A had exactly the same selection criteria with two corresponding patients in group B. To reduce bias selection, both of them were included in this group, and group B therefore includes four supplementary patients.

To evaluate the impact of BC on HRQoL in a consistent way and decrease the statistical bias, two parallel studies were designed. HRQoL was compared (a) prospectively in the same cohort of patients before (group A1) and at least 6 months after BC (group A2) and (b) between patients of group A2 and group B.

All subjects in the study originally presented BMI more than 40 kg/m² (mean BMI 46.4 kg/m² [41–48]). After an initial EBWL of 68.4% [52.8–80.7%] with RYGBP in group A and 64.2% [52.8–76.2%] in group B the mean BMI was 29.9 [26–34 kg/m²]) and 31.2 [28.34 kg/m²] respectively, with non-significant differences between groups A and B up to 2 years post-RYGBP (p > 0.05), the mean time point when BC was proposed. All other criteria (e.g. age, gender, follow-up time) were also comparable between groups A and B (Table 1).

Experimental Design

Demographic, personal and weight data were prospectively collected for all patients. In group A, HRQoL was assessed during two interviews: before BC and at least 6 months (mean 26 months [18–84]) after BC. In group B, subjects were submitted to the HRQoL questionnaire only once, 18 months to 8 years post-RYGBP (mean 24 months [18–96 months]). The study protocol was reviewed and approved by the local Clinical Ethics Committee.
Table 1 Data of groups A and B: no significant difference between these two groups during the period pre-gastric bypass (RYGBP) to 2 years post-RYGBP when body contouring (BC) was proposed to patients in group A

<table>
<thead>
<tr>
<th></th>
<th>Group A, Bypass and BC (N=98)</th>
<th>Group B, Bypass only (N=102)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (SD), IQR</td>
<td>42.6 (11.1), (34–55)</td>
<td>38.6 (10.1), (31–48)</td>
<td>NS</td>
</tr>
<tr>
<td>Women, N (%)</td>
<td>88 (89.8 %)</td>
<td>83 (81.4 %)</td>
<td></td>
</tr>
<tr>
<td>Pre-RYGBP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²), mean (SD), IQR</td>
<td>46.0 (5.1), (42–48)</td>
<td>46 (7.7), (41–48)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg), mean (SD), IQR</td>
<td>122.6 (17.5), (110–132)</td>
<td>125.3 (24), (109–140)</td>
<td>NS</td>
</tr>
<tr>
<td>2 years post-RYGBP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²), mean (SD), IQR</td>
<td>29.9 (5.1), (26–34)</td>
<td>31.2 (6.6), (28–34)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg), mean (SD), IQR</td>
<td>79.7 (15.9), (68–90)</td>
<td>82.7 (19.8), (71–93)</td>
<td>NS</td>
</tr>
<tr>
<td>EBW (%), mean (SD), IQR</td>
<td>113.0 (23.5), (94–126)</td>
<td>111.5 (36.7), (89–126)</td>
<td></td>
</tr>
<tr>
<td>EBW loss (%), mean (SD), IQR</td>
<td>68.4 (16.3), (58.2–80.7)</td>
<td>64.2 (17.7), (52.8–76.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

SD standard deviation, IQR interquartile range, NS non-significant (p>0.05), BMI body mass index, EBW excess of body weight

Surgical Procedures

Bariatric Surgery

After multidisciplinary evaluation, a fully standardized RYGBP (gastric pouch ≤ 30 ml, alimentary loop 150 cm, bilio-pancreatic loop 50 cm) was performed by the surgeons of our surgical department by laparotomy until 2001 and laparoscopically thereafter.

Plastic Surgery

BC was performed on group A patients only: 97% abdominoplasties (with 47% incisional hernia repair), 32% mammoplasties (51% mastopexy alone, 33% breast reduction and 16% breast augmentation with or without breast lift), 19% europlasties and 14% brachioplasties; 45% of the patients had a combined procedure in one or several operation sessions.

Quality of Life Investigation

HRQoL was assessed at each time point by using the Moorehead–Ardelt [23] questionnaire, which is the HRQoL part of the “Bariatric Analysis and Reporting Outcome System”. This questionnaire was created to specifically evaluate the outcome of bariatric surgery, and it is widely used by national and international bariatric surgery associations. It evaluates five domains of HRQoL: self-esteem, physical activity, social life, work ability and sexual activity. For each domain, patient evaluates “much better”, “better”, “same”, “worse” or “much worse” his status on the time that he answers to the questionnaire in comparison to his status before bariatric surgery. Results are summarized in a total score (-3.0 to +3.0) which is the sum of the self-esteem score (-1 to +1) and the four other domains (-0.5 to +0.5 for each). Total score is estimated as “much better” (scores +2.25 to +3), “better” (+0.75 to +2), “same” (+0.5 to –0.5), “worse” (-0.75 to -2 points) and “much worse” (-2.25 to –3).

Statistical Analysis

Values are shown as the mean ± SD. Comparisons before and after, firstly RYGBP and secondly plastic surgery, were done by paired two-tailed Student’s t test to a significance level of 5 % (p<0.05).

Results

RYGBP Alone Improves HRQoL

The quality of life was evaluated as “better” by 65 % of patients and “much better” by 22 % after RYGBP alone (groups A1 and B) (Fig. 1). This improvement was essentially important for self-esteem (89 %) and physical activity (88 %). Social life and work ability were improved in 63 and 61 % of patients, respectively. Only 38 % of patients evaluated their sexual activity as improved.

HRQoL Improvement Is Directly Related to EBWL

Of patients who had more than 75 % EBWL, 97.8 % estimated that their quality of life improved (mean total score 1.84) (Fig. 2). In comparison, among patients with 51–74 % EBWL, HRQoL improved by 87.6 % as estimated (mean total score 1.47). The corresponding figures for the groups 26–50 % EBWL and <25 % EBWL were 72.7 and 50 %, respectively (mean total scores 1.1 and 1.0). The differences between these four groups were statistically significant (p<0.01).
Plastic Surgery Further Improves HRQoL

After plastic surgery, in comparison to the scores achieved after RYGBP alone, the total score was significantly improved as in all domains of HRQoL (Fig. 3, Table 2). In group A2, 98% of patients estimated that their quality of life improved after BC ("much better" 58%, "better" 40%) in comparison to 85% (group A1) before BC ("much better" 22% and "better" 63%) with a mean total score of 1.95 vs. 1.5 (p<0.001) (Table 2, Fig. 3).

This improvement was significant after BC in all domains of HRQoL comparing group A2 to A1: self-esteem (98 vs. 89%, mean score 0.85 vs. 0.71, p<0.001), social life (87 vs. 62%, mean score 0.3 vs. 0.2, p<0.001), work ability (76 vs. 66%, mean score 0.24 vs. 0.19, p<0.001), physical activity (92 vs. 88%, mean score 0.38 vs. 0.32, p<0.05) and sexual activity (65 vs. 43%, mean score 0.18 vs. 0.07, p<0.001).

Discussion

The WHO defines quality of life as "the individuals' perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns" [24]. It is a broad concept affected in a complex way by the person's physical health, psychological state, level of independence, social relationships, personal beliefs and their relationship to salient features of their environment.

For bariatric-surgery-seeking patients, the quality of life is an important concern: Psychosocial impairment is the main motivation for bariatric surgery in 66% of cases, in
comparison to 10% only for medical reasons [25]. And most of these psychosocial variables, including self-esteem and HRQoL, improve dramatically in the first years after bariatric surgery [26]. However, these parameters remain lower than those of the general population with the same weight but who were never obese. This could be explained partially by the excess skin resulting from massive surgical weight loss, which may be corrected by BC. Some previous publications of uncontrolled series, including small numbers of patients with short follow-up, have already suggested the benefits of BC on HRQoL [21, 22, 27, 28]. Our study, with a rather longer follow-up comparing between pre- and post-BC and including a matched control group without BC, showed that (1) RYGBP improves HRQoL, (2) HRQoL improvement is directly related to weight loss, and (3) BC further improves HRQoL in comparison to RYGBP alone.

Self-esteem is Significantly Improved by Body-Contouring Surgery

Self-esteem is the most affected domain of quality of life in patients with BMI > 40 kg/m², especially in women between 35 to 64 years old [29]. This dissatisfaction motivates diverse behaviours, including weight loss, exercise and cosmetic surgery [22]. It could explain partially why women more frequently seek bariatric surgery, even if obesity is more prevalent in men. However, despite the clear improvement of self-esteem achieved after bariatric surgery, it remains still relatively low after the weight loss. This study shows that self-esteem is further corrected by the addition of plastic surgery to bariatric surgery: 85% of patients after BC surgery estimate that their self-esteem was "much better", versus only 48% after bariatric surgery alone.

Improvement of Physical Activity Implies Partially Improvement of Work Ability

Although physical activity is significantly improved by bariatric surgery and further by plastic surgery, the domain of work ability presented a lower improvement after weight loss, with minimal further effect by plastic surgery. This could be explained partially by the huge difficulties encountered by these patients, often in disability for years before RYGBP, to find employment in a difficult job market.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Comparison of mean total score (range -3 to +3) and mean scores of health-related quality of life (HRQoL) sub-domains (self-esteem score -1 to +1, others -0.5 to +0.5) between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total score</td>
</tr>
<tr>
<td>A1</td>
<td>1.5</td>
</tr>
<tr>
<td>A2</td>
<td>1.95</td>
</tr>
<tr>
<td>B</td>
<td>1.48</td>
</tr>
<tr>
<td>p A1 vs. A2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>p A2 vs. B</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>p A1 vs. B</td>
<td>NS</td>
</tr>
</tbody>
</table>

A1 before body contouring, A2 after body contouring, B gastric bypass alone, NS non-significant (p > 0.05)
Adipose tissue: obesity vs regeneration

Even with an Improved Social Life, Sexual Activity Is Only Partially Improved by Plastic Surgery

Despite a lower frequency of sexual relations than the general population (6.5 vs. 5.6 per month), most overweight patients declare themselves satisfied with their sexual life both before and after bariatric surgery. The margin of potential improvement is hence narrower than in the other domains of the HRQoL. This could explain that sexual activity is the only domain where the majority of patients (48 %) feel no change after RYGGB, and the improvement is only partial by BC. Some patients (11 %) declare themselves even less satisfied with their sexual life than before RYGGB, and the gain in sexual activity is only marginal after BC. One of the explanation could be that partners have some difficulty to adapt themselves to the new image of the other after weight loss [30].

We have demonstrated that social life is considerably improved after RYGGB and even more after BC. Hafner et al. had also concluded that after bariatric surgery, women considered themselves more attractive and more sociable, but estimated that their husbands are less attractive than before. Symmetrically, the husbands considered their wives too sociable after surgery, in contrast to their pre-operative expectation [31]. Furthermore, at least one study found a higher than anticipated divorce rate following bariatric surgery [32].

High Satisfaction of Body-Contouring Surgery Despite Important Scars

The vast majority of patients report satisfaction with their post-BC results. Nevertheless, it was reported that after breast reduction [22], for example, the most common reason for dissatisfaction was the scars. Interestingly, after BC which leaves numerous and visible scars, 84 % of our patients indicated that they would undergo BC surgery again.

BC should not be considered as part of the treatment for morbid obesity, but as a reconstructive surgery for sequel of massive weight loss. A perfect silhouette will never be achieved; therefore, patients have to be informed about esthetical outcomes, including unavoidable scars left by BC. As demonstrated by Warner et al., before bariatric surgery, only 54 % of bariatric surgeons inform patients about potential functional and morphologic consequences of massive weight loss, and minority of them refer patients thereafter to a plastic surgeons [33]. As proposed, plastic surgeons can provide written, electronic and video material that can be integrated to the multidisciplinary schedule that takes place before RYGGB.

We are still obviously in the early stages of the increase in body-contouring surgery following massive weight loss. Given the recognized importance of psychosocial factors in bariatric and plastic surgery separately, it is important to assess these issues in this new common area. The increasing interest of our health systems for cost-efficiency of treatments requires outcome studies. In addition to morbidity and mortality analysis, the evaluation of HRQoL opens fascinating perspectives for the estimation of the benefits of surgery. It was shown that bariatric surgery is economically efficient [34, 35], decreasing by 45 % the direct and indirect medical costs of operated morbidly obese patients in comparison to non-operated [30, 36]. This positive balance would be even more important if we were to take into consideration HRQoL improvement. Our study demonstrates clearly the benefit of the plastic surgery by a net improvement of HRQoL and a high rate of patient’s satisfaction. Further studies are needed to estimate the direct and indirect cost/benefit ratios of BC.

It has been demonstrated that 74–85 % of patients desire a BC after a bariatric surgery [17, 39]. But as, in most cases, the BC is not covered by health insurances, majority of patients don’t achieve this procedure because they can’t afford it (54.7 %) or need a payment plan (28.5 %) [39]. In our study, only 32 % of patients underwent a BC procedure after RYGGB. In our country, health insurances do not cover BC as long as the excess skin does not achieve “a value of somatic or psychic disease”. According to the definition of the WHO, any perceived limitation of HRQoL has to be considered as a disease. As it is able to normalize HRQoL scores, BC after RYGGB should be considered as an effective therapy and reimbursed by the health system.

Even if this was a prospective matched group, there is a statistical bias limitation. It can be supposed that some patients in group B were looking for BC. But they did not undergo the procedure because they were turned down by plastic surgeons for some reasons (e.g. weight instability, lack of motivation, body dysmorphia) or because BC was not covered by health insurance. This refusal could have a negative impact on patients self-esteem and therefore on their HRQoL. However, as demonstrated, the HRQoL of patients before BC (group A1) was similar to those of group B (Table 2).

We demonstrated that BC has a contributive role to help achieve the main goal of patients who seek bariatric surgery: quality of life improvement. According to Kalarchian et al., any interventions improving psychosocial functioning could also strengthen the weight loss [37]. Considering that plastic surgery improves psychosocial status, it could also encourage patients to keep their weight stable over years and prevent the 10–15 % of weight gain observed in the long-term follow-up after bariatric surgery [38]. To demonstrate, in an irrefutable way, this positive effect of plastic surgery on the long-term weight, more specific studies are necessary.

Conclusion

With increasing number of bariatric surgeries reflecting increasing prevalence of morbid obesity, candidates for BC are certainly going to increase massively too.
Excess skin after massive weight loss is evidently extremely annoying in daily life. In our knowledge, this is the first group-matched study with a long-term follow-up, demonstrating that BC, in spite of important scars, significantly improves the satisfaction and HRQoL after massive surgical weight loss.

Excellent centres of bariatric surgery should already include plastic surgeons during the pre-operative visits in their multidisciplinary team to evoke, firstly, the possible cutaneous excess after a massive weight loss and, secondly, to discuss possibilities of its corrections by BC.

Indeed, the treatment of the morbid obesity should not be considered achieved as long as the plastic surgery is not finished. We hope that our results will be used as an argument in favour of BC and its coverage by health insurances.

Acknowledgments The authors would like to thank Mr. Christophe Combescure (Biostatistician from the Epidemiology Department) for his contribution to the statistical analysis and Mrs. Pascale Koutny-Fong for database management.

Disclosure Statement The authors declare that they have no conflict of interest for this paper.

References

6.2 Paper 2: Plastic surgery improves long-term weight control after bariatric surgery

Plastic Surgery Improves Long-Term Weight Control after Bariatric Surgery

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Background: The positive impact of Roux-en-Y gastric bypass on weight, comorbidities, and health-related quality of life is well documented. However, 50 percent of patients regain some of the lost weight after 2 years with Roux-en-Y gastric bypass and present a mean weight regain of 10 to 15 percent after several years, partially losing the previously obtained benefits. The authors hypothesize that body contouring could decrease weight regain, leading to better long-term weight control after Roux-en-Y gastric bypass.

Methods: In a matched control study, variations in weight for 98 patients with body contouring after Roux-en-Y gastric bypass were compared with those of 102 matched control patients with Roux-en-Y gastric bypass alone. Data were collected prospectively at 1, 3, 6, 9, 12, and 18 months after Roux-en-Y gastric bypass and then yearly until 7 years.

Results: After a massive mean weight loss of 45.2 kg during the first 2 years after Roux-en-Y gastric bypass, patients with Roux-en-Y gastric bypass alone presented a higher continuous mean weight regain than those with Roux-en-Y gastric bypass and body contouring (1.78 kg/year versus 0.51 kg/year of weight regain, respectively; p = 0.001). After 7 years, patients with Roux-en-Y gastric bypass presented significantly higher mean weight regain than patients with Roux-en-Y gastric bypass and body contouring (i.e., 10.8 percent versus 3.6 percent mean weight regain, respectively; p < 0.001). Netting out mean skin excision weight of 2.04 kg by body contouring, the weight regain was 22.9 kg for patients with Roux-en-Y gastric bypass alone and only 6.2 kg for those with Roux-en-Y gastric bypass and body contouring.

Conclusions: The authors demonstrated that patients with body contouring present better long-term weight control after Roux-en-Y gastric bypass. Therefore, body contouring must be considered as a reconstructive operation in the treatment of morbid obesity. (Plast. Reconstr. Surg. 132: 926, 2013.)

CLINICAL QUESTION/LEVEL OF EVIDENCE: Therapeutic, III.

Most obese individuals suffer from impaired health-related quality of life and other forms of psychosocial distress.1,2 A majority of them pursue obesity treatment seemingly for improvement in their physical image, their self-esteem, and their health-related quality of life rather than the loss of weight and related health improvement.3,4 However, to the public health system and physicians, comorbidity control is the main goal of the treatment.

Presently, bariatric surgery, especially Roux-en-Y gastric bypass, has become the criterion standard treatment for morbid obesity,5–9 with more than 100,000 operations performed each year in the United States alone.10 Indeed, the procedure results in the best weight loss10 and comorbidity improvement,11–13 with the lowest complication rate in both the short term and the long term in comparison with other bariatric procedures (e.g., gastric banding) or nonsurgical treatment. Roux-en-Y gastric bypass also leads to a 30 to 40 percent decrease in mortality14,15 and, moreover, improves health-related quality of life.2,16–20

Disclosure: The authors have no financial interest to declare in relation to the content of this article.
However, even though Roux-en-Y gastric bypass offers a fast, massive weight loss within the first 18 months after surgery, as many as 50 percent of patients may unfortunately regain some of the lost weight, with a mean weight regain of 5 to 10 percent within the first 18 to 36 months after surgery and 10 to 15 percent over the course of the next 10 years. This weight regain can be associated with a recurrence of comorbidities, such as hypertension, diabetes, and hyperuricemia.

Furthermore, more than two-thirds of patients who have undergone bariatric surgery consider the resulting excess skin to be a negative consequence of surgery. This excess skin presents problems for the patient in their daily life and provokes important psychosocial disturbances that could compromise the beneficial effects of the weight loss. This dissatisfaction motivates 74 percent of patients to seek body contouring procedures, but only 21 percent undergo at least one such procedure.

The basic principle of body contouring is to tighten the cutaneous tissue to eliminate physical or psychological handicaps linked to the massive amount of excess skin. We demonstrated in a previous study that body contouring after Roux-en-Y gastric bypass improves health-related quality of life more than Roux-en-Y gastric bypass alone. This improvement was significant after body contouring in all evaluated domains of health-related quality of life: self-esteem, social life, work ability, physical activity, and sexual activity.

Kalarchian et al. concluded that any interventions that improved the psychosocial functioning of a patient would also strengthen the weight loss maintenance. Likewise, we hypothesize that body contouring, which improves health-related quality of life, could also help patients to maintain previously obtained weight loss after Roux-en-Y gastric bypass.

In this study, we intended to evaluate the benefit of plastic surgery on body weight control. It is the first study that addresses the role of body contouring in maintaining a stable weight and decreasing the typical 10 to 15 percent weight regain following Roux-en-Y gastric bypass.

PATIENTS AND METHODS

In the context of a multidisciplinary obesity program, from the beginning of use of Roux-en-Y gastric bypass in our General Surgery Department in 1997, a database was created. Demographic and personal data were collected from candidates for Roux-en-Y gastric bypass. Those who underwent Roux-en-Y gastric bypass were followed-up, and weight, body mass index, and excess body weight loss were collected during the follow-up appointments at 1, 3, 6, 9, 12, and 18 months after surgery and then each year after Roux-en-Y gastric bypass, and added to the database.

For this study, to assess the hypothetical benefit of body contouring on weight control after Roux-en-Y gastric bypass, two groups were formed from patients who underwent Roux-en-Y gastric bypass between 1997 and 2007 (n = 538). To reduce patients’ insurance and economic status bias, in both groups, only patients who had public health insurance coverage, and for whom Roux-en-Y gastric bypass and body contouring were reimbursed by this insurance, were included. Then, the data from these groups were compared at each time point. The study protocol was reviewed and approved by the local clinical ethics committee.

Study Groups

Group A

Group A consisted of 98 patients with Roux-en-Y gastric bypass and body contouring. Among 538 patients who underwent Roux-en-Y gastric bypass, 136 had a body contouring procedure. All had undergone Roux-en-Y gastric bypass at least 18 months before plastic surgery and maintained a stable body weight through the prior 6 months. Ninety-eight consecutive patients (i.e., women, 89.8 percent; mean age, 42.6 years; range, 34 to 55 years) of those who had body contouring after Roux-en-Y gastric bypass with a complete follow-up for more than 2 years after body contouring were included in this group. The total weight of excised skin during body contouring was collected for each patient in group A.

Group B

Group B consisted of 102 patients with Roux-en-Y gastric bypass only. For each patient in group A, among 402 patients of the database who had undergone only Roux-en-Y gastric bypass, a matched patient was selected blindly by means of computer. The following criteria, in decreasing order of importance, were applied to find a matched patient in this group for each patient of group A: body mass index, excess body weight loss, sex, and age before and 2 years after Roux-en-Y gastric bypass. Four patients in group A had exactly the same selection criteria with two corresponding patients in group B; therefore, to reduce bias selection, both were included in this group. Therefore, 102 patients were included in this group (i.e., women, 89.1 percent; mean age,
38.6 years; range, 31 to 48 years). These patients had all demanded a plastic surgery consultation but had not undergone body contouring because they either had been turned down by plastic surgeons or, more frequently, their health insurance did not cover the cost.

**Surgical Procedure**

**Bariatric Surgery**

After a multidisciplinary consultation, a fully standardized Roux-en-Y gastric bypass (i.e., gastric pouch, ≤30 ml; alimentary loop, 150 cm; biliopancreatic loop, 50 cm) was performed on morbidly obese patients (i.e., body mass index > 40 kg/m²) by general surgeons in our surgical department. These operations were performed by means of laparotomy until 2001 and by means of laparoscopy thereafter.

**Plastic Surgery**

Group A patients underwent the following procedures: abdominoplasty, 97 percent (with incisional hernia repair in 47 percent); mammoplasty, 32 percent (i.e., mastopexy alone, 51 percent; breast reduction, 33 percent; and breast augmentation with or without breast lift, 16 percent); cruroplasty, 19 percent; and brachioplasty, 14 percent. Moreover, 45 percent of patients underwent combined procedures through one or several operations.

**Statistical Analysis**

Patient characteristics were described as the mean ± SD or by percentages. The weight, body mass index, and excess body weight loss of group A were compared with those of group B by means of a t test for paired data as the patients were matched.

The change in weight, body mass index, and excess body weight loss decreased significantly more after Roux-en-Y gastric bypass was analyzed with a mixed linear regression model adjusted for age and sex (i.e., a random effect was introduced in an effort to account for the repeated measures). The group and the time were used as predictors in the model, and an interaction term was added to test whether the change over time was different in both groups. The goodness-of-fit was checked by plotting residuals (not shown).

The percentage of patients who achieved greater than or equal to 50 percent of excess body weight loss was assessed at various times, and the 95 percent confidence intervals were obtained using the exact method of Clopper-Pearson. The statistical analyses were performed using S-Plus version 8.0 software for Windows (Tibco Software, Inc., Palo Alto, Calif.), and the significance level was set at 5 percent.

**RESULTS**

**Roux-en-Y Gastric Bypass Induces Fast Massive Weight Loss in the First 18 Months**

Before Roux-en-Y gastric bypass, patients presented with a mean body mass index of 46 kg/m² (range, 41 to 48 kg/m²) and a mean weight of 125 kg (range, 109 to 140 kg). Roux-en-Y gastric bypass alone resulted in an initial massive mean weight loss of 45.2 kg. Then, the patients reached a plateau approximately 12 to 18 months after surgery, thereby allowing them to obtain a minimal mean weight of 78.3 kg (range, 65 to 92 kg), a mean excess body weight loss of 68.4 percent (range, 58.2 to 80.7 percent), and a mean body mass index of 29.9 (range, 26 to 34 kg/m²) ($p < 0.001$) (Fig. 1). During this period, 88.52 percent of the patients achieved greater than 50 percent excess body weight loss (i.e., 87.67 percent in group A and 88.52 percent in group B; $p > 0.05$). Similar kinetics of weight loss were observed in both groups, with nonsignificant differences between groups A and B up to 2 years after Roux-en-Y gastric bypass ($p > 0.05$), just before the mean time point when body contouring was achieved (Table 1).

**Secondary Weight Regain after Roux-en-Y Gastric Bypass Is Prevented by Body Contouring Surgery**

In group A, body contouring was performed within 2 years on average after Roux-en-Y gastric bypass. At the time just before body contouring in group A and at the matched time in group B, the weight lost, body mass index, and excess body weight lost were similar for both groups.

Total mean weight of excised skin by body contouring procedure in group A was 2.04 kg (range, 0.45 to 6.3 kg). Beyond the second year after Roux-en-Y gastric bypass, patients without body contouring (group B) started to regain significant weight. The weight differences between groups gradually became more significant over time. The yearly weight and body mass index increase was significantly more important in group B than in group A: 1.78 kg/year versus 0.51 kg/year ($p = 0.001$) of weight regain and 0.60 kg/m²/year versus 0.16 kg/m²/year ($p = 0.006$) of body mass index increase, respectively. The excess body weight loss decrease was also significantly higher.
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in group B compared with group A (i.e., $p < 0.001$ for $-2.91\% \text{ year}^{-1}$ versus $-0.86\% \text{ year}^{-1}$, respectively) (Fig. 1).

From the minimum weight achieved by Roux-en-Y gastric bypass, the mean weight regain at 7 years after Roux-en-Y gastric bypass was 3.6 percent of pre-Roux-en-Y gastric bypass weight (range, 0 to 6.34 percent) in group A and 10.8 percent (range, 7.4 to 20 percent) in group B ($p < 0.001$). This resulted in a higher final weight in group B as compared with that of group A (i.e., 101.2 kg versus 82.5 kg, respectively; $p = 0.01$). Even considering some weight lost because of skin excision in group A, the difference between these groups remained significant. Netting out skin excision weight, patients in group A gained only 4.8 kg during the 5-year period between 2 and 7 years after Roux-en-Y gastric bypass, whereas patients with Roux-en-Y gastric bypass alone regained 20.1 kg during this time. The mean body mass index increased significantly more in group B than in group A (i.e., 3.2 percent (range, 0 to 21 percent) versus 16 percent (range, 8.7 to 22 percent), respectively; $p < 0.001$) to achieve a body mass index of 37.2 and 30.6 kg/m$^2$, respectively.

At 7 years after Roux-en-Y gastric bypass, 58 percent of patients in group B and 25 percent of those in group A presented with at least a 10 percent weight regain. The mean excess body weight loss was 67 percent in group A and 38.5 percent in group B. Therefore, 75 percent of patients from group A had maintained greater than or equal to 50 percent excess body weight loss, in comparison with only 29.2 percent in group B (Table 2).

The multivariate models also indicated a significant gender effect in both groups: at 7 years after Roux-en-Y gastric bypass, men had a higher weight regain [i.e., 25.1 kg of weight regain ($p < 0.001$) and 3.3 kg/m$^2$ of body mass index elevation] and a lower excess body weight loss (−12.4 percent; $p < 0.001$) than women. Age was not significantly associated with weight ($p = 0.20$) or body mass index ($p = 0.10$), but the excess body weight loss decreased with age (−0.25 percent/year; $p = 0.04$).

**DISCUSSION**

Body image dissatisfaction, low self-esteem, and reduced health-related quality of life motivate many behaviors among obese people, including participation in diet programs and cosmetic surgery.\(^\text{32}\) For patients seeking bariatric surgery, health-related quality of life is very important too. In 66 percent of cases, psychosocial impairment is the main motivation for their desire to have bariatric surgery; in contrast, only 10 percent of patients indicate a medically motivated desire for bariatric surgery.\(^\text{33}\)

Unfortunately, because of excess skin that appears after the quick, massive surgical weight loss, patients’ health-related quality of life
remains impaired after bariatric surgery. In fact, previous research indicates that 74 to 85 percent of patients want body contouring after Roux-en-Y gastric bypass, but in most cases, body contouring is not covered by health insurance. Therefore, more than 80 percent of patients do not undergo this procedure because they cannot afford it (54.7 percent) or need to establish a payment plan (28.5 percent). Finally, only 12 to 21 percent of patients will undergo body contouring after massive weight loss. In our study, 32 percent of Roux-en-Y gastric bypass patients underwent body contouring as well (Fig. 2).

In many cases, insurance companies do not consider excess skin to be a disease, and body contouring is not viewed as a cost-effective treatment because, until now, no research had investigated whether patients who have undergone bariatric and plastic surgery experience a better long-term result in terms of weight. Our previous study demonstrated that body contouring after Roux-en-Y gastric bypass significantly improves health-related quality of life, specifically, self-esteem. Indeed, 85 percent of patients who have had Roux-en-Y gastric bypass and body contouring feel that their self-esteem is very improved as compared with only 48 percent of patients after Roux-en-Y gastric bypass alone. We believe that body contouring contributes to achieving the main goal for patients seeking bariatric surgery (i.e., a better quality of life). This improvement may therefore encourage patients to maintain a stable weight over the years. However, health-related quality-of-life improvement could also be explained by better weight control after body contouring. We have demonstrated that health-related quality-of-life improvement after Roux-en-Y gastric bypass is related directly to excess body weight loss (i.e., 97.8 percent of patients who had achieved >75 percent excess body weight loss estimated that their quality of life improved, but among those who had achieved <25 percent excess body weight loss, only 50 percent felt their quality of life improved).

Previous studies have clearly demonstrated that Roux-en-Y gastric bypass per se appears to be a cost-effective intervention for moderately to severely obese people compared with nonsurgical approaches. The surgical treatment decreases 45 percent of direct costs (e.g., the number of consultations, medical treatments, and hospitalizations) and also indirect costs (e.g., unemployment rate and sick leave) for morbidly obese patients who undergo bariatric surgery compared with the morbidly obese who do not have weight loss surgery. The decrease in these costs is mainly related

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<td>Mean EBWL ± SD, %</td>
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BC, body contouring; IQR, interquartile range; RYGBP, Roux-en-Y gastric bypass; NS, not significant; BMI, body mass index; EBW, excess body weight; EBWL, excess body weight loss.

* p > 0.05.

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<th>Table 2. Percentage of Patients Presenting with Greater Than 50% Excess Body Weight Loss in Groups A and B over Time after Roux-en-Y Gastric Bypass*</th>
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EBWL, excess body weight loss; RYGBP, Roux-en-Y gastric bypass; BC, body contouring.

*Weight lost of more than 50 percent EBWL at more than 5 years after Roux-en-Y gastric bypass in more than 75 percent of patients has been defined as a criterion of an effective and successful Roux-en-Y gastric bypass (Balataris A, Boui R, Bengoecha M, et al. Duodenal switch: An effective therapy for morbid obesity. Intermediate results. Obes Surg 2001;11:54–56). At 7 years after Roux-en-Y gastric bypass, 75 percent of patients from group A had maintained ≥50 percent EBWL in comparison with only 29.2 percent of patients in group B.

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to a decrease in comorbidities, which is linked directly to weight loss. Previous research has concluded that even small weight changes (i.e., as little as 5 percent) can dramatically change comorbidities. In this article, we demonstrated that patients with body contouring presented 18.8 percent less weight at 7 years after Roux-en-Y gastric bypass than those with only Roux-en-Y gastric bypass. Thus, it may be considered that plastic surgery prevents the secondary worsening of comorbidities and plays a role as a cost-effective treatment plan for obesity. However, as in the beginning of this prospective study, these endpoints have not been addressed directly; we cannot draw any conclusion concerning this hypothesis. Therefore, more clinical research focused on improvement of comorbidity and cost-effectiveness of plastic surgery are needed in the future.

Furthermore, as defined previously, among other criteria, an effective and successful Roux-en-Y gastric bypass should achieve more than 50 percent excess body weight loss that is maintained for at least 5 years in more than 75 percent of patients. As we demonstrated (Table 2), these criteria are achieved mainly during the long-term follow-up period in patients who had body contouring after Roux-en-Y gastric bypass; indeed, those who had Roux-en-Y gastric bypass alone do not meet these criteria starting in the third year after surgery.

Even though this was a matched group study, some statistical bias limitations can still be identified. Indeed, a reasonable supposition suggests that patients who underwent plastic surgery had an initially stronger motivation and were more determined to control their body weight. Second, because some patients seeking body contouring were turned down by plastic surgeons or not covered by health insurance, a selection bias may be present between groups (i.e., wealthier patients have more access to body contouring surgery). Finally, it can be considered that body contouring procedures per se reduce the total body weight by removing some adipocutaneous tissue. However, this quantity was minimal (i.e., mean weight, 2.04 kg; range, 0.45 to 6.3 kg) and nonsignificant. Furthermore, as the weight changes become more obvious during long-term follow-up, this weight reduction could not by itself explain the continuous weight difference between these two groups, year after year. Even netting out the skin excision weight, the difference between the two groups remains significant and, essentially, the most important goal for patients and physicians is the final weight obtained. Weight improvement with body contouring could be attributable to excised skin partially, improved health-related quality of life, or other unknown mechanisms.

We demonstrated for the first time that patients who underwent body contouring after massive surgical weight loss presented better long-term weight control. Therefore, we suggest that body contouring should be encouraged by bariatric surgeons. As concluded by Warner et al., patients seeking bariatric surgery are insufficiently informed of the possibilities offered by plastic surgery after gastric bypass; indeed, only 7 percent of bariatric surgeons always refer their patients to a plastic surgeon, and only 33 percent refer patients occasionally. Plastic surgeons should be included in the multidisciplinary team for bariatric surgery before Roux-en-Y gastric bypass to inform patients about the likely development of excess skin following this procedure and to discuss all of the
possibilities offered by plastic surgery thereafter. However, no excessive promises about the results should be made, and insurance conditions and restrictions should also be evoked.

CONCLUSIONS

With the increasing number of bariatric operations occurring today, the number of candidates for plastic surgery will certainly increase as well. However, in the absence of cost-effectiveness studies, insurance companies do not currently cover the costs of these operations provided that the excess skin does not achieve “a value of somatic or psychic disease.” For the first time, our study demonstrates that patients who have undergone body contouring present significantly improved long-term body weight control after Roux-en-Y gastric bypass, in comparison with those without body contouring. This could suggest that body contouring after massive surgical weight loss may improve comorbidities that can relapse over the long-term period after Roux-en-Y gastric bypass alone. These improvements offered by body contouring are probably associated with a decrease in direct and indirect costs for morbibly obese patients, which is thus an important argument in favor of this type of treatment and coverage by health insurance. Indeed, in some cases, the treatment of the morbibly obese should not be considered successful as long as plastic surgery has not been performed. If we consider morbid obesity as a real disease, global care should be accepted by insurance companies. Because plastic surgery after massive weight loss is mandatory for improvement of health-related quality of life and weight loss maintenance in many patients, body contouring must be considered as a reconstructive operation for those who have achieved massive weight loss.

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The mission of the American Society of Plastic Surgeons® is to support its members in their efforts to provide the highest quality patient care and maintain professional and ethical standards through education, research, and advocacy of socioeconomic and other professional activities.
Platlet Rich Plasma (PRP) Improves Fat Grafting Outcomes

Ali Modarressi

ABSTRACT

Autologous fat transfer offers many qualities of a ideal soft tissue filler. Main advantages of fat grafting ensue from the fact that the lipospirate tissue is an abundant source of regenerative pluripotent cells. However, the reported rates of fat cell survival vary greatly in the medical literature (10-90%). Different techniques of harvesting, processing, and reinjecting the fat cells are so claimed to be responsible for these differences, without any agreement concerning the best way to process. To address this important disadvantage, we propose the addition of autologous platelet rich plasma (PRP) which is known as a natural reservoir of growth factors stimulating tissue repair and regeneration. This approach is completely autologous and immediately employed without any type of preconditioning. Platelets rich plasma (PRP) preparation included bleeding of 8 ml of blood from patient’s peripheral vein in Regen Lab® tubes containing sodium citrate anticoagulant. The whole blood was centrifugated at 1500 g during 3 min. As Regen-tubes contained a special gel separator, 99% of red blood cells were discarded from the plasma at the bottom of the gel, and >90% of platelets were harvested in 4 ml of plasma on the top of the gel, called the platelet-rich plasma (PRP). The purified fat prepared by Coleman technique was mixed with different amount of PRP for in vitro, in vivo (mice) and clinical experiments: >50% of PRP for skin rejuvenation, superficial scars correction, infraorbital region, ..., and for 20% of PRP with 80% of purified fat for deep filler indication (nasolabial folds, lips, or soft tissue defect). In vitro studies demonstrated that PRP increased fat cells survival rate and stem cells differentiation. Animal models showed that fat graft survival rate was significantly increased by addition of PRP. Several clinical cases confirmed the improvement of wound healing and fat grafting survival in facial reconstruction and aesthetic cases by association of fat grafting with PRP. The addition of PRP to fat grafts represented many advantages with a simple, cost-effective and safe method. In addition to its booster effect on fat grafts, PRP had a rejuvenation capacity per se. It is also used on nappage technique, on mask and as a temporary regenerative filler in combination with thrombin. So we consider the addition of 20% PRP to fat grafts offers a better fat grafting survival, a less bruising and inflammation reaction, and easier application of fat grafts due to liquefaction effect of PRP.

KEYWORDS

Platlet rich plasma; PRP; Fat; Graft; Outcome
Adipose tissue: obesity vs regeneration

INTRODUCTION

The importance of addressing the volume loss is becoming increasingly evident to cosmetic surgeons and patients. This volume loss can be corrected through several means, including tissue repositioning (e.g. facial lifting), implants (e.g. malar implants), synthetic fillers (e.g. hyaluronic acid) or autologous tissue.

More recently, autologous fat grafting has come to be considered an ideal filler, becoming a clinical reality in aesthetic medicine and surgery. The success of fat grafting is thought to originate the abundant source of regenerative multi-potent cells in particular, Adipocytic derived Stem Cells (ADCs). These cells are all capable, of integrating into host tissue and to secrete an important orchestrated quantity of cytokines and growth factors including vascular growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), platelet derived growth factor (PDGF), and transforming growth factor-beta (TGFβ).1

Main advantages of fat grafting include (i) A long lasting result contrary to the synthetic resorbable products, (ii) Prevention of granuloma and allergic reactions which could be provoked by the permanent products injection, (iii) A natural consistency and (iv) An improvement of cutaneous and subcutaneous trophicity.

On the other hand, disadvantages of autologous fat grafting include (i) Its complexity of use, requiring a more important learning curve with regard to the prepared products, (ii) The morbidity and the necessity of donor site, that sometimes could not be enough, and mainly (iii) The unpredictability of the remaining volume by partial uncontrolled absorption of the fat transplant.5 To address this latter important disadvantage, we propone the addition of autologous, platelet rich plasma (PRP), which is known as a natural reservoir of growth factors.

AUTOLOGOUS FAT GRAFTING

The transplant of autologous fat tissue, so-called lipomodelage, liposculpture or lipofilling has been known since the early of 20th century. The principle of the technique is to transfer patient’s own fat tissue from a donor site (e.g. abdomen, flanks, thighs) to a site where there is a volume deficit. Its first indications were for aesthetic surgery of the face,3 and more recently in hands.4 Fat grafting is also useful for tissue loss due to an accident, operation, congenital disease or lipodystrophy.5,6 In addition to a volumizing effect, the injected fat leads to a neoangiogenesis effect7 improving the cutaneous elasticity, and to an antiaging effect.8 This technique is thus also recommended for wound healing,9,10 radiodermatitis treatment11 and correction of acne scars.12 During the last decade, fat grafting has been used more and more frequently for breast reconstruction and augmentation.13,14

Despite Neuber’s use of fat transfer in 1893, the first description of the fat transplantation by infiltration dates to 1962 by Miller. Given the little satisfactory long-term results, this technique did not have the expected success and other numerous methods were described. At present, the most used method for the fat harvesting, purification and infiltration is the one described in detail by Coleman in 1986.5,16 This method takes into account the fragility of fat cells during the various steps of the treatment.

The survival rate and longstanding results depend partially on indications and patients but mostly on surgical technique. However, even with the best surgical technique, the fat grafting survival is unpredictable, with a variable resorption rate reported throughout the literature (10% to 90%). Different techniques have been proposed to improve the survival rate of fat grafting and its predictability. The most efficient technique proposed until today is to highly enrich the grafted tissue on mesenchymal stem cells. However most of these techniques are time consuming, expensive with significant harvested fat loss, and the results still remain unpredictable.

Another approach to improve the fat grafting results could be stimulation of transplanted tissue by growth factors. Unfortunately, the exogenous and synthetic growth factors treatment have not provided the desired expected results in clinic (e.g., wound healing treatment, bioengineering). One of the reasons is the protein fragility and instability of growth factors. Recently, autologous plate-
lets considered as a natural reservoir of growth factors has been used for different pathologies. So, we suggest that its addition to fat grafts could be a solution to boost stem cell survival, multiplication and differentiation to improve the long standing results of lipofilling efficiently and simply.

PLATELETS RICH PLASMA (PRP)

Platelets are enucleated circulating blood particles that derive from the fragmentation of megakaryocytes. They circulate in an inactivate state until they come into contact with endothelia damage areas. Platelets work via the degranulation of the α-granules in platelets, which contain the synthesized and pre-packed growth factors. The most potent ones in restoring damaged tissues are PDGF, TGFβ, IGF, VEGF and endothelial growth factor (EGF).

The synthesized growth factors directly bind to the surface of cell membranes to stimulate hemostasis and normal healing. They induce internal cellular signaling that activates angiogenesis, cell proliferation, cell differentiation and new matrix formation for tissue repair. Platelets are therefore a natural reservoir of growth factors that could be used to regenerate tissues. Previous topical growth factor studies have shown that synthetic human platelet-derived growth factors could be an efficacious treatment for wound healing. However, as those synthetic proteins presented some limits for clinical use, a newer treatment, autologous platelet-rich plasma (PRP), has been developed. It represents a greater similarity to the natural healing process as a composite of multiple growth factors. It is safe due to its autologous nature, and is produced simply as needed from the patient’s blood.

After 30 years of PRP clinical application to stimulate bone regeneration and wound healing, autologous PRP is actually recognized as a new tissue engineering element and a developing area for clinicians and researchers that helps healing of soft and supportive connective tissues.

The benefit and safety of PRP is documented in more than 5,000 studies where the authors observed enhancement of bone regeneration, wound healing, tendon and cartilage healing, corneal healing and skin rejuvenation. PRP is so used more and more often in the plastic, reconstructive and aesthetic surgery fields.

FAT GRAFT AND PRP

Recently, there has been increased interest in the co-application of PRP and fat grafts. The live fat tissue is revascularized at the transplantation site within 48 hours. During this time, it is fed by diffused material in the plasma. In contrast, non-viable tissue is removed by macrophages, leaving behind fibrotic and cystic changes. The main obstacles preventing permanent augmentation are partial absorption and ischemia of the transplanted fat tissue, which often necessitate multiple transplantations. Then, the quality of transplanted tissue becomes highly dependent on the healing process, restoration of vascularization and adipocyte differentiation. The reported rates of fat cell survival vary greatly in the medical literature, and different techniques of harvesting, processing, and reinjection of fat cells are claimed to be responsible for these differences.

However, there is no consensus concerning the best way to process the harvested fat before reinjection. Based on recent literature, we hypothesized that adding PRP to fat preparation may be a reliable way to bring appropriate nutrient to the early moments of transplantation to improve fat survival and render the result more predictable. PRP releases the native growth factors in their biologically determined ratios at the treatment site. Released growth factors stimulate angiogenesis, cell differentiation and proliferation leading to the reconstitution of the tridimensional matrix that allows the rearrangement of adipocytes into the correct 3D organization. This approach is completely autologous and immediately employed without any type of in vitro preconditioning or media complement.

In a series of in vitro studies, it has been demonstrated that PRP increases fat cells survival rate and stem cells differentiation. Nakamura et al. showed that fat graft survival rates are significantly increased in rats. Finally, several clinical cases have been reported to improve wound healing by association of fat grafting with PRP. There is also some successful cases of facial reconstruction with fat grafting and PRP. This association has recently been also described for aesthetic cases.
FAT GRAFTING WITH PRP TECHNIQUE

Firstly, patients must be assessed correctly during initial consultation. Volume loss occurs in various patterns according to its cause; diffuse volume loss after massive weight loss is different from localized volume loss in nasolabial, infraorbital region or lips due to aging. Skin texture and thickness is another point to be evaluated before treatment. Patient information is very important because the agreement between patient’s expectation and the result that can be offered by the treatment is a guarantee of satisfaction.

Adipocytes have short lifespans once removed from the body, and they do not react well to excessive handling, refrigeration or major trauma during tissue collection or processing. The fat graft resorption is the main drawback, which could be dramatically reduced by using good technique.

The method described by Coleman advances the principle that the fat transplants have to survive and be revascularized. Coleman et al. recommends a small quantity of fat injection in fine layers to increase the proportion of fat graft surface area to receptor bed. The total procedure could be realized with local or general anesthesia, according to patient/physician preference and the importance of fat volume previewed to be grafted.

DONOR SITES

The most common donor site in clinical practice is the abdomen, but the fat could be harvested from any location that presents adequate non-fibrous fat flank, thigh, and medial knee which is patient-specific, and dependent on patient/physician preference. There is no compelling evidence regarding harvest site and efficacy of fat grafting.

FAT HARVESTING

Adipocyte viability decreases with increasing negative suction pressure. Thus, mechanical liposuction by machine should be avoided (~500 mmHg), and only manual harvesting offers a satisfied fat graft quality. Low pressure vacuum, created by a 2 ml withdrawing plunger of a 10 ml Luer Lock® syringe, gives the best result. The fat harvesting is performed with a blunt cannula connected to 10 ml Luer Lock® syringe. The ideal cannula combines efficient collection of fat parcels with minimal neurovascular damage. The most used is a blunt tip cannula with a single distal opening of 3 mm diameter. For small and precise fat grafting (e.g. suborbital region), we suggest the use of 1.65 mm cannula.

FAT PURIFICATION

The ideal method for fat purification would separate blood, infiltration fluid, and cell debris from healthy adipocytes with minimal trauma (Figure 1). This particular step is the most debated part of the fat grafting procedure, subjected to intense scrutiny without, however, a definitive solution. While various methods for separating out fat have been described, none has been determined to be superior to the others, but it is accepted that techniques involving less manipulation may have better outcomes including (i) Sedimentation: Aspirate material stands for 30 minutes to 1 hour, which separates it into its various components; (ii) Washing: aspirate fat is washed with 5% glucose solution, 0.9% normal saline, or sterile water; (iii) Filtration: Harvested fat is placed on sterile gauze over sterile cup, washed with ringer’s lactate and dried before loading into syringes; (iv) Centrifugation: Harvested fat is centrifuged 3 minutes at 3000 rpm. This method separates fat from substances that increase degradation, and concentrates adipocytes and stem cells per milliliter of fat transplanted. It is the most rapid and clean method.

Fig. 1: Harvested fat after centrifugation: 1) Upper part: Oil from damaged adipocytes, 2) Middle part: Purified fat, and 3) Lowest part: Red cells, cell’s debris and liquids.
PRP in fat grafts

Various studies assessed the impact of centrifugation on fat transfer, and most of have concluded that centrifugation, unless conducted at very high speeds, does not adversely affect adipocyte viability.\textsuperscript{45,46} Coleman et al. suggests 3000 rpm for 3 minutes, but 1 minute of centrifugation is as efficient with less harm to fat cells.\textsuperscript{15}

PRP PREPARATION

Today, there are different techniques for PRP preparation in the market. Since 2003, RegenLab has developed a new technique to prepare autologous PRP from whole patient blood. This is a simple and safe method to realize in the operating room while maintaining low cost. It requires no specialized skill and a small amount (8 ml) of patient’s blood is enough. In comparison to other methods, this technique has also been shown to offer the best platelet concentration and survival, with highest growth factor secretion.\textsuperscript{47,48} This method of PRP preparation has already shown good results for bone regeneration\textsuperscript{57} and skin rejuvenation\textsuperscript{49}. As it is a safe, efficient, simple and cheap system for a better and predictable fat grafting, in our clinical practice, we use this RegenLab PRP preparation method.

Eight ml of blood is withdrawn from patient’s peripheral vein in Regen-tubes containing sodium citrate anticoagulant. The whole blood is centrifuged at 3000 rpm during 5 min. As Regen-tubes contain a special gel separator, 99% of red blood cells are discarded from the plasma at the bottom of the gel. Platelets and white blood cells are pellet on top of the gel and re-suspended in plasma by gently mixing the tube (Figure 2). The 4 ml of cell suspension is called the PRP.

FAT AND PRP MIXTURE

The purified fat by centrifugation is mixed through a 3-ways connector with 20% of PRP. According to our in vitro experiments, the 80% fat/20% PRP seems to be the optimal rate for cell proliferation and survival.

FAT/PRP INJECTION

The fat/PRP mixture is transferred from 10 ml Luer Lock\textsuperscript{®} syringes to 1 ml or 3 ml Luer Lock\textsuperscript{®} syringes via a 3-ways connector (Figure 3). It is important to use smaller syringe, because the fat placement is more precise. For fat placement, special blunt cannula (0.75 mm to 1.65 mm) is connected to the 1 or 3 ml syringes.

As suggested by Coleman et al.,\textsuperscript{15} fat is injected in small parcels and thin strips in several layers. Before injection, it is recommended to create some tunnels, especially in nasolabial region or in scars, to release fibrotic tissues. The fat graft is then placed by a withdrawing way.

![Fig. 2: PRP preparation with RegenLab kit: 1) 8 ml of blood vena puncture in Regen-tube: Upper part=total blood, lowest part=gel separator; 2) Regen-tube after 5 minutes of centrifugation on 3000 rpm: Upper part=PRP, middle part=gel separator, lowest part=red cells and debris.](image-url)
CONCLUSION

A basic principle of aesthetic surgery is to replace “like with like”. Autologous fat transfer offers many qualities of an ideal soft tissue filler: It is biocompatible, inexpensive, readily available, non-migratory, and long term results. However, even with the best technique, the survival rate is still quite variable and unpredictable. The addition of PRP to fat grafts represents several advantages with a simple, cost-effective, and safe method. We recommend this combination for all fat grafting, but especially for aesthetic purposes. In addition to its booster effect on fat grafts, PRP has a rejuvenation capacity per se. It is also used on nappage technique for skin or hair regeneration. To summarize, we conclude that the addition of PRP to fat grafts offers several advantages including: (i) Better fat grafting survival, (ii) Less bruising and inflammation, and (iii) Easier application of fat grafts due to liquefaction effect of PRP.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


The Role of Reactive Oxygen Species in Mesenchymal Stem Cell Adipogenic and Osteogenic Differentiation: A Review

Fatemeh Atashi,1 Ali Modarressi,1 and Michael S. Pepper2–4

Mesenchymal stromal cells (MSCs) are promising candidates for tissue engineering and regenerative medicine. The multipotent stem cell component of MSC isolates is able to differentiate into derivatives of the mesodermal lineage including adipocytes, osteocytes, chondrocytes, and myocytes. Many common pathways have been described in the regulation of adipogenesis and osteogenesis. However, stimulation of osteogenesis appears to suppress adipogenesis and vice versa. Increasing evidence implicates a tight regulation of these processes by reactive oxygen species (ROS). ROS are short-lived oxygen-containing molecules that display high chemical reactivity toward DNA, RNA, proteins, and lipids. Mitochondrial complexes I and III, and the NADPH oxidase isoform NOX4 are major sources of ROS production during MSC differentiation. ROS are thought to interact with several pathways that affect the transcription machinery required for MSC differentiation including the Wnt, Hedgehog, and FOXO signaling cascades. On the other hand, elevated levels of ROS, defined as oxidative stress, lead to arrest of the MSC cell cycle and apoptosis. Tightly regulated levels of ROS are therefore critical for MSC terminal differentiation, although the precise sources, localization, levels and the exact species of ROS implicated remain to be determined. This review provides a detailed overview of the influence of ROS on adipogenic and osteogenic differentiation in MSCs.

Introduction

Reactive oxygen species (ROS) are oxygen-derived small molecules, which react readily with a variety of chemical structures such as proteins, lipids, sugars, and nucleic acids. Most ROS that have been described in living organisms include the superoxide anion (O2−), hydrogen peroxide (H2O2), hydroxyl radical (‘OH), hydroxyl ion (OH−), and nitric oxide (NO). ROS are often termed free radicals; this does not apply to H2O2 and ONOO−, which are nonradical ROS.

Chemists first discovered free radicals and described their highly reactive nature. Biologists then investigated the role of free radicals in biological systems. In 1956 Harman, a radiation biologist, introduced his noteworthy observations on the role of ROS in the aging process that were similar to his findings on radiation damage [1]. Shortly afterward the concept that emerged was that ROS lead to cellular damage in aging [2]. Nowadays, it is increasingly recognized that ROS are involved in the regulation of cell function despite the fact that for many years they were considered to be harmful elements in biological systems. Indeed high levels of ROS cause cell damage by oxidation and nitration of macromolecules including DNA, RNA, proteins, and lipids. The concept that ROS are harmful was confirmed by the discovery of ROS detoxifying enzymes (e.g., superoxide dismutase SOD, catalase, etc.), scavengers (e.g., vitamin C and E) and the bactericidal activity of neutrophils, which is strongly dependent on the generation of large amounts of ROS in the phagosome. However, this concept was challenged following the description of a family of enzymes called NADPH oxidases (NOX-es) at the beginning of the 20th century. NOX enzymes generate ROS by oxidizing intracellular NADPH to NADP+ and the transfer of electrons through membranes to reduce molecular oxygen and generate the superoxide anion as a primary product.

It is currently believed that only unregulated levels of ROS are harmful, while regulated ROS production promotes essential signaling pathways, which regulate cell functions [3] such as cell proliferation, differentiation, survival, and

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apoptosis [4–6]. Redox regulation or controlled ROS generation or neutralization of ROS-generated reactive oxygen species is critical for cell survival. Many oxidative stress conditions are associated with increased ROS levels, which can lead to cellular damage and dysfunction. The responses of adipose tissue to different stress stimuli such as oxidative stress, heat shock, and γ-radiation have been widely studied in the context of tissue repair, tissue engineering, and transplantation [7]. Mesenchymal stromal cells (MSCs) are a heterogeneous population of cells that can be isolated from the mesenchyme or stroma of several tissues including bone marrow and adipose tissue [8–10]. Within this heterogeneous population is a subpopulation of cells with self-renewal and multipotent differentiation capacity that can be qualified as stem cells. Other sources include dental pulp, umbilical cord (Warton’s jelly), umbilical cord blood, placenta, peripheral blood, and amniotic fluid [11–14]. There is an ongoing and intense debate regarding the precise origin, nature, and therapeutic potential of MSCs [15]. However, an experimental perspective, MSCs display the following features: (1) following isolation, primary cultures of MSCs are plastic adherent and remain plastic adherent during subsequent propagation; (2) depending on their source, MSCs express several cell surface markers such as CD73, CD90, CD105, and CD44, but not CD31 or CD45, although there are many other markers that may be considered and may also be used to differentiate MSCs from other sources; (3) MSCs have the ability to differentiate into adipocytes, osteoblasts, and chondrocytes in vitro [16,17]; and (4) confirmation of the existence of the stem cell subpopulation requires in vitro clonogenic assays and demonstration that the cells have the ability to differentiate along the desired lineage in vivo [18].

MSCs from several sources are being assessed in a large number of clinical trials in a wide range of settings based on the following assumptions: (1) MSCs have the ability to home to sites of injury and inflammation; (2) MSCs have the ability to differentiate into cells of the mesodermal lineage; (3) MSCs secrete trophic factors that promote proliferation and differentiation of local progenitor cells [19]; (4) MSCs induce or increase neovascularization; (5) MSCs have immunomodulatory properties [20]; and (6) MSCs produce survival factors in ischemic tissues and have antioxidant properties [21]. However, despite the enormous effort that has thus far been invested into clinical trials, very few if any therapies have become part of routine clinical practice.

MSCs are known to have low levels of intracellular ROS and high levels of glutathione, a key antioxidant. They also constitutively express high levels of enzymes required to manage oxidative stress. For example, when compared with the pancreatic beta cell line INS-1, expression of SOD1, SOD2, CAT, and GPX1 was significantly higher in MSCs. These enzymes are able to scavenge peroxide and peroxynitrite (ONOO−). Thus, it has been proposed that the high antioxidant capacity of MSCs makes them ideal for the treatment of pathologies in which tissue damage is linked to oxidative stress [21].

In terms of redox regulation, numerous recent reports describe the importance of oxidants on MSC differentiation toward adipocytes [22], osteocytes [23], chondrocytes [24], and myocytes [25] through activation of signaling cascades involved in differentiation [26–32]. Increased adipogenic fate suppresses the osteogenic lineage, while upregulation of osteogenic signaling attenuates adipogenic terminal differentiation. Many signaling pathways such as Wnt, FOXO, Hh, NEL-like protein 1 (NELL-1), insulin-like growth factor (IGF), and bone morphogenetic protein (BMP) determine MSC terminal fate. Among the pathways that favor either adipogenic or osteogenic differentiation via their activation or suppression, IGF and BMP have a dual effect. These pathways positively regulate both adipogenesis and osteogenesis [28,33–36].

There is evidence for the role of ROS in MSC survival, proliferation, and terminal differentiation, and ROS affect adipogenesis or osteogenesis by stimulating or inhibiting several MSC differentiation signaling cascades. Recently, the impact of ROS on MSC differentiation has generated a great deal of interest due to its potential application in the clinic (e.g., diabetes [37], hypertension [38], atherosclerosis [39], carcinogenesis, and aging [40]. Understanding the impact of ROS on MSC terminal fate will increase our knowledge of the nature and behavior of these cells and how this may be harnessed for therapeutic purposes. This includes the use of ROS inhibitors/activators as pharmaceutical agents. This review focuses on the various potential sources of ROS in MSCs and how they might influence adipogenic and osteogenic differentiation. We will also summarize the studies that have applied exogenous ROS to MSCs and the studies that measure intra and extracellular ROS during MSC differentiation.

**Cellular Sources of ROS**

ROS generation can be physiological, pathological, and tissue specific and varies under different circumstances [41]. ROS can be generated in mitochondrial electron transport systems, by NADPH oxidases, xanthine oxidase, cytochrome P450, nitric oxide synthases, lipooxygenases, heme oxygenase, cyclooxygenases, myeloperoxidase, and monoamine oxidases [42,43]. ROS in mammalian cells can be localized in (1) mitochondria [44]; (2) peroxisomes [45]; (3) endoplasmic reticulum (ER) [46]; (4) lipid peroxides, lipooxygenases [47,48]; (5) plasma membrane (i.e., NADPH oxidases, lipooxygenase) [49]; and (6) the extracellular space [50] (Fig. 1). It has been suggested that NADPH oxidases are localized on the ER and possibly mitochondria [51,52].

**Mitochondria**

Mitochondria are the main source of ROS and the mitochondrial electron transport machinery is thought to be a primary generator of ROS. A small fraction of oxygen escapes from mitochondria during the generation of adenosine triphosphate (ATP) and water. This fraction is then implicated in the formation of ROS. O$_2^*$ is the first ROS produced by mitochondria. The main sources of mitochondrial O$_2^*$ are complex I [53] and complex III [54]. Complex I (NADH ubiquinone oxidoreductase) produces O$_2^*$ in the matrix, whereas complex III (co-enzyme Q, bcc 1 complex, ubiquinone/cytochrome c reductase) induces O$_2^*$ production either in the matrix or the inter-membrane space (Fig. 2). O$_2^*$ is then transformed into a more stable form, H$_2$O, through the activity of Mn, Cu, and Zn-SOD in the inter-
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induces ROS generation by the respiratory chain machinery. Other potential sources of ROS within mitochondria could be α-ketoglutarate dehydrogenase [58] located in the matrix and monoamine oxidase [59] at the outer membrane. Recently, some investigators have suggested that NOX4 is localized in mitochondria, although this remains to be confirmed [60,61].

**NADPH oxidases**

Nonmitochondrial ROS production was first described in neutrophils and macrophages during phagocytosis [62]. NOX consists of a family of seven isoforms that catalyze the reduction of oxygen to superoxide using the pyridine nucleotide NADPH as an electron donor and molecular oxygen as electron acceptor, with the secondary production of other ROS (Fig. 3). In phagocytes, NOX activity requires the cytosolic regulators p47phox, p40phox, p67phox, and the small GTPase RAC. An active complex is formed when the catalytic (gp91phox) and the regulatory subunits are assembled [5].

While it has been known since the seventies [63] that phagocytes contain an NADPH oxidase activity, a number of studies performed in the nineties described low amounts of ROS in nonphagocytic tissues such as smooth muscle cells [64,65]. In 1999 the first nonphagocytic NOX, open reading frame was described in the colon [66]. This isoform is nowadays called NOX1. Other NOX enzymes were then described in other tissues. Seven NOX isoforms are detected in most mammals: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2. However, NOX5 is absent in mice and rats for reasons that are not understood. It has been reported that levels of NOX4 and NOX5 expression are higher than NOX1, NOX2, and NOX3 in adipose-derived MSCs [67]. Most NOX isoforms produce O$_2$•⁻ as a primary product. However, H$_2$O$_2$ is the dominant ROS detected for

![Figure 1](image1.png)

**FIG. 1.** Sources of reactive oxygen species (ROS). ROS can be intracellularly generated by mitochondria and diverse NOX isoforms, peroxosome, endoplasmic reticulum (ER), xanthine oxidase, and lipopigase. It can also be applied from exogenous sources. Irrespective of its source, it may cause cell proliferation, differentiation, and/or cell cycle arrest, and this effect appears to be concentration dependent. Color images available online at www.liebertpub.com/scd

![Figure 2](image2.png)

**FIG. 2.** Mitochondrial ROS production. The production of the superoxide anion, O$_2$•⁻, by complex I and complex III in the matrix or the inter-membrane space forms H$_2$O$_2$ through the activity of SOD catalase dismutation. H$_2$O$_2$ can then be converted to H$_2$O and O$_2$ by glutathione peroxidase (GPX) and catalase or may play a second messenger role in essential signaling pathways. Color images available online at www.liebertpub.com/scd

![Figure 3](image3.png)

**FIG. 3.** NADPH oxidases. NOX enzymes reduce oxygen to O$_2$•⁻ by using pyridine nucleotide NADPH as an electron donor and molecular oxygen as an electron acceptor. ROS will be generated as the secondary product. A part of O$_2$•⁻ can directly react with nitric oxide (NO) to form a toxic peroxynitrite. It can also be dismutated by superoxide dismutase to form hydrogen peroxide to induce cell signaling cascades or directly react with Fe$^{3+}$ to form hydroxyl radical. Color images available online at www.liebertpub.com/scd
NOX4, DUOX1, and DUOX2 [68,69]. This is generally explained by the rapid dismutation of $O_2^-$ into $H_2O_2$.

**Role of ROS in Regulating MSC Fate**

There is a large difference in energy metabolism and cellular redox status between pluripotent stem cells and terminally differentiated (stem) cells. For example, in the proliferative phase (early passages), embryonic stem (ES) cells express high levels of glycolytic enzymes and mitochondria consume low amounts of oxygen. However, differentiated ES cells show a lower glycolytic flux, less than half of that predicted in proliferating ES cells [70]. In addition, the degree of stemness of adult stromal stem cells is linked to the intracellular distribution of mitochondria; stem cell differentiation competence could be defined by a perinuclear arrangement of mitochondria, a low ATP/cell content and a high rate of oxygen consumption, whereas lack of these characteristics was an indication of stem cell differentiation [71].

It is believed that MSCs derived from diverse sources (mostly from adipose tissue and bone marrow) implanted at a site of tissue injury are able to survive, proliferate, and differentiate into various cell types. However, several tissue regeneration-based studies have reported that the majority of grafted MSCs die after several days and only a small percentage survive, which are hardly enough to replace lost tissue [72,73]. This low cell survival rate is due to local hypoxia. Eto et al. demonstrated that adipose-derived MSCs are very sensitive to oxygen concentrations and that only those cells implanted less than 300 μm from an oxygen source would survive, the others undergo apoptosis [74]. Therefore, most transplanted MSCs experience oxidative stress and the excessive ROS produced either by host tissues or by MSCs themselves is believed to account for cell cycle arrest and cell death. ROS can induce the activation of MAPK pathways such as JNK and p38MAPK and ERK along with activation of apoptotic proteins and suppression of antiapoptotic pathways [75]. On the other hand, many investigations have claimed that mitochondrial metabolism and ROS generation regulate MSC differentiation into adipocytes, chondrocytes, osteocytes, and neuronal cells [23,52,75,76]. ROS induce micro RNA-210 (miR-21) expression via PDGFR-b, Akt, and ERK pathways. Micro RNAs act primarily at the post-transcriptional level. Consequently, MSC proliferation and migration increase as a result of miR-210 expression. Micro RNAs suppress mRNA translation and/or promote degradation [77]. Additionally, NADPH oxidase complex induced ROS was reported to induce cell survival cascades through activation of PI3K/Akt pathways and inhibition of p38 MAPK [78]. Therefore, based on previous studies, there is evidence for a role for ROS in MSC survival, proliferation, and terminal differentiation. The impact of the oxidative environment on the regulation of osteogenesis and adipogenesis is described in the following sections (Fig. 4).

**Oxidative Stress and Antioxidant Regulation During Osteogenesis**

Several studies have suggested a link between oxidative stress, osteogenic differentiation and bone formation. It is known that oxidative stress impairs skeletal integrity, and reduces osteogenic differentiation of murine preosteoblastic (MC3T3-E1) and bone marrow-derived stromal (M2-10B4) cell lines [79]. On the other hand, by using antioxidants such as pyro-lidinethiocarbamate, a thiol-containing antioxidant and Trolox, a hydrophilic vitamin E analogue, osteogenic differentiation could be restored [79] suggesting that antioxidants may play a role in preventing age-related osteoporosis [80,81].

A study on mitochondrial metabolism revealed that osteogenic induction in human bone marrow-derived MSCs in vitro is associated with an upregulation of mtDNA copy number, protein subunits of respiratory enzymes, oxygen consumption rate and antioxidant enzymes, but a reduction in the levels of intracellular ROS [51]. The authors reported a dramatic reduction in intracellular levels of $H_2O_2$ and $O_2^-$ on the second day of osteogenic induction. In addition, they reported that 14 days after induction the protein levels of the antioxidant enzymes Mn-SOD and catalase were upregulated three and four fold, respectively.

When compared to untreated cells, addition of exogenous $H_2O_2$ (125–500 μM) to human bone marrow-derived MSCs reduced activity of alkaline phosphatase [82], a marker of

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**FIG. 4.** ROS control signaling cascades involved in osteogenesis/adipogenesis. Wnt/β-catenin, MAPK (NELL-1), and Hh signaling induce osteogenesis while FOXO, PPARγ, and CEBPα signaling stimulate adipogenesis. BMP and IGF signaling have a dual effect in inducing both of these terminal fates. Induction of osteogenesis is optimal in the absence of ROS while induction of adipogenesis is optimal in the presence of ROS. Color images available online at www.liebertpub.com/scd
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<th>Source</th>
<th>Osteogenic differentiation</th>
<th>Adipogenic differentiation</th>
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<td><strong>Intracellular</strong></td>
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<td>Mitochondria</td>
<td>mtDNA copy number, protein subunits of respiratory enzymes and oxygen consumption rate are upregulated while intracellular ( \text{H}_2\text{O}_2 ) and ( \text{O}_2^{•−} ) are reduced after osteogenic induction [51]</td>
<td>Inhibition of the mitochondrial electron transport chain suppresses MSC adipogenic differentiation [76]</td>
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<td>Oxidative stress in aged mice results in reduced osteoblast and bone formation, increased osteoblast and osteocyte apoptosis and decreased bone density [84]</td>
<td>ROS produced by mitochondrial complex III are required for activation of adipogenesis. Intracellular ROS increase after exposure of MSCs to an adipogenic cocktail [139]</td>
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<td>NADPH oxidase</td>
<td>NOX4 knockout mice display higher bone density. NOX4 is involved in the transformation of osteoblasts to osteoclasts and is thus responsible for reduced bone density [112]</td>
<td>NOX4 mRNA expression is decreased while NOX2 mRNA expression is unchanged during adipogenic differentiation [92]</td>
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<td>Extracellular</td>
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<td>( \text{H}_2\text{O}_2 ) reduces Alp activity in osteogenic induced hMSC [51]</td>
<td>Inhibition of the mitochondrial electron transport chain suppresses MSC adipogenic differentiation [76]</td>
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<td>( \text{H}_2\text{O}_2 ) abolishes osteogenesis in osteoblast progenitors [84]</td>
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<td>( \text{H}_2\text{O}_2 ) induced oxidative stress reduces Gli protein levels thus preventing Hh signaling and reducing osteogenesis. The level of Alp mRNA expression is reduced [111]</td>
<td>NOX4 mRNA expression is decreased while NOX2 mRNA expression is unchanged during adipogenic differentiation [92]</td>
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<td>( \text{H}_2\text{O}_2 ) inhibits expression of osteogenic differentiation markers in MC3T3-E1 and M2-10B4 cell lines. Alp activity is also reduced [79]</td>
<td>Elevated oxidative stress and consequently elevated NADPH oxidase in accumulated fat is related to obesity-associated metabolic syndrome in humans and mice [85]</td>
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<td>eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; MSC, mesenchymal stromal cell; ROS, reactive oxygen species.</td>
<td>NOX4 induces adipogenesis in adipose-derived MSCs [87]</td>
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<td>Knock down of NOX4 inhibits MSC adipogenic differentiation even in the presence of an adipogenic cocktail [92]</td>
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<td>Reduced expression of NOX4 is a hallmark of adipogenesis in 3T3-L1 cells [85,92,141]</td>
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<td>H(_2)O(_2) induced oxidative stress induces MSC adipogenesis [22]</td>
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<td>Treating 3T3-L1 cells with H(_2)O(_2) results in adipogenesis even in the absence of insulin [85]</td>
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<td>H(_2)O(_2) increases adipogenesis in 3T3-L1 cells in a dose-dependent manner [86]</td>
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<td>H(_2)O(_2) diminishes expression of adipo-cytokines [87]</td>
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<td>eNOS rather than iNOS governs adipogenesis. NO stimulates rat preadipocyte differentiation [93]</td>
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Oxidative Stress and Antioxidant Regulation During Adipogenesis

Schröder et al. have suggested that stimulation of murine 3T3-L1 cells and human preadipocytes with exogenous \( \text{H}_2\text{O}_2 \) (30 \( \mu \)M, every other day) results in adipogenic differentiation even in the absence of insulin [85]. A dose-dependent role for \( \text{H}_2\text{O}_2 \) in regulating adipogenesis in 3T3-L1 preadipocytes has been observed, with higher doses of \( \text{H}_2\text{O}_2 \) (1 and 10 \( \mu \)M) increasing adipogenesis [86].
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ROS AND MSC DIFFERENTIATION

Additionally, mimicking oxidative stress by addition of exogenous H$_2$O$_2$ (100 μM) for 8 days was shown to induce adipogenesis in human adipose-derived MSCs [22]. In contrast, one study has reported that incubation with H$_2$O$_2$ (0.1–0.5 mM) diminished expression of adipocyte mRNAs such as the fat-derived hormone adiponectin and the transcription factor PPARγ in murine 3T3-L1 cells in a dose-dependent manner [87].

Recently, several studies have focused on antioxidant levels during adipogenesis in an oxidative stress environment [86,88–91]. Application of an antioxidant such as N-acetyl-L-cysteine (NAC) inhibited the expression of transcription factors such as C/EBPα (days 2 and 4) and PPARγ (day 4) in rat bone marrow MSCs and murine 10T1/2 cells [92]. In agreement with these findings, increased levels of ROS production in adipose tissue are accompanied by decreased expression of antioxidant enzymes such as Cu, Zn-SOD, and catalase. ROS production was significantly decreased by the antioxidants aconitin or NAC in fully differentiated 3T3-L1 adipocytes [87]. Recently, it was demonstrated that a concomitant increase in the expression of SOD3 mRNA and protein occurs with the differentiation of human bone marrow-derived MSCs into adipocytes [90] and during the early stages of adipogenesis in 3T3-L1 cells [89]. Using siRNA interference to knock down Mn-SOD, it was observed that the expression of late adipogenesis markers such as adiponectin and fatty acid-binding protein 4 (FABP4) was reduced, demonstrating that Mn-SOD knockdown impairs adipogenesis in 3T3-L1 cells [88]. Similarly, upregulation of antioxidant enzymes such as SOD, catalase, and GPX have been observed during adipogenesis in human adipose-derived MSCs [22].

Several studies have looked at the role of other free radicals in adipogenesis. For example, a stimulatory role for endogenous nitric oxide (NO) on adipogenesis in preadipocytes derived from rat white adipose tissue has been reported [93]. A 50% increase in basal levels of NO was observed on the first two days after adipogenic differentiation. As inducible nitric oxide synthases (iNOS) inhibitors such as 1400W and amiloridine had a minor impact on differentiation and NO production [93], endothelial nitric oxide synthases (eNOS) rather than iNOS may be the major isoform of nitric oxide synthase that modulates adipogenesis [93].

Taken together, these findings demonstrate that an oxidized intracellular environment favors murine and human MSC and preadipocyte differentiation into mature adipocytes. ROS increases the expression of genes associated with adipogenesis. Additionally, adipocytes contain higher levels of intracellular ROS compared with progenitors. The adipogenic process increases the expression of antioxidants, an event that could become a hallmark of adipogenesis. Table 1 summarizes the effects of ROS on MSC adipogenic differentiation.

REGULATION OF DIFFERENTIATION TOWARD AN OSTEOGENIC/ADIPOGENIC FATE

Bone is produced by two mechanisms: (1) intramembranous ossification, the direct differentiation of mesenchymal progenitors into osteoblasts; and (2) endochondral ossification, bone formation via a cartilage anlagen, a mechanism initiated by the formation of MSC clusters [94]. Adipogenic differentiation occurs via two phases: (1) commitment of MSCs to a preadipocyte stage; and (2) terminal differentiation of preadipocytes into mature adipocytes [95].

The interplay of several extracellular signals such as hormones (glucocorticoid and parathyroid hormones) and ligands of the wingless/Indian1 (Wnt), BMP, fibroblast growth factor (FGF), transforming growth factor β (TGFβ), and hedgehog signaling pathways are required for osteogenic differentiation [96,97].

The main signaling pathways that determine MSC terminal fate are reviewed in the following sections.

EVIDENCE FOR A POTENTIAL ROLE FOR ROS IN INHIBITING BONE FORMATION

WNT SIGNALING

Wnt is a molecular switch for adipogenic/osteogenic differentiation. The Wnt canonical pathway (β-catenin dependent) is initiated through the binding of extracellular Wnt ligands to the frizzled seven pass transmembrane receptors (FzRs). Consequently, intracellular signaling of the complex of axin, glycogen synthase kinase 3 (GSK3), and adenomatous polyposis coli (APC) protein will be inhibited. Upon Wnt signaling, β-catenin degradation is inhibited by the Axin/APC/GSK3 complex, which results in the translocation of β-catenin from the cytoplasm to the nucleus [29]. Nuclear β-catenin binds to the T-cell factor lymphoid-enhancing factor (TCF/LEF), which then forms a transcriptional effector for activating Wnt target genes [32] (Fig. 5).

Currently available data suggest that Wnt/β-catenin positively regulate osteoblast and osteoclast activity. In line with this notion, disruption of the Wnt/β-catenin pathway impairs osteogenesis [29]. β-catenin induces essential signals for osteogenic initiation [98] and conditional inactivation converts osteoblasts into chondrocytes and thus delays skeletal mineralization. Wnt/β-catenin signaling suppresses adipogenesis and thus favors osteogenesis by reducing the expression of C/EBPα and PPARγ mRNAs; these molecules are key regulators of adipogenesis and suppressors of osteogenesis [32,99]. Recently, several studies have reported the adverse effect of oxidative stress on osteoblastogenesis [100]. Interestingly, the suppressive effect of H$_2$O$_2$ on Tcf-mediated transcription was abolished by overexpression of β-catenin. An in vivo study reported that ROS increases with increasing age, which in turn decreases the expression of Wnt target genes such as Axin2 and Opg in 31-month-old mice when compared with 4-month-old mice, and thus diminishes osteogenesis [83]. Taken together, these findings suggest that ROS inhibit the osteoinductive effect of Wnt signaling, although under normal circumstances this pathway positively stimulates osteogenesis.

FOXO SIGNALING

Bone involution such as occurs during decreased bone formation and increased bone marrow adiposity is associated with increased oxidative stress and decreased growth factor production. This results in the activation of the FOXO family of transcription factors [101]. Indeed, the defense mechanism against oxidative stress is governed by the FOXO family of transcription factors [102–104]. The FOXO family consists of four members: FOXO1 (or Fkhr),
FIG. 5. ROS suppress important osteogenic signaling pathways while they promote adipogenic signaling pathways. Wnt/β-catenin and Hh signaling cascades induce osteogenesis and this is inhibited in the presence of high levels of ROS, which favors adipogenesis. MAPK signaling induces osteogenesis and is stimulated by ROS. In response to oxidative stress, FOXOs are phosphorylated and translocate to the nucleus where they attenuate the transcription of osteogenic genes while inducing adipogenic differentiation. The active form FOXO also induces the regulation of antioxidant and cell cycle arrest genes. The expression of antioxidants also increases adipogenic differentiation. Color images available online at www.liebertpub.com/scd

FOXO3a (or Fkhrl1), FOXO4 (or Afx), and FOXO6 [105]. β-catenin is also required for FOXO-mediated transcriptional downstream effectors of the Wnt/β-catenin pathway [103]. In osteoblast progenitors and many other cell types, the association of β-catenin with FOXOs increases in the presence of oxidative stress [103]. In the absence of growth factors or in the presence of high levels of ROS, FOXO is activated. It then translocates to the nucleus and induces the transcription of a variety of target genes such as antioxidants (Fig. 5). It is known that FOXO represses osteogenic differentiation [101]. An in vivo investigation in both female and male C57BL/6 mice reported that FOXOs impair bone formation by antagonizing Wnt signaling [83].

Iyer et al. have demonstrated that mice deficient in FOXO1, -3, and -4 in osteoblast progenitors exhibit increased osteoblast number and a higher bone mass [101]. In line with this observation, treating murine osteoblastic cells with 100 μM H2O2 for 1 h enhances β-catenin and FOXO3 association. β-catenin in turn is essential for FOXO target gene stimulation by H2O2. FOXO transcription is promoted by H2O2 while Wnt/Tcf mediated transcription and osteoblast differentiation is reduced [83]. In response to oxidative stress, FOXOs induce cell cycle arrest and dormancy [104,106]. Under such conditions, FOXOs regulate transcription of antioxidant enzymes (eg, catalase, Mn-SOD) and also genes that play role in the cell cycle and cell longevity [102,107] (Fig. 5). Collectively, this suggests that activation of FOXO signaling by oxidative stress attenuates the osteogenic process.

**Hedgehog signaling**

At least three members of the Hedgehog signaling, Hh, family have been described in vertebrates: sonic hedgehog (Shh), Indian hedgehog (hh), and desert hedgehog (Dhh) [108]. Hedgehog signaling is activated by the binding of an Hh ligand to the receptor patched (PTCH), a 12 pass transmembrane protein that inhibits smoothened (Smo), a 7 pass transmembrane protein. This inhibition leads to transcription of the glioblastoma gene product [109] family of DNA-binding proteins to the nucleus where transcription of Hh target genes follows [29,110,111] (Fig. 5). Osteoinductive [111] and anti-adipogenic roles have been ascribed to Hh signaling (99, 135–140). Oxidative stress inhibits Hh-induced osteogenic differentiation in murine primary bone marrow-derived and other MSC cell lines [111] (Fig. 5). Addition of nonphysiological levels of H2O2 (0.5–1 mM) for 72 h suppressed Hh signaling thus inhibiting Hh-mediated osteogenic differentiation in bone marrow stromal cells [111]. The expression of osteogenic differentiation markers such as Alp, OSX, and BSP was significantly reduced, indicating that in MSCs, Shh-induced osteogenesis is inhibited by H2O2-induced oxidative stress (0.5–1 mM H2O2 for
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48 h). Moreover, H2O2-induced oxidative stress impaired the proliferation of MSCs. Taken together, these data suggest that under normal conditions, Hh signaling induces osteogenesis. However, ROS inhibits the osteoinductive effect of Hh signaling.

NADPH oxidase

The impact of NOX isoforms on MSC osteogenic differentiation has been less well studied. NOX4 induced ROS has been implicated in bone disease. NOX4, as a constitutively active source of ROS, is involved in osteoclastogenesis. It has recently been reported that NOX4 knockout mice display higher bone density. Interestingly, a specific single nucleotide polymorphism (SNP) in the human NOX4 gene has been shown to contribute to the greater expression of bone turnover markers and reduced bone density in women. NOX4 expression could be thus responsible for reduced bone density [112]. Understanding the role of NOX isoforms in osteoblast formation is therefore essential in studies on bone loss and regeneration. Future studies should determine the levels of ROS required for osteogenic attenuation and should also clarify the role of the different sources of ROS in osteogenic signaling pathways.

Evidence for a Potential Role for ROS in Promoting Adipogenesis

WNT signaling

A large body of experimental evidence, both in vitro and in vivo, has demonstrated the inhibitory role of Wnt molecules during adipogenic differentiation of mesenchymal or preadipocyte cells [99,113–115]. It is known that Wnt inhibits the early stages of adipogenesis. Inhibiting the Wnt pathway stimulates the generation of adipocytes in 3T3-L1 preadipocytes [99]. From a pathophysiological perspective, several genetic studies have revealed that polymorphisms in genes of the WNT signaling pathway are linked to the development of obesity and type 2 diabetes in humans [113]. It has also been shown that Wnt signaling promotes MSC osteogenic, myogenic, and chondrogenic differentiation and abrogates adipogenic differentiation [116–119]. β-catenin activation suppressed PPARγ expression and impaired murine 3T3-L1 adipogenic differentiation [120]. Taken together, these findings indicate that Wnt has an adiporepressive effect. However, in an oxidative environment, β-catenin diverts to FOXO instead of Tcf to suppress Wnt, and thus favors adipogenesis [83]. Further studies should reveal the role of ROS, their optimum level, and the role of different ROS generators in regulating the Wnt pathway.

FOXO signaling

FOXO negatively regulates adipogenesis. The expression and transcriptional activity of PPARγ, the master transcription factor for adipogenesis, is suppressed by FOXO1 [121]. Insulin induces phosphorylation of Akt, which then activates adipogenic transcription factors, specifically PPARγ. Akt promotes PPARγ expression by FOXO1 exclusion from the nucleus [122]. Akt tightly governs the function of FOXO proteins through Akt-mediated phosphorylation mechanisms [123,124]. FOXO plays a key role in maintaining cellular redox homeostasis. FOXO1 limits oxidative stress in human adipocyte-derived MSCs by upregulating antioxidant enzymes [22]. Additionally, mice lacking FOXO1, FOXO3, and FOXO4 showed decreased adiposity in aged bone marrow, although osteoblast number was increased and these mice had a greater bone mass in old age [101]. Using siRNAs against FOXO1 in murine 3T3-L1 preadipocytes, decreased lipid droplet formation was observed after adipogenic induction. Adipogenesis was more severely inhibited when cells were exposed to FOXO1 siRNA before induction of adipogenic differentiation. Downregulation of FOXO1 in 3T3-L1 cells resulted in a decrease in expression of the adipogenic transcription factors, PPARγ and C/EBPα [123]. However, another study in 3T3-F442A cells and murine embryonic fibroblasts suggested that FOXO1 prevents adipogenic differentiation [124]. Similarly, SIRT2, a cytoplasmic sirtuin, indirectly inhibits PPARγ by reducing FOXO1 acetylation and phosphorylation; this increased the amount of FOXO1 in the nucleus and consequently PPARγ transcription was repressed. These results propose a cell type and context-dependent role for FOXO expression in cellular signaling during adipogenesis [22]. Additionally, the exact role of FOXO, its regulation by phosphorylation and its effect on antioxidant expression requires further investigation.

Hedgehog signaling

An antiadipogenic role has been suggested for the Hh pathway upon its activation [31,112,113]. Hedgehog signaling inhibits adipogenic differentiation of murine 3T3-L1, NIH-3T3, and C3H10T1/2 cells, but when this pathway is inhibited adipogenesis is promoted. Hedgehog signaling induces antiadipogenic transcription factors (eg, Gata2 and Gli2) to repress adipogenesis. Consequently, the antiadipogenic factors downregulate PPARγ expression [127]. Under normal conditions, Hh signaling induces osteogenesis while inhibiting adipogenesis. However, oxidative stress inhibits Hh-induced osteogenic differentiation, and may thus favour adipogenesis. To our knowledge, there is no study reporting the role of oxidative stress in the regulation of adipogenic differentiation via Hh signaling. It would therefore be of interest to assess this possible relationship in future investigations.

Transcription factors

Several transcription factors such as CCAAT/enhancer-binding proteins (C/EBPs) and PPARγ play a key role adipogenesis [29]. Transcription factors such as C/EBPβ and C/EBPδ are the major adipogenesis regulators during early phases of differentiation. Thus, C/EBPδ is expressed during the early phase of adipogenesis and disappears in the late phase [131]. C/EBPα and PPARγ regulate terminal differentiation stages [132]. Coordinated activity between these two transcription factors functions as a positive feedback loop in which PPARγ activates the promoter of the gene encoding C/EBPβ and vice versa. This induces the expression of adipocyte specific genes such as glucose transporter GLUT4 (also known as SLC2A4), lipoprotein lipase, fatty acid-binding protein 4 (FABP4, also known as aP2), adiponectin, and leptin [133–135]. Many factors affect the ability of PPARγ to influence the adipogenic process. Sirtuin 1 (SIRT1), a histone/protein deacetylase, directly
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bonds to PPARγ and impairs adipogenesis [136]. It also acts as a PPARγ co-repressor [137]. SIRT2, a cytoplasmic sir-tuin, indirectly inhibits PPARγ. It has been shown that SIRT2 is downregulated during adipogenesis in murine 3T3-L1 cells [138].

Mitochondrial ROS

Mitochondrial ROS production appears to be critical in promoting the differentiation of human bone marrow-derived MSCs and an increase in ROS levels supports an unrestricted oxidative environment for launching signaling events leading to adipogenic differentiation [139]. Not only do ROS modulate adipogenic differentiation, but they also impact on MSC proliferation. Mitochondrial ROS was suggested to inhibit proliferation of murine 3T3-L1 pre-adipocytes [140]. Similarly, it was demonstrated that ROS produced by mitochondrial complex III is required for activation of adipogenic gene transcription in human bone marrow-derived MSCs. Intracellular H₂O₂ was increased after two days upon exposure of MSCs to adipogenic induction medium (containing indomethacin, dexamethasone, isobutylmethylxanthine, and insulin). However, mitochondrial targeted antioxidants (500 nM MitoCP) attenuated the amount of intracellular H₂O₂ and consequently impaired lipid accumulation during adipogenesis. In relation to this observation, protein levels of major adipogenic transcription factors such as C/EBPα and PPARγ were dramatically decreased. However, adipogenesis was rescued when cells were treated with D-galactose (0.5 mM) to deliberately generate exogenous H₂O₂ [139]. In agreement with these findings, it has recently been suggested that elevated mitochondrial activity is an essential requirement for human MSC adipogenic differentiation. siRNA-based knockdown of the mitochondrial transcription factor A (TFAM), which suppresses mitochondrial activity, inhibited adipogenic differentiation. Under hypoxic conditions or by inhibition of the mitochondrial electron transport chain, mitochondrial respiration was reduced. As a result, adipogenic differentiation was significantly suppressed [76]. Taken together, these findings indicate that superoxide generated by the mitochondrial electron transport chain is converted to H₂O₂, which initiates the PPARγ transcriptional machinery that regulates adipocyte differentiation.

NADPH oxidase

NADPH oxidase was demonstrated to be the central source of ROS in adipocyte precursors [87,141]. NOX4, for instance, is highly expressed in preadipocytes and is emerging as a hallmark of preadipocyte proliferation and differentiation [85,92,141]. Recently, Schröder et al. reported that the effect of NOX4 expression on proliferation and differentiation of murine 3T3-L1 and human adipose-derived MSCs is mediated by the MEK/ERK pathway. They suggested that NOX4 controls proliferation by activating the ROS-dependent phosphorylation of ERK1/2. In addition, both knockout and siRNA studies demonstrate a role for NOX4 in governing MSCs differentiation. siRNA directed against NOX4 resulted in inhibition of ERK1/2 thus promoting proliferation and impairing adipogenic differentiation of 3T3-L1 preadipocytes. siRNA against NOX4 resulted in inhibition of insulin-induced accumulation of lipid droplets in 3T3-L1 cells [85]. Similarly, RNA interference knockdown of NOX4 inhibited the adipogenic differentiation of rat bone marrow-derived MSCs and murine 10T1/2 cells, even in the presence of an adipogenic cocktail. NOX4 expression was decreased; NOX2 expression was, however, constant after adipogenic differentiation [92]. On the other hand, in the experiments reported by Schröder et al., overexpression of NOX4 increased the accumulation of lipid droplets even in the absence of insulin, demonstrating that NOX4 is a direct mediator of insulin-induced differentiation in human preadipocytes [85]. Similarly, overexpression of NOX4 was shown to induce adipogenesis in human adipose-derived MSCs [22]. A further study demonstrated that the level of production of ROS such as H₂O₂ increased in adipose tissue of obese mice when compared to control mice and was accompanied by increased expression of NADPH oxidase (gp91phox and p22phox), and cytosolic components p47phox, p67phox, and p40phox and a reduction in the levels of antioxidant enzymes. ROS production was significantly decreased by the NOX inhibitor diphenyleneiodonium in murine 3T3-L1 preadipocytes [87]. Collectively, these data suggest a positive role for the NOX4 isoform of NADPH oxidase in the differentiation of adipogenic progenitors. Generation of ROS by NADPH oxidase appears to be necessary for positive regulation of MSC proliferation and adipogenic differentiation. Targeting various NOX isoforms may elucidate their role in the effect of ROS on MSC differentiation.

Therapeutic Role of ROS

Oxidative stress plays a key role in the pathogenesis of many diseases [87] such as diabetes [37], hypertension [38], atherosclerosis [39], carcinogenesis, metabolic, cardiovascular, pulmonary, and neurological diseases [142]. Recently, NOX inhibitors that target NOX1 and NOX4 enzymes have been used in patients with diabetic nephropathy [142]. The role of exogenous or endogenous ROS on osteogenesis and adipogenesis in the clinical setting has been less intensely investigated. However, ROS is known to be a critical factor in aging [40]. Increased oxidative stress, mainly associated with ageing, has been implicated in the pathogenesis of age-related bone loss in humans and mice [83]. Recently, it was reported that NOX4 is involved in osteoclastogenesis. Subsequently increased human bone resorption has been linked to NOX4, as a constitutively producer of H₂O₂ [112]. SNP analysis of NOX4 in middle-aged woman has revealed a link to altered bone density and plasma markers for bone turnover. In addition, NOX4 is highly expressed in osteoporotic bone in humans [112]. Thus, application of NOX inhibitors should be considered in the context of osteoporosis treatment and possibly bone-related disorders.

Studies in humans have revealed that accumulation of adipose tissue in obese patients is associated with increased systemic oxidative stress, which might therefore present an interesting target for the development of new therapies for obesity-associated metabolic syndrome [87]. In addition, several studies have reported elevated systemic oxidative stress in obesity [143]. A greater understanding of ROS signaling and the consequences thereof might therefore open new therapeutic avenues for the treatment of obesity and its comorbid entities.
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Conclusion

ROS have for many years been regarded as having a negative effect on cell function and survival. However, it is becoming increasingly recognised that ROS also mediate important physiological functions. The findings reviewed here demonstrate a pivotal role for ROS in MSC differentiation. Regardless of the sources of ROS, it has been shown that osteogenic and adipogenic differentiation is partly ROS dependent. We conclude that osteogenesis is blunted by elevated ROS while ROS positively induces adipogenesis in MSCs and other adipogenic progenitors. The activity of the ROS generating NOX4 isofrom most likely increases adipogenesis. However, mitochondrial ROS also appears to be necessary for MSC adipogenic differentiation. Taken together, these findings highlight the need to further investigate the role of ROS in regulating MSC differentiation. Further studies should clarify the role of ROS in each signaling cascade, the role of different sources of ROS and their concentration, the period required for treating cells with exogenous ROS and finally the impact of various antioxidants exogenously applied and/or produced in stem cell-based studies and also in the pathogenesis and treatment of relevant diseases.

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Author Disclosure Statement

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6.5 Paper 5: Autologous PRP: a biological supplement to enhance AT-MSC expansion

Autologous Platelet-Rich Plasma: A Biological Supplement to Enhance Adipose-Derived Mesenchymal Stem Cell Expansion

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Currently the use of non-autologous cell culture media (e.g., animal-derived or allogeneic serum) for clinical applications of mesenchymal stem cells (MSCs) is criticized by regulatory agencies. Autologous platelet-rich plasma (PRP) is proposed as a safer alternative medium supplement for adipose-derived mesenchymal stem cells (AT-MSC) culture. To study its efficiency on cell proliferation, AT-MSCs were cultured for 10 days in media supplemented with different concentrations of autologous non-activated PRP (nPRP) or thrombin-activated PRP (tPRP) (1–60%). AT-MSC proliferation, cell phenotype, multipotency capacity, and chromosome stability were assessed and compared to AT-MSCs expanded in a classical medium supplemented with 10% of fetal bovine serum (FBS). Culture media supplemented with nPRP showed dose-dependent higher AT-MSC proliferation than did FBS or tPRP. Twenty percent nPRP was the most effective concentration to promote cell proliferation. This condition increased 13.9 times greater AT-MSC number in comparison to culture with FBS, without changing the AT-MSC phenotype, differentiation capacity, and chromosome status. We concluded that 20% autologous nPRP is a safe, efficient, and cost-effective supplement for AT-MSC expansion. It should be considered as an alternative to FBS or other nonautologous blood derivatives. It could serve as a potent substitute for the validation of future clinical protocols as it respects good manufacturing practices and regulatory agencies’ standards.

Introduction

Mesenchymal stem cells (MSCs) currently represent a promising cell source for regenerative medicine and tissue engineering strategies,12,13 in particular for bone, cartilage, and soft tissue regeneration.5,6 These multipotent cells principally have the ability to differentiate to mesodermal lineages such as adipocytes, osteocytes, and chondrocytes.6–10 Bone marrow has been used as the main source of MSCs for many years. Presently, an increasing interest is devoted to MSC isolated from adipose tissue (AT-MSC).11,12 This source presents several advantages in comparison to bone marrow: (i) adipose tissue is easier to harvest, (ii) it is widely available, and (iii) it contains higher MSC concentration.12,15

Ex vivo cell culture is mandatory for most clinical applications of MSCs. Cell expansion requires a basal medium supplemented with proteins, growth factors, and enzymes to support cell attachment and proliferation. Classical protocols use culture media supplemented with xenogeneic additives (e.g., fetal calf serum or fetal bovine serum [FBS]),14,15 which present a potential risk of infection and immunological reaction. To reduce these risks, efforts are devoted toward the development of human allogeneic supplements (e.g., human serum, human platelet derivatives).16–19 The use of these nonautologous culture protocols still presents at least three main limitations: (i) potential risks of contamination (e.g., virus, prion),20 (ii) immune reactions due to nonautologous proteins internalization by MSCs,21–24 and (iii) the suboptimal rate of cell proliferation.25–29 Therefore, a safe and effective culture supplement is urgently needed to comply at best with national and international regulatory agencies’ requirements for clinical applications of MSCs.

Platelets are a natural reservoir of growth factors, which are efficient in promoting cell proliferation, differentiation, and tissue regeneration. When platelets are physiologically activated, their α-granules gradually secrete growth factors and cytokines such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor-β (TGFβ),1 vascular endothelial growth factor (VEGF), and endothelial growth factor (EGF).30,31 However, platelet activation by thrombin or Ca2+1 provokes complete nonorchestrated release of growth factors within the first few hours only.29,30 Currently, plasma rich in platelets obtained from patients’ own blood is already used efficiently for wound healing, bone regeneration, or skin rejuvenation.31–33 We thus postulate that autologous platelet-rich plasma (PRP)
can serve as a safe and effective biological supplement, substituting current nonautologous products for cell expansion. To define an autologous system for AT-MSC proliferation, we assessed the efficiency of autologous PRP on AT-MSC proliferation in comparison to the classical FBS-supplemented medium. We investigated the optimal PRP concentration and compared nonactivated PRP (nPRP), containing intact platelets, to thrombin-activated PRP (tPRP). Furthermore, we assessed the platelet viability over time in PRP. We postulated that live platelets, delivering continuous growth factors to the media, could eliminate the need for medium changes during up to 10 days of AT-MSC culture.

Materials and Methods

Adipose tissue harvesting and PRP preparation

For each experiment, adipose tissue and blood were collected from the same patient who underwent abdominoplasty. All experiments were done in accordance with the established ethical standards, local ethics committee agreement, and patient consent.

Adipose tissue harvesting. Fat tissue was collected and purified from the subcutaneous abdomen layer of patients according to the Coleman technique as previously described.34 Briefly, 20 mL of fat tissue was harvested manually from each patient with a 3 mm cannula (Mentor, Santa Barbara, CA) connected to 10 mL Luer-Lok<sup>19</sup> syringes (BD Biosciences, Franklin Lakes, NJ). The pure fat tissue was separated from blood, oil, and liquid after 3 min centrifugation at 3200 rpm at 120G.

PRP and autologous thrombin preparation. For PRP preparation, specific tubes containing sodium citrate as anticoagulant and a specific gel separating platelets and plasma from other blood components (e.g., red and white blood cells) were used. Briefly, 8 mL of human peripheral blood was collected into a Regen-BCT tube (RegenKit<sup>15</sup>; RegenLab, Le Mont-sur-Lausanne, Switzerland). The collected blood was centrifuged for 5 min in a standard laboratory centrifuge at 150G. Subsequently, the white and red blood cells accumulated in the bottom of the tube under the separator gel, whereas the plasma and platelets remained above the gel layer. Plasma containing platelets was homogenized by returning the tube five times to obtain 4 mL of nPRP, which was collected in a polycarbonate tube (Becton-Dickinson, Franklin Lakes, NJ) until use. Platelets, red and white blood cells in whole blood were counted (KX-21N; Sysmex, Lincolnshire, IL) before centrifugation and in the prepared nPRP before addition to culture media.

For tPRP experiments, autologous thrombin was obtained with an ATS tube, a similar tube to that used for PRP preparation, but without the added anticoagulant. Briefly, 8 mL of blood was collected into the Regen-ATS tubes (RegenKit, RegenLab) and centrifuged for 10 min at 150G. Similarly to the PRP preparation, red and most of the white blood cells were sequestered below the separating gel, whereas the plasma over the gel formed a clot due to the lack of anticoagulant. The serum extracted from the clot, rich in thrombin, was added 1:10 to PRP to activate the platelets and obtain tPRP.

AT-MSC isolation

Pure fat was digested with 0.01% collagenase type I (Sigma-Aldrich, St. Louis, MO) for 45 min at 37°C with gentle agitation. The nondigested adipose tissue was removed after centrifugation at 1400 rpm for 10 min. The remaining pellet, called the stromal vascular fraction (SVF), was suspended in erythrocyte lysis buffer for 5 min (Qiagen, Hilden, Germany). It was then washed with the basal medium: Dulbecco’s modified Eagle’s medium (DMEM)-low glucose containing 1 g/L glucose, 1-glutamine, 25 mM HEPES (Invitrogen, Carlsbad, CA), supplemented with penicillin and streptomycin 10,000 μg/mL (Bioconcept, Salem, NH), and 2 units/mL heparin (Liquemin 5000; Roche, Basel, Switzerland). After centrifugation at 1200 rpm for 5 min, SVF was then resuspended in DMEM and supplements, and filtered through a 100 μm nylon cell strainer (BD Biosciences). The mean cell density in the isolated SVF was 30 × 10⁶ cells/mL.

AT-MSC culture

SVF cells were plated at 5000 cell/cm² in a 48-well plate (BD Biosciences) and cultured in different media culture conditions: 10% FBS (Gibco, Carlsbad, CA) as control or 1%, 5%, 10%, 20%, 40%, and 60% of either nPRP or tPRP added to the basal DMEM and supplements (1 mL medium for each condition). The resulting plastic-adherent cell population after 24–48 h of culture was determined as AT-MSCs. Cells were cultivated at 37°C for 10 days in a standard incubator with 5% CO₂ without changing the culture media for FBS and PRP conditions.

AT-MSC viability and proliferation

Cell viability and number was assessed after 10 days of culture using two different techniques, hemocytometer (Marienfeld, Emmendingen, Germany) and an image-based cytometer (Tali; Invitrogen). After dissociation with trypsin (trypsin-EDTA [1 × 0.05%; Invitrogen]), cell viability was determined for each condition by Trypan blue exclusion (Sigma-Aldrich) or Propidium iodide staining and fluorescence analysis (R&D Systems, Minneapolis, MN), respectively. The doubling time for the AT-MSC population in 10 days (240 h) was quantified for each patient.

To assess cell proliferation, an EdU cell proliferation assay (Click-it EdU; Invitrogen) was performed. Briefly, 5000 cells at P1 were cultured per condition for 24 h and then incubated with EdU solution for 24 h. Thereafter, cells were fixed with 10% formalin, stained according to the manufacturer’s instructions and scored for EdU-labeled proliferating cells. EdU dye integrates with newly formed DNA strands. The EdU assay thus provides the proliferation ratio expressed as a percentage; the number of EdU bright cells divided by the number of Hoechst bright cells (total number of cells) × 100.

Cell phenotype assessment by flow cytometry

To confirm the bona fide MSC phenotype in 20% nPRP versus 10% FBS cultures after 10 days, immunophenotypic analysis was performed by flow cytometry, according to whether they show positive for CD73, CD105, CD90, and absence of CD45, CD19, and HLA-DR. Briefly, cells were trypsinized, resuspended in phosphate-buffered saline (PBS) containing 1% FBS, and marked with mouse anti-human
antibodies: CD45-FITC (Diatec, Oslo, Norway), CD73-PE, CD90-Cy5, HLA-DR-FITC (Immunotech-Coulter, Marseille, France), CD105-FITC (ABD SeroTek, Kidlington, United Kingdom), CD19-FITC (Life Technologies, Carlsbad, CA). Cell viability was quantified by 7-Aminoactinomycin D (Sigma-Aldrich). Mouse isotype antibodies (Beckman Coulter, Brea, CA) were used as a control. Ten thousand labeled cells were analyzed by a FACSCaliber flow cytometer using the CellQuest software (Becton-Dickinson).

**AT-MSC differentiation analysis**

To assess the influence of nPRP on conserving the multipotentiality of AT-MSCs, we induced adipogenic, osteogenic, and chondrogenic differentiation on P3. Cells were cultured on P0–P2 with either 20% nPRP or 10% FBS containing medium, and passed at 80% of confluence. For the third passage, a fresh nPRP was prepared from the same blood donor as initially used. Adipogenic, osteogenic, or chondrogenic differentiation was achieved by adding specific differentiation media: Stemprio, Adipogenesis, Osteogenesis, or Chondrogenesis Differentiation Kits (Life Technologies), at the third passage and the medium was changed every 3 days.

Adipocytes were marked by Red Oil O (Sigma-Aldrich) staining lipid droplets after 2 weeks of culture. Osteocytes were stained by Alizarin Red (Sigma-Aldrich), which marks calcium deposits after 3 weeks of culture, whereas chondrocytes were stained by Alcian Blue (Sigma-Aldrich), which reveals the presence of acid mucopolysaccharides and glycosaminoglycan after 2 weeks of culture.

**Chromosome stability assessment and cytogenetic analysis**

The stability of the chromosomes in 20% nPRP cultured cells was compared to 10% FBS cultured cells. The samples were prepared on the first passage from a 60% to 80% confluent culture flask of 25 cm². Colcemid (Invitrogen) treatment was done for 15 h with concentration of 0.05 μg/mL as described previously. The colcemid was removed and the cultures were trypsinized with trypsin-EDTA (Invitrogen) to recover the cells. The cells were treated with 0.075 mM hypotonic lysis solution (KCI) at 37°C for 20 min and fixed in methanol-acetic acid solution (3:1, v/v). The metaphase spreads were analyzed after a G-banding (GTG) as previously described with an average resolution of 350 bph.

**Platelet viability analysis**

Platelet viability was assessed by Calcein/AM (Biotium, Inc., Hayward, CA), which marks viable platelets by producing a green fluorescent signal. Time course platelet viability (day 0, 4, 7, and 10) was established in the basal DMEM medium and supplements with 20% nPRP with and without AT-MSC culture at 37°C in the incubator. Briefly, at each time point 50 μL of 1 μM Calcein/AM in PBS was added to PRP followed by incubation for 30 min at 37°C.

The fluorescence was measured on a fluorescence plate reader at 485 nm excitation and 530 nm emission wavelengths. Platelet viability in each time point was normalized by the platelet viability on day 0.

**Statistical analysis**

The Mann–Whitney U test was used for comparison between groups, with values of $p<0.05$ being regarded as significant. Data are presented as mean±SEM.

**Results**

**Platelet and blood cells counting**

The platelet recovery rate in nPRP from the whole blood was 98%. Mean platelet concentration stuck over the separator gel (buffy coat) was $1.12 \times 10^{11} \pm 120.6$ platelets/μL. As for these experiments, the platelets were resuspended in all 4 mL of plasma over the gel, the final mean platelet concentration of nPRP used in this study was $2.41 \times 10^{11} \pm 20.36$ platelets/μL. This concentration is 1.7 times more than the whole blood before centrifugation ($1.44 \times 10^{11} \pm 9.10$ platelets/μL) (Fig. 1). Conversely, the mean white blood cell concentration was significantly lower in nPRP in comparison to whole blood ($0.72 \times 10^{11} \pm 0.11$ cells/μL vs. $4.87 \times 10^{10} \pm 0.46$ cells/μL); the mean red blood cell concentration was similarly

**FIG. 1.** Analysis of number of platelets (A), white blood cells (B), and red blood cells (C) in whole blood compared to purified platelet-rich plasma (PRP). $n=14$ patients ($**p<0.001$).
Adipose tissue: obesity vs regeneration

**FIG. 2.** Bright-field micrographs of adipose-derived mesenchymal stem cell (AT-MSC). In the presence of 10% fetal bovine serum (FBS) (A), and 1–60% non-activated PRP (nPRP) (B–G) after 10 days culture (P0). Magnification 20×. Pictures are representatives of one donor. High density of platelets produces a darker background.

PRP enhances AT-MSC proliferation dose dependently

In all conditions, AT-MSCs kept their typical spindle fibroblast shape during the culture period. After 10 days culture, all media supplemented with different nPRP concentrations presented a higher AT-MSC number when compared to FBS-containing media (Fig. 2). This positive effect of nPRP followed a dose-dependent bell-shape curve. Media supplemented with 20% nPRP offered the optimal condition, with AT-MSC number being 13.9 times higher than in 10% FBS (n = 14, p < 0.001) after 10 days of culture. In comparison, other conditions were less effective [e.g., 10% and 40% nPRP media increased respectively 5.6 and 10.9 times the AT-MSC number when compared to 10% FBS (n = 14, p < 0.001)] (Fig. 3A).

The tPRP-containing media showed also a higher AT-MSC number in comparison to media with FBS. Compared to 10% FBS, the AT-MSC number was 5.9, 5.8, 5.2, and 5.6 times higher in 10%, 20%, 40%, and 60% tPRP (n = 6, p < 0.05), respectively (Fig. 3B). However, all tPRP conditions were consistently less effective than 20% nPRP.

In comparison with nPRP media, where a concentration of 20% presented the best and reproducible condition for all tested patient cells (n = 14), tPRP-treated AT-MSC showed inconsistent results over six patients: three patients had the best AT-MSC proliferation rate with 40% tPRP, whereas two other patients had the highest AT-MSC number with 10% tPRP and one patient with 60% tPRP (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tec).

In summary, over 10 days culture, 20% nPRP and 10% tPRP produced the highest stimulatory effect on AT-MSC, causing them to multiply 230- and 155-fold versus 16.5-fold expansion in 10% FBS, respectively.

The EdU assay over 24 h demonstrated that 20% nPRP induces a higher proliferation rate of AT-MSCs (68%) in comparison to 10% FBS (54%) and other nPRP concentrations (Fig. 4). The population doubling time was significantly shorter for 20% nPRP, in comparison to other conditions (Fig. 5). AT-MSCs doubled their population every 28 h in 20% nPRP condition: twice as fast than with 10% FBS media condition (56 h, n = 6, p < 0.05) (Fig. 5).

Cell characterization by surface marker expression

To confirm the stability of AT-MSC characteristics after 10 days culture, the typical surface marker expression of

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**FIG. 3.** Effect of different concentrations of nPRP (A, n = 14) or thrombin-activated PRP (tPRP) (B, n = 6) on the proliferation of AT-MSC cultured over 10 days without medium change. Ten percent FBS is used as control. *p < 0.05; **p < 0.01 and ***p < 0.001.
ADIPOSE TISSUE: OBESITY VS REGENERATION

AT-MSCs cultured with nPRP or FBS was studied. Regardless of the supplement used in the medium, the AT-MSC marker profile remained unchanged; cells were positive for CD90 (82.5% of cells in FBS condition vs. 91.3% in nPRP), CD105 (82.5% vs. 91.3%), and CD73 (91.3% vs. 95.1%), whereas they were negative for CD45 (0.103% vs. 0.058%), CD19 (0.027% vs. 0.096%), and HLA-DR (1.35% vs. 0.81%) (Fig. 6).

Differentiation capacity

We verified that the intrinsic differentiation potential of AT-MSCs into adipocytes, osteocytes, or chondrocytes was kept intact regardless of the supplements used for their proliferation. Indeed, we observed that nPRP did not adversely affect the differentiation capacity of AT-MSCs tested at passage 3. Adipogenic (Fig. 7A, B), chondrogenic (Fig. 7C, D), and osteogenic differentiation (Fig. 7E, F) were confirmed and were qualitatively comparable to 10% FBS.

Karyotyping analysis

Cytogenetic analysis of cells cultured in either 10% FBS or 20% PRP conditions, did not show any abnormal karyotype. FBS and nPRP culture conditions had numerical and structural stability. Thus, treating cells with nPRP do not modify the chromosomal stability (Fig. 8).

Platelet viability

We then measured the time-dependent platelet survival rate in 20% nPRP-supplemented medium in the presence or absence of AT-MSCs. On day 4 and in the presence of AT-MSCs, more than 80% of platelets were viable when normalized to day 0. This percentage decreased to 64% platelet viability on day 7 and remained as high as 57% on day 10 (Fig. 9). The survival rate in the absence of AT-MSCs was instead lower: 60%, 58%, and 43% on days 4, 7, and 10, respectively.

Discussion

The culture protocols for clinical application of AT-MSCs have to comply with good manufacturing practices (GMP) and regulatory agencies’ standards. All steps including manufacturing, sampling, testing, storage, packaging, and distribution need to be standardized, safe, and efficient. In vitro cell expansion is one of the key steps of manufacturing. However, so far, none of the available protocols completely satisfy these requirements. Currently FBS, a complex mixture of proteins and nutrients, is typically used to isolate and expand MSCs. This xenogeneic media supplement is regarded critically by regulatory agencies because of its high risk of contamination (e.g., virus positivity reported to be as high as 20–50%) and its allergic reactions due to xenogeneic proteins internalization by MSCs. Obviously, all these disadvantages can compromise the therapeutic success.

Several researchers have made efforts in substituting xenogeneic MSC culture media by human blood-derived products. As reviewed recently by Bieback, a range of human products have been tested: plasma, serum, umbilical cord blood serum, and platelet, derived from either a blood bank or fresh blood, but rarely autologous. Bieback et al. demonstrated that pooled human platelet lysate derived from several patients could be a substitute for FBS in

FIG. 4. Assessment of 24 h-proliferation efficiency of AT-MSCs by EdU staining. Different concentrations of nPRP (1–60%) were compared to 10% FBS on AT-MSCs (P1) at the end of 10 days culture period. (A) Active proliferating cells are revealed by EdU-based green fluorescence nucleus staining, compared to total cells stained by Hoechst dye (blue nucleus staining). (B) Percentage of EdU positive cells was estimated by the formula: (green bright AT-MSC nucleus/total blue AT-MSC nucleus) x 100. n=4.
Adipose tissue: obesity vs regeneration

FIG. 5. Population doubling time in AT-MSC. Cells were cultured in media supplemented with 10% FBS as control (white bar), and increasing concentrations of nPRP (gray bars) or tPRP (black bars). *p < 0.05 and **p < 0.01 (n = 6).

Bone marrow–derived MSC expansion. Kocaoemer et al. claimed that 10% nonautologous PRP activated by shock freezing or thrombin increased the AT-MSC proliferation after 11 days culture. Instead of directly adding platelets, others reported that the supernatant of activated PRP could increase MSC proliferation rate over other medium supplements. Others concluded that the addition of growth factors per se (e.g., PDGF, TGF, EGF) can greatly increase the cell migration and proliferation rate. However, as all of these studies applied nonautologous products, the potential risk of contamination and immunologic reactions remains.

Therefore, considerable efforts are presently devoted to defining an ideal medium which can substitute the current nonautologous systems. Recently, a few studies have suggested that media supplemented with autologous PRP could be used for AT-MSC expansion. In these studies, only the supernatant of PRP was added to the cell culture, and the media were free of platelets. Li et al., demonstrated that the frozen supernatant of autologous PRP activated with bovine thrombin had a potent effect on AT-MSC proliferation and neurogenic differentiation. Kakudo et al. compared the effect of different media supplemented with frozen supernatant obtained from whole blood, nonactivated or thrombin–calcium-activated autologous PRP on AT-MSC proliferation as well. They concluded that the media with 5% of activated-PRP supernatant offers the best effect on AT-MSC proliferation by increasing cell numbers of five-folds within 7 days of culture.

In conformity with regulatory agencies and GMP standards, culture media should be, amongst other criteria, free from contamination risk, nonimmunogenic, nononcogenic, and effective in increasing cell proliferation rate. Importantly, it should maintain the MSC phenotype unmodified and retain its differentiation capacity over an extended time period.

According to these needs, our study demonstrates that nPRP can be used as an autologous biological medium supplement for AT-MSC proliferation in vitro. It could efficiently substitute current nonautologous products. In comparison with FBS used traditionally for cell culture, our data demonstrated that a medium supplemented with 20% of autologous nPRP can significantly promote AT-MSC expansion, without affecting AT-MSC phenotype and multipotency.

FIG. 6. Analysis of surface marker expression. Phenotype of AT-MSCs cultured in 10% FBS or 20% nPRP was assessed by flow cytometry at passage 0. Isotype control antibody is shown by the black empty lines whereas the expressed markers are in red. “M bars” indicate the percentage of cells expressing the surface marker.
In addition, to our knowledge, our study demonstrates for the first time the advantage of AT-MSC coculture with platelets, where the culture media is directly supplemented with autologous functional fresh and nonactivated platelets, instead of their supernatant.

Our data show that nPRP has a dose-dependent effect on AT-MSC proliferation. Media supplemented with 20% nPRP (2.41 × 10^5 ± 20.36 platelets/μL) was the optimal condition for each patient, where AT-MSCs grew up to 230-fold within 10 days culture, significantly higher than with FBS (i.e., 16.5-fold) or other supplements. Furthermore, unlike other studies using chemically activated PRP with calcium or thrombin, 26,12 our study reveals nPRP as more efficient than the nonphysiologically activated PRP such as tPRP.

We were also able to demonstrate that a majority of platelets remained viable after 10 days of culture, particularly in the presence of AT-MSCs. Therefore, it circumvents the need for a medium change every 3 days as required in classical cell culture protocols using FBS or PRP supernatant. So, as nPRP application decreases the need of medium replacement up to 10 days, we postulate that it would enable a safer system, thereby reducing risks inherent to cell manipulation, and also providing a more cost-effective and manpower-saving operational method.

We hypothesize that nonactivated platelets present in nPRP get gradually activated and secrete orchestrated growth factors up to 10 days. This could closely mimic the physiological activity of platelets in blood stream, where they gradually release their growth factors during their 10 days lifespan. This avoids high burst growth factors release at culture start and ensures the activity of secreted growth factors during longer time periods.

As growth factors are essential for MSC proliferation and differentiation, a precise dose combination and time delivery are critical for optimal results. 51,52 For instance, high growth factor concentrations may lead to unwanted differentiated cell phenotypes, 28 as well as downregulation of surface receptors rendering cells insensible to such factors. This could explain why the highest nPRP concentrations (e.g., 40% and 60%), by containing excessive growth factor amounts, were less effective than 20% nPRP. Several studies

FIG. 7. Representative qualitative evaluation of AT-MSC differentiation capacity. Differentiation toward adipocyte (A, B), chondrocyte (C, D), or (E, F) osteocyte phenotype after culture in 10% FBS or 20% nPRP.

FIG. 8. Representative chromosome karyotype by G-bandung. (A) 10% FBS and (B) 20% nPRP cultured MSCs were compared for chromosome aberration.

FIG. 9. Assessment of platelet viability by Fluorescence microscopy using calcein dye. (A–D) Fluorescence micrograph of viable, calcein-stained platelets monitored over a 10 days period in media containing 20% nPRP in contact with AT-MSC without medium change (magnification: 40 ×). (E) Mean viable platelet in the presence or absence of AT-MSC. Percentage of platelet viability in 20% nPRP was normalized to day 0 (n = 5; *p < 0.05, **p < 0.01).
have also proved that applying excessive platelet concentrations may bring paradoxical inhibitory effects. Graziani et al. concluded that the PRP containing a platelet concentration 2.5× higher than the whole blood had the maximum effect on fibroblasts and osteoblasts proliferation; whereas higher concentrations would decrease the proliferation effect. Weibrich et al. claimed that a concentration of 106 platelets/μL stimulates bone regeneration, whereas higher concentrations might reveal inhibitory effects. Similarly, we can speculate that physiological and controlled growth factor release by live platelets in nPRP may be more effective than hPRP, where a burst of growth factors are released at start. In line with this, Scherer et al. also observed that nPRP was more efficient than hPRP in wound healing.

Our study demonstrates that nPRP did not change AT-MSCs marker phenotype and multipotency, important criteria for an ideal expansion medium. Unlike other studies claiming that platelet lysate or thrombin-activated platelets may change the differentiation capacity of MSCs, we proved, in this study, that AT-MSC's differentiation capacity toward adipogenic, osteogenic, and chondrogenic lineages was not reduced in 20% nPRP, in comparison to FBS.

Furthermore, unlike several studies reporting some concern about genetic stability throughout cell expansion and the investigations that report the chromosomal aberration due to culture contamination, we demonstrated that media supplemented with nPRP do not modify the chromosome stability of AT-MSCs. This is a biosafety criterion that is mandatory for MSC's clinical administration and required by regulatory agencies.

Obviously, for the clinical application of PRP, there is still a lack of standardization and agreement on the efficiency of PRP and its method of preparation and application. Each autologous PRP could have different characteristics (e.g., platelet number and growth factor concentrations, platelet survival rate), thus achieving variable results. This variability is due to variations between individual donors (e.g., age, thrombocytemia) and to different PRP preparation techniques available on the market (e.g., device type, centrifugation number); therefore, efforts have to be made to standardize PRP products.

Clearly, further investigations are required to address the signaling pathways involved in PRP-dependent AT-MSCs proliferation and differentiation.

Conclusions

In this study, we demonstrate for the first time that, when compared to activated PRP or a xenogeneic supplement like FBS, autologous nPRP is a safe and efficient biological culture supplement capable of promoting AT-MSC proliferation while maintaining their phenotype, differentiation potential, and chromosome stability. The use of fresh autologous nPRP easily complies with regulatory agencies' standards for clinical protocols.

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