Redox changes in obesity, metabolic syndrome, and diabetes

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ABSTRACT

"Life is an instantaneous encounter of circulating matter and flowing energy" (Jean Giaja, Serbian physiologist), is one of the most elegant definitions not only of life but the relationship of redox biology and metabolism. Their evolutionary liaison has created inseparable yet dynamic homeostasis in health, which, when disrupted, leads to disease. This interconnection is even more pertinent today, in an era of increasing metabolic diseases of epidemic proportions such as obesity, metabolic syndrome, and diabetes. Despite great advances in understanding the molecular mechanisms of redox and metabolic regulation, we face significant challenges in preventing, diagnosing, and treating metabolic diseases. The etiological association and temporal overlap of these syndromes present significant challenges for the discrimination of appropriate clinical biomarkers for diagnosis, treatment, and outcome prediction. These multifactorial, multiorgan metabolic syndromes with complex etiopathogenic mechanisms are accompanied by disturbed redox equilibrium in target tissues and circulation. Free radicals and reactive species are considered both a causal factor and a consequence of disease status. Thus, determining the subtypes and levels of free radicals and reactive species, oxidatively damaged biomolecules (lipids, proteins, and nucleic acids) and antioxidant defense components as well as redox-sensitive transcription factors and fluxes of redox-dependent metabolic pathways will help define existing and establish novel redox biomarkers for stratifying metabolic diseases. This review aims to discuss diverse redox/metabolic aspects in obesity, metabolic syndrome, and diabetes, with the imperative to help establish a platform for emerging and future redox-metabolic biomarkers research in precision medicine. Future research warrants detailed investigations into the status of redox biomarkers in healthy subjects and patients, including the use of emerging 'omic' profiling technologies (e.g., redox proteomes, lipidomes, metabolomes, and transcriptomes), taking into account the influence of lifestyle (diet, physical activity, sleep, work patterns) as well as circadian ~24h fluctuations in circulatory factors and metabolites.

1. Introduction

Modern human medicine faces numerous challenges, despite the explosive progress of diagnostic and therapeutic approaches. Our ability to gain an in-depth understanding of the molecular basis of various pathological conditions has opened diverse research avenues and many questions that require urgent answers. If we were to allow ourselves some scientific skepticism, we would ascertain the fact that even after almost a hundred years since the discovery of insulin and despite much scientific progress, we still live in a pandemic of diabetes, especially type 2. The forecasts for the coming decades are not promising either. In parallel with type 2 diabetes mellitus (T2DM), another two conditions strongly associated with it – obesity and metabolic syndrome, paint the same picture of a pandemic with predictions of exponential growth in the future.

Each of these three conditions is complex in itself. The challenges we face are further compounded because these conditions can occur concomitantly while their underlying mechanisms often overlap. The complexity is multifactorial, whether we consider the etiology, clinical manifestations, or complex therapeutic approaches. For these reasons, we do not regard diabetes and obesity as classic diseases but rather as

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Abbreviations

4-HNE	4-hydroxynonenal
8-epi-PGF2α	8-epi-prostaglandin F2α
8-oxo-dG	8-oxo-2`-deoxyguanosine
8-oxo-Guo	8-oxo-7,8-dihydroguanosine
AGE	advanced glycation end products
AREs	antioxidant response elements
BMI	body mass index
CA	carnosic acid
CAT	catalase
CEBPα	CCAAT enhancer binding protein α
CS	carnosol
CuZnSOD	copper-zinc superoxide dismutase
DAG	diacylglycerol
ETC	electron transport chain
ERK	extracellular signal-regulated kinase
F2-IsoPs	F2-Isoprostanes
FABP4	fatty acid-binding protein 4
FFA	free fatty acids
FPG	fasting plasma glucose
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidized GSH
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
HbA1c	glycosylated hemoglobin
HDL	high-density lipoprotein
HFD	high-fat diet
HO$^•$	hydroxyl radical
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IFG	impaired fasting glucose
IFN-γ	interferon-γ
IGT	impaired glucose tolerance
iNOS	inducible nitric oxide synthase
IR	insulin resistance
IRS	insulin receptor substrate
JNK	NH_2-terminal Jun kinases
KEAP1	Kelch-like ECH-associated protein 1
KD	knockdown
KO	knockout
LDL	low-density lipoprotein
MDA	malondialdehyde
MnSOD	manganese superoxide dismutase
mtDNA	mitochondrial DNA
NF-κB	nuclear factor-kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOD	non-obese diabetic
NOS	nitric oxide synthase
NRF2	nuclear factor (erythroid-derived 2)-like 2
NQO1 NAD(P)H	quinone oxidoreductase 1
$O_2^{•-}$	superoxide anion radical
OGTT	oral glucose tolerance test
p38 MAPK	p38 mitogen-activated protein kinases
PCO	protein carbonyl
PKC	protein kinase C
PPARγ	peroxisome proliferator activated receptor γ
PPP	pentose phosphate pathway
Prdx	peroxiredoxin
RAGE	receptor for advanced glycation end products
RCS	reactive carbonyl species
RNS	reactive nitrogen species
RO(N)S	reactive oxygen and nitrogen species
ROS	reactive oxygen species
SAPK	stress-activated protein kinases
SAT	subcutaneous adipose tissue
SCN	suprachiasmatic nucleus
SNF	sulforaphane
SOD	superoxide dismutase
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TAG	triacylglycerol
TBARS	thiobarbituric acid reactive substances
TR	thioredoxin reductase
Trx	thioredoxin
VAT	visceral adipose tissue
WAT	white adipose tissue
WC	waist circumference
WHR	waist-to-hip ratio

syndromes, including metabolic syndrome. When we take a comprehensive look at this triad, the scope of the problem reaches the level of multi-metabolic disorder/syndrome. Systemic metabolic dysregulation is initiated by the disturbances in several physiological systems, primarily those with endocrine functions. This includes various organs, predominantly white adipose tissue (WAT, specific morpho-functional depots), brown adipose tissue, pancreas, liver, and muscles.

The modern concept of redox biology and medicine also faces a number of challenges. The traditional concept where reactive species are viewed primarily in the light of oxidative damage they inflict on biomolecules is "distant" history. Indeed, reactive species are essential signaling molecules involved in almost every physiological process, from cell division and differentiation to metabolic regulation. Through direct and indirect action, they modulate the activity and function of biomolecules (proteins, lipids, nucleic acids) and redox-sensitive transcription factors, inducing an endogenous adaptive response in cells, including the antioxidant defense. The redox homeostasis of cells and their extracellular environments is determined by the degree of reactive species production and neutralization that are firmly coupled with (oxidative) metabolism. At the regulatory level, the relationship between reactive species production and metabolism is bidirectional and

dynamic. Setting the redox state of cells is vital in health as much as in disease and opens redox-based therapeutic avenues for treating metabolic diseases such as obesity, metabolic syndrome, and diabetes.

2. "Clinical" versus redox biomarkers

Ever since Claude Bernard postulated the "constancy of the internal environment" [1], we have been searching for (bio)markers of homeostasis whose change indicates disease. Many of them in obesity, metabolic syndrome, and diabetes (i.e., levels of insulin, glucose, free fatty acids, triacylglycerol, cholesterol, glycosylated hemoglobin-HbA1c) are clinical biomarkers that are routinely used in diagnostics in human medicine. Their changes are a reflection of complex metabolic disturbances, predominantly in the most affected tissues. Because their circulatory levels are dynamic in healthy individuals, it is not always straightforward to relate their circulatory changes in patients to pathological conditions.

The search for redox biomarkers that are clinically relevant is an imperative task of redox biology in regards to human medicine. However, when we discuss the utility of redox biomarkers, some considerations deserve attention. First of all, what is considered under the term

redox biomarkers? Precisely what and where do we measure? What can we feasibly measure in humans? Redox biomarkers can include a diverse range of measurements from reactive species (that are often difficult to determine due to their nature and short life) and antioxidant defense components (enzymatic and non-enzymatic) to stable end-products of the oxidative modifications of proteins, lipids, and nucleic acids as well as concentrations of iron (free and bound) and those of other transition metals. They are primarily determined in the circulation (plasma/ serum, erythrocytes, monocytes, platelets), urine, or saliva. Changes in their status are a reflection of corresponding changes in their concentration, expression, activity, or localization in affected tissues. Outstanding reviews of methodological aspects and theoretical considerations for potential redox-dependent biomarkers are provided by Halliwell [2], Poulsen et al. [3], and Wang et al. [4].

When it comes to metabolic diseases, we face many difficulties in defining and validating redox biomarkers. To precisely define existing and discover novel redox biomarkers, it is necessary to achieve a better understanding of the molecular mechanisms of redox-dependent metabolic processes in target tissues and organs as well as those involved in inter-organ communication.

3. Clinical criteria to define a complex landscape of obesity, metabolic syndrome, and diabetes

Body mass index (BMI) is a useful population-level measure of overweight state and obesity in medical practice. However, metabolic alterations at the core of insulin resistance (IR) and T2DM pathophysiology may develop over the broad continuum of BMI. Nevertheless, it is now well accepted that obesity becomes a metabolic disease once the adaptive lipid-storing function of adipose tissue fails [5]. To emphasize the importance of lipid-storage malfunction, as much as the overall excess of adipose tissue in metabolic disorders, overweight state and obesity are now defined as "excessive or abnormal fat accumulation that presents a health risk" by the World Health Organisation [6]. Heterogeneity of the obese state was attributed to the differences in adipose tissue accumulation back in 1947 when it was first noticed that apple-shaped (mostly men) subjects are more likely to develop diabetes and other health complications than their pear-shaped (mostly women) counterparts [7]. Today still, the anthropometric measurements of BMI, waist circumference (WC), and waist-to-hip ratio (WHR) are used by most healthcare institutions to define abdominal obesity with high metabolic risk [8]. Apart from the genetic background, the upsurge in abdominal obesity during the last several decades is a direct result of an excess in available energy that impairs the metabolic function of adipose tissue. Exceeded functional capacity of highly insulin-sensitive adipose tissue (subcutaneous) to store lipids causes visceral and ectopic lipid deposition in muscle, liver, and other organs, further aggravating insulin resistance and perpetuating prediabetic conditions (Fig. 1).

According to the American Diabetes Association, a prediabetic state is clinically defined by impaired fasting glucose (IFG) level and impaired glucose tolerance (IGT) test. IFG level of 100–125 mg/dL and IGT of 140–199 mg/dL plasma glucose, respectively, indicate prediabetes. However, the prediabetic state is characterized by a combination of different and overlapping clinical conditions that often congregate, thereby increasing the risk of diabetes onset. The gathering of interrelated metabolic risk factors such as atherogenic dyslipidemia (elevated serum triacylglycerol and apolipoprotein B), increased small low-density lipoprotein (LDL) particles, reduced level of high-density lipoprotein (HDL) and cholesterol, and elevated blood pressure and plasma glucose, is known as metabolic syndrome. This pleiad of metabolic risk factors often develops due to another set of underlying risk factors – obesity and insulin resistance. Although the risk of T2DM generally increases with increasing insulin resistance and obesity, it has been widely accepted that T2DM development depends on adipose tissue function
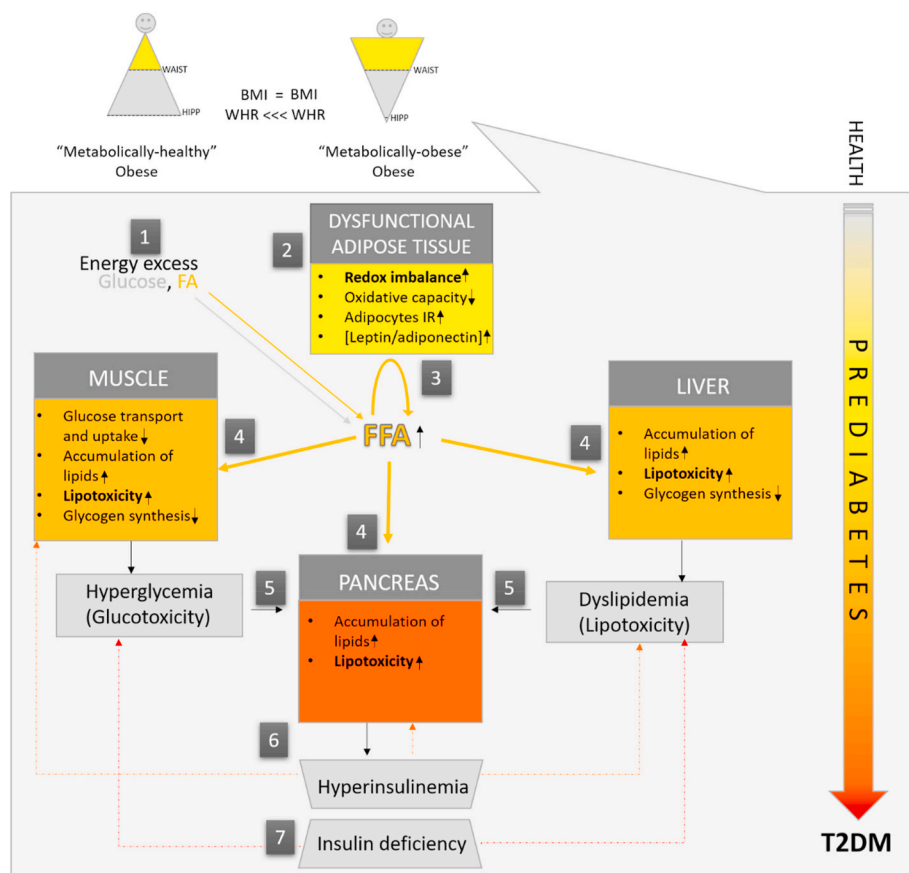

Fig. 1. Adipose tissue - centric pathogenesis of type 2 diabetes. In health, postprandially, excess fat is stored in adipose tissue, limiting its deposition in other tissues. In obesity (1), due to energy excess, the number of hypertrophic adipocytes increases when counteractive metabolic and hyperplastic responses are surpassed, and the expanding and lipid-buffering capacity of adipose tissue fails (2). Unrestrained postprandial lipolysis and low oxidative capacity drive systemic free fatty acid (FFA) (3) and pro-inflammatory adipocytokine delivery to non-adipose tissues (4). Consequently, muscles, liver, and pancreas are faced with ectopic lipid deposition and lipotoxicity (5), which ultimately interferes with multiple regulatory mechanisms of glucose storage in muscles and liver. In parallel, the pancreas releases more insulin to sustain normoglycaemia (6). If such conditions persist, compensatory pancreatic hypersecretion of insulin fails, and signs of hyperglycemia occur. Initially only transiently, in postprandial state (defined as impaired glucose tolerance), and later persistently, regardless of the nutritional state, resulting in overt type 2 diabetes mellitus (T2DM) (7). Subjects with an excess of upper-body or visceral adiposity (right) are more prone to develop metabolic syndrome and type 2 diabetes for a given body mass index (BMI), as these "metabolically obese" individuals suffer from a low expanding capacity of lower-body or subcutaneous adiposity. In clinical practice, this state is crudely defined by a higher waist-to-hip ratio (WHR).

rather than obesity *per se*. Accordingly, some people that appear normal-weight are insulin resistant and are thereby described as "metabolically obese" [9]. Similarly, obese people that are insulin sensitive are described as "metabolically healthy obese" [10]. These transient metabolic phenotypes - with or without IR, and with or without obesity (defined by BMI) complicate metabolic syndrome diagnostics and T2DM risk predictions (Fig. 2).

Diabetes mellitus is a chronic metabolic disease characterized by hyperglycemia, defined either by HbA1c $\geq$ 6.5%, or fasting plasma glucose (FPG) FPG $\geq$126 mg/dL or the 2h plasma glucose after a 75 g oral glucose tolerance test (OGTT) $\geq$200 mg/dL. If left untreated, hyperglycemia leads to severe health complications increasing all-cause mortality. Diabetes can be broadly classified into two main categories: type 1 diabetes mellitus (T1DM), which occurs due to autoimmune or idiopathic β-cell destruction, usually leading to absolute insulin deficiency (accounts for ~24% cases), and T2DM, which develops due to a progressive insulin secretory defect in the background of insulin resistance, from a "stealthy" prediabetic state (accounts for ~76% cases) [11].

Obesity-associated T2DM, as well as the other obesity-associated diseases, can be prevented. The earlier the disease is recognized, the greater the successes of prevention and treatment. However, early symptoms are often missed. The global prevalence of diabetes is rising, currently affecting ~451 million adults worldwide, while according to the Center for Disease Control and Prevention, more than 84% of prediabetic patients are not even aware of their condition [12]. Since T2DM is strongly related to obesity, the risk of diabetes is easily overlooked in subjects who appear to be non-obese (according to BMI). Approximately 23% of the non-obese population is, however, metabolically obese [13]. These non-obese subjects are metabolically obese since they have visceral obesity, lower energy expenditure, dyslipidemia, insulin resistance, and higher diabetic risk than some obese counterparts, but may escape detection because they have normal BMI [14]. Therefore, new diagnostic approaches should be implemented in diabetes diagnostics, especially in complex prediabetic conditions.

4. Redox basis of glucotoxicity and lipotoxicity

Physical inactivity, aging, and obesity present major risk factors for T2DM development. Continuous high-calorie food intake creates chronic hyperglycemia and hyperlipidemia that gradually deteriorates insulin sensitivity and secretion, leading to overt T2DM. During T2DM pathogenesis, especially in advanced stages of T2DM, hyperglycemia-related oxidative stress is one of the leading causes of metabolic and endocrine disturbances as well as cellular damage in affected tissues. Glucose overload increases all pathways of glucose breakdown, leading to the accumulation of NADH and $FADH_2$. This puts pressure on the electron transport chain (ETC) complexes and increases their reduction state. Subsequently, the electrochemical potential across the inner mitochondrial membrane causes electron leakage at complexes I, II, and III, leading to superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) production (Fig. 3). The elevated levels of reduced NADH as well as reactive oxygen and nitrogen species (ROS and RNS, respectively) inactivate glyceraldehyde 3-phosphate dehydrogenase (GAPDH), resulting in blockage of the glycolytic pathway and glyceraldehyde 3-phosphate accumulation. Glyceraldehyde 3-phosphate is a glycolytic intermediate and the main precursor for methylglyoxal synthesis. The accumulation of antecedent glycolytic metabolites, in turn, activates alternative pathways of glucose metabolism (polyol-, hexosamine- and advanced glycation pathways), additionally increasing reactive oxygen and nitrogen species (RO(N)S) production. The polyol pathway causes NADPH depletion associated with low glutathione reductase (GR) activity [15], while diminished levels of reduced glutathione (GSH) compromise cellular antioxidant capacity resulting in elevated levels of RO(N)S and oxidative damage. Therefore, reductive stress followed by oxidative stress is a leading hypothesis on the mechanism of hyperglycemia-induced metabolic syndrome [16]. Oxidative, carbonyl, and glycation stress induced by advanced glycation end products (AGE) and reactive dicarbonyls foster pathogenesis and complications of T2DM. AGEs bind to specific cell surface receptors of AGE (RAGE), causing alterations in respective signaling pathways and promoting intracellular RO(N)S production. Methylglyoxal is a strong glycating agent that reacts with arginine residues in proteins. Higher plasma concentrations of methylglyoxal underlie carbonyl stress-induced cardiovascular complications in patients with advanced diabetes [17] and "metabolically healthy obese" subjects [18]. High levels of methylglyoxal result from increased production and lower levels/activity of glyoxalases, GSH-dependent enzymes that metabolize methylglyoxal, and other reactive oxoaldehyde metabolites. This is usually accompanied by a decrease in aldehyde reductase, the enzyme responsible for the detoxification of carbonyl compounds [19]. In general, glucotoxicity implies activation of alternative glucose oxidation pathways that induce oxidative, carbonyl, and nitrosative stresses by enhancing free radical production or impairing antioxidant defense. Detailed mechanisms of impaired redox homeostasis in T2DM due to hyperglycemia are reviewed in recent papers [16,19–21].
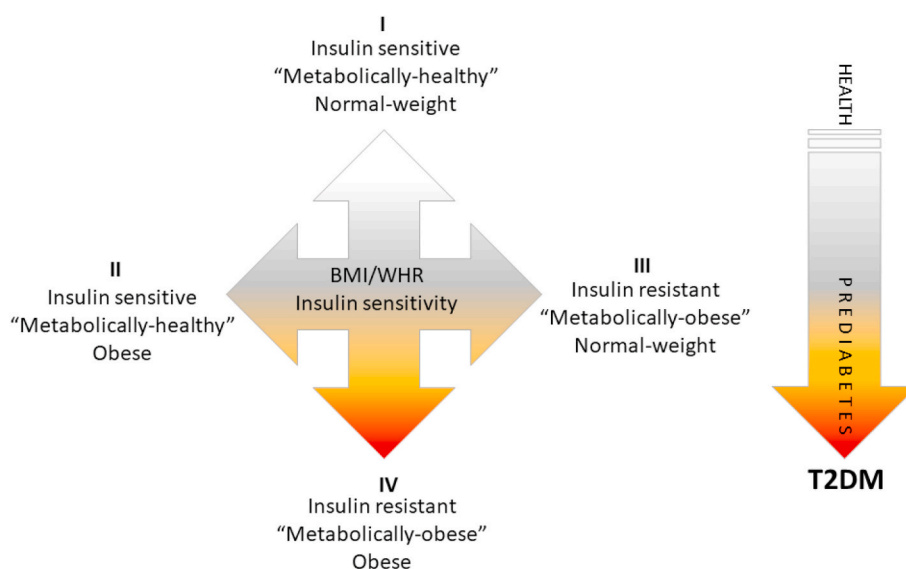
I
Insulin sensitive
"Metabolically-healthy"
Normal-weight

BMI/WHR
Insulin sensitivity

II
Insulin sensitive
"Metabolically-healthy"
Obese

III
Insulin resistant
"Metabolically-obese"
Normal-weight

IV
Insulin resistant
"Metabolically-obese"
Obese

HEALTH

PREDIABETES

T2DM

Fig. 2. Types of obesity in respect to metabolic phenotype and respective risk of type 2 diabetes. According to body composition (defined by BMI and WHR) and metabolic profile (defined by HOMA-IR index and lipid profile), at least 4 types of metabolic states/phenotypes can be defined (I-IV). People who appear normal-weight can be insulin resistant and therefore designated as "metabolically obese" normal-weight (III). In addition, obese people who are insulin sensitive are designated as "metabolically healthy" obese (II). Transient metabolic phenotypes - with or without insulin resistance and with or without obesity should be considered in predictions of T2DM development. T2DM: type 2 diabetes mellitus; BMI: body mass index; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; WHR: waist-to-hip ratio.
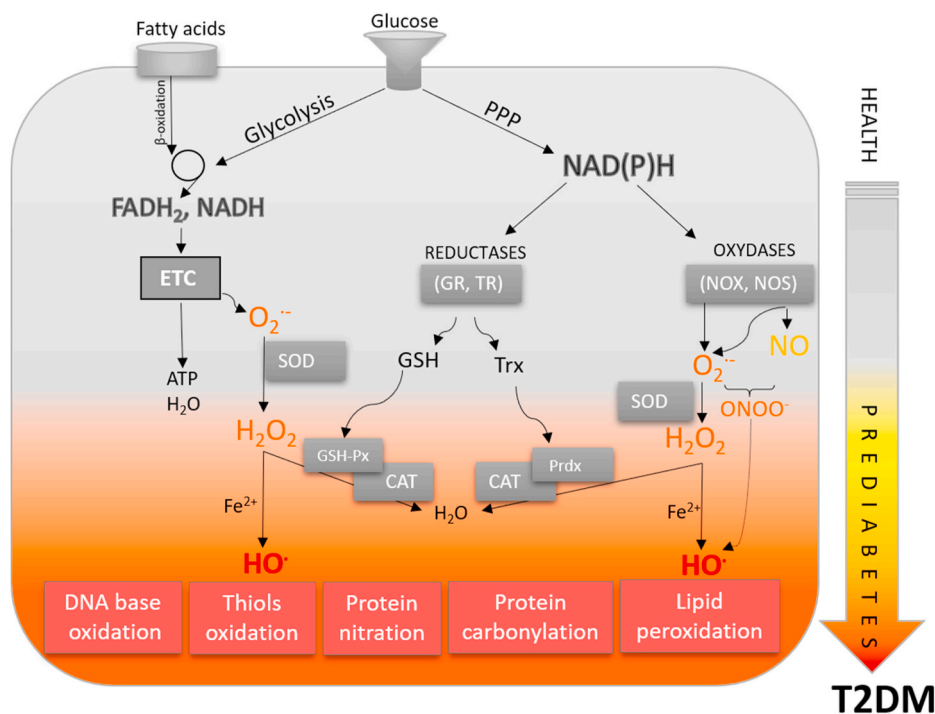
Fig. 3. From redox physiology to redox medicine in obesity, metabolic syndrome, and type 2 diabetes. In physiological states, the production of reactive species, metabolic processes, antioxidant defense responses, and repair/removal of oxidatively damaged biomolecules are highly synchronized processes. In metabolic diseases, this homeostasis is disturbed. Nutrient overload disrupts redox/metabolic equilibrium. Gluco- and lipotoxicity are an integrative part of the overproduction of reactive species, forming a vicious circle in which oxidative stress is central. Induction of secondary pathways of glucose and lipid metabolism strongly enhances these processes, leading to oxidative stress and oxidative modification of all biomolecules, further inducing alterations in redox-metabolic regulation. T2DM: type 2 diabetes mellitus; ETC: electron transport chain; GSH: glutathione; GSH-Px: glutathione peroxidase; GR: glutathione reductase; NOS: nitric oxide synthase; NOX: NADPH oxidase; PPP: pentose phosphate pathway; Prdx: peroxiredoxin; SOD: superoxide dismutase; TR: thioredoxin reductase; Trx: thioredoxin.

As a consequence of adipose tissue lipid storage dysfunction in obesity-associated metabolic syndrome, increased circulatory FFA reposit in visceral fat, liver, muscle, and pancreatic β-cells, forming ectopic lipid deposits and causing lipotoxicity [22]. More than 50 years ago, it was postulated that plasma FFA delivery and fatty acid oxidation in muscles increase the mitochondrial acetyl CoA/CoA and NADH/NAD$^+$ ratio, which in turn inactivates rate-controlling enzymes in glycolysis, ultimately resulting in the accumulation of glucose 6-phosphate [23]. It has been hypothesized that glucose 6-phosphate inhibits hexokinase II activity, increasing intracellular glucose concentrations and finally leading to decreased glucose uptake and glycogen synthesis. The subsequent studies gave complementary evidence that impaired glucose transport activity precedes fatty acid oxidation and represents the primary defect in insulin insensitivity and impaired glycogen synthesis in muscles [24–27]. It appears that fatty acids taken from the circulation are first esterified and deposited as triacylglycerols and, after that, retrieved and oxidized [28]. When this ectopic pool of intracellular fat in muscles and other organs like the liver or pancreas exceeds critical levels, it causes lipotoxicity. In lipotoxicity, fatty acids and other lipid metabolites, such as diacylglycerol (DAG), ceramide, long-chain acyl-coenzyme A, lipid aldehydes, increase oxidative stress via numerous intracellular mechanisms. DAGs act as direct activators of protein kinase C (PKC) and other stress-sensitive serine/threonine kinases that impair tyrosine phosphorylation of insulin receptor substrate (IRS) and insulin signaling [29–31]. PKC and other stress-activated protein kinases (SAPK) increase the overproduction of nitric oxide (NO) via inducible nitric oxide synthase (iNOS), resulting in nitrosative/nitrative stress [32]. Notably, one of the main features of lipotoxicity implies the accumulation of reactive lipid aldehydes, including the 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) and subsequent protein carbonylation [33]. In addition to the described mechanisms, there are numerous cellular sources of oxidative stress, including the endoplasmic reticulum stress, several stress-activated kinases, AGEs, iNOS, NADPH oxidases, xanthine oxidase, mitochondrial ETC, peroxisomes, lipid peroxidation, carbonylation related to lipotoxicity, and calcium signaling. Intensive research indicates a strong bidirectional link between calcium levels and RO(N)S production, pointing out that this interplay is disrupted in various pathophysiological conditions. Likewise, several

oxidative stress sources converge and foster each other, creating a vicious cycle that results in insulin insensitivity and impaired insulin secretion (in the case of β cells). A comprehensive discussion of these potential mechanisms has been provided elsewhere [34–45].

In addition to chronic elevations in circulating glucose and free fatty acid (FFA) levels, T2DM patients suffer from late diabetic complications across peripheral tissues [46,47]. Recent evidence suggests that common stress-activated signaling pathways such as nuclear factor-kappa B (NF-κB), p38 mitogen-activated protein kinases (p38 MAPK), and NH2-terminal Jun kinases (JNK)/SAPK underlie the development of most diabetic complications. Moreover, a chronic increase of FFA in obesity instigates similar redox-dependent stress pathways impairing insulin sensitivity and secretion [31].

5. Redox diseases need redox biomarkers

Advances in redox biology unequivocally indicate that all metabolic diseases, obesity, and related metabolic syndrome, as well as diabetes, can be considered redox diseases. Impaired redox regulation is coupled with metabolic dysregulation and *vice versa*. Both are at the core of the disease pathogenesis, initiators of dysfunction, and mediators of progression that contribute to further complications. It is necessary to develop functional redox biomarkers that would reflect adipose tissue function to screen a "crime scene" where many morbidities actually begin. Current research focuses on identifying redox biomarkers, specific for etiology and pathogenesis of distinct metabolic diseases, standardization of their measurement, and selection as clinically acceptable biomarkers. Underlying oxidative stress in metabolic diseases is often evident as concomitant changes in both levels of RO(N)S and lipid, protein, and nucleic acid oxidation products as well as changes in levels of antioxidant defense components [48–52]. Therefore, all redox parameters that determine the level of prooxidants - RO(N)S and their biological effects can potentially be relevant redox biomarkers of metabolic diseases. Measurement of these parameters in biofluids such as serum, plasma, urine, and saliva can help predict, diagnose, treat, and monitor the progression of metabolic diseases as well as differentiate and provide a functional link between obesity, metabolic syndrome, and diabetes [53].

Altered activity, expression, and specific gene variants of antioxidant enzymes, and oxidative modifications of mitochondrial DNA (mtDNA) have been investigated as potential biomarkers of metabolic diseases. Reduced activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and a significant decrease in the GSH/GSSG ratio, were found in peripheral blood mononuclear cells in obese individuals [54]. Additionally, these redox parameters are associated with metabolic functions of adipose tissue and the overall risk of metabolic syndrome. Higher basal GSH level and GSH-dependent enzyme activities were found in visceral adipose tissue (VAT) than in subcutaneous adipose tissue (SAT), while these intercepted differences in thiol-redox parameters reflect a higher triacylglycerol turnover in VAT depots [55]. In obese individuals, lower GSH levels in VAT were coupled with lower manganese superoxide dismutase (MnSOD) and higher NADPH oxidase protein expression. Interestingly, low CAT protein expression in VAT was specific to "metabolically obese" individuals (based on the BMI, Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index, triacylglycerol, total-, LDL- and HDL-cholesterol levels), in both lean and obese subjects with high metabolic risk. In contrast, increased MnSOD activity associated with a higher degree of mtDNA oxidative damage was found in peripheral blood mononuclear cells of T2DM patients with late diabetic complications [56]. Increased glutathione S-transferase (GST) activity was found in the plasma of individuals with T2DM [57], while increased plasma GSH-Px activity was associated with improved renal function in T2DM patients with hypertension as a result of vitamin E and/or selenium supplementation [58]. Copper-zinc superoxide dismutase (CuZn-SOD) polymorphisms were found to be associated with increased plasma copper concentrations in patients with newly diagnosed impaired glucose regulation and T2DM [59]. Furthermore, studies of the MnSOD, extracellular SOD, and CAT polymorphisms showed specific molecular association with diabetic neuropathy in T1DM patients [60].

Oxidation products of lipids, fatty acids, and nucleic acids are useful in characterizing and monitoring obesity as well as detecting prediabetic state and predicting associated health complications [61]. Higher levels of MDA in monocytes and F2-Isoprostanes (F2-IsoPs) and 8-oxo-2'-deoxyguanosine (8-oxo-dG) in urine were found in obese patients, where 8-oxo-dG showed potential for metabolic status monitoring since it correlated directly with glucose, insulin, HOMA index, and body weight and inversely with weight loss and antioxidant activities in morbidly obese patients undergoing bariatric surgery [54]. Moreover, serum levels of 8-oxo-dG and MDA were found to be increased in the serum of prediabetic subjects with high sensitivity for C-reactive protein and reduced GSH levels, implicating these redox markers as candidates for the diagnosis of prediabetes, as they correlated with glucose intolerance [62]. Similar disturbances in redox homeostasis are found in T2DM. High levels of oxidative DNA damage in lymphocytes of diabetic patients were found to be directly associated with serum lipid peroxidation and protein oxidation levels and inversely with SOD activity and protein thiol group content [63]. In another study, increased excretion of urinary markers of DNA (8-oxo-dG) and RNA (8-oxo-Guo) oxidation was found in children and adults, which particularly correlated with age and female sex. The authors reported a very interesting finding that obesity in children with insulin resistance correlates with higher levels of oxidation RNA markers, which could be predictive of T2DM [64].

Other redox biomarkers showed promise in monitoring the development of insulin resistance and diabetes progression. Long-term treatment of human primary preadipocytes from obese SAT with physiological concentrations of 4-HNE caused increased oxidative stress, inhibition of cell growth, loss of adipogenic capacity, and impairment of insulin sensitivity [65]. A higher level of 4-HNE was found in VAT of overweight/obese premenopausal women, in comparison to normal-weight women, irrespectively of metabolic syndrome risk factors [55]. Such changes were associated with lower levels of GSH and MnSOD and high NADPH oxidase protein expression levels. In contrast, a significant increase in 4-HNE was found in the SAT of morbidly obese

premenopausal women, but again irrespective of metabolic syndrome risk factors [65]. Therefore, 4-HNE in VAT is related to VAT dysfunction and oxidative stress even at low BMI, leading to restricted lipid buffering function of adipose tissue and increased lipid peroxidation as a function of BMI increase. In T2DM patients, higher serum levels of 4-HNE were found to directly correlate with clinical prognostic parameters (glycated hemoglobin, fasting glucose) [66]. However, the role of 4-HNE in metabolic diseases has not yet been fully elucidated and is probably concentration-dependent. At low physiological concentrations, it plays a crucial role in redox homeostasis, acting as a signaling molecule and inducing an adaptive response in diabetes, while at high concentrations, it exhibits cytotoxicity due to its high reactivity and can cause adipocyte dysfunction and insulin resistance [67,68]. This is true not only in diabetes but in other pathophysiological conditions, such as Rett syndrome and aging. For example, high levels of plasma 4-HNE protein adducts were found to increase with disease progression in subjects affected by Rett syndrome [69]. Moreover, our group previously showed that high levels of 4-HNE correlate with degenerative cutaneous tissue changes and oxidative DNA damage with advanced age, which is not the case in young animals [70].

Many of the commonly analyzed lipid peroxidation products are often found in different metabolic diseases. However, additional research will be necessary to assess their utility in differentiating between obesity, metabolic syndrome, and diabetes or their potential to predict the development of diabetes in patients with obesity and metabolic syndrome. Importantly, several emerging studies highlight that specific parameters of lipid peroxidation could be used to achieve such stratifications. The development of metabolic syndrome in obese individuals can be predicted from a linear combination of visceral fat thickness and serum thiobarbituric acid reactive substances (TBARS): cholesterol ratio [50]. Low levels of plasma vitamin C and nitrosoglutathione were found to be inversely correlated with the grade of hypertension in patients with metabolic syndrome, possibly discriminating between patients with low and high risk for diabetes and cardiovascular complications [50]. Interestingly, the results from the Framingham study indicated that urinary 8-epi-prostaglandin F2α (8-epi-PGF2α) levels were highly associated with BMI and cardiovascular complications, but not with diabetes [71]. Moreover, these redox changes seem to be closely related to the type of obesity. Furukawa et al. were the first to demonstrate that obesity exacerbates systemic markers of oxidative stress in a feedback loop leading to metabolic syndrome [72]. Several studies since then found correlations of lipid peroxidation markers with abdominal [73], but not with general obesity [74]. Moreover, it was found that increased fat mass with visceral distribution is specifically associated with systemic chronic low-grade inflammation and oxidative stress markers [50,71,75], highlighting the importance of redox and inflammatory pathways cross-talk, a phenomenon recently termed "OxInflammation" [76]. For example, urinary 8-epi-PGF2 levels were particularly associated with the visceral fat area, even in non-obese subjects. Similarly, a significant correlation of urine 8-epi-PGF2α and inflammation associated with lipid peroxidation-mediated platelet activation was seen in obese women with the android type of obesity, indicating a higher risk of developing hypercholesterolemia, diabetes mellitus, and vascular complications [77].

In a hyperglycemic environment, reactive carbonyl species (RCS) are readily generated, and subsequently, advanced glycation end products and protein carbonyl (PCO) content represent important biomarkers in diabetes and associated syndromes [78]. High levels of PCO, advanced oxidation protein products, and lipid hydroperoxides accompanied by a lower total thiol content in T2DM patients with poor glycaemic control were identified as important prognostic redox biomarkers for the development of diabetic complications [79]. In an exciting study of nutrition-mediated redox control in T2DM, a high-protein diet was found to reduce plasma MDA and protein carbonyls levels but increase nitrotyrosine levels [80]. A direct association of carbonyl stress with increased risk of the acute coronary syndrome was found in T2DM

patients, evident as an increase in methylglyoxal, methylglyoxal derived hydroimidazolones-1, and N$^{\varepsilon}$-carboxymethyl-lysine serum levels [81]. Additionally, lower levels of GAPDH, glyoxalase 1, and aldehyde reductase suggest that metabolic disturbance precedes the development of acute coronary syndrome through RCS, while elevated levels of AGEs and protein carbonyls in vitreal fluid of T1DM and T2DM patients suggests their role in proliferative diabetic retinopathy development [82].

Furthermore, reduced NO bioavailability is recognized as one of the key factors that contribute to disturbed redox homeostasis in metabolic diseases, especially diabetes. In an excellent review of human studies investigating NO metabolism in metabolic diseases, Kobayashi summarizes the effects of endothelial nitric oxide synthase (eNOS) polymorphisms in a wide range of conditions (T2DM, IR, obesity) with modifications at the molecular level [83]. There is a clear association between eNOS polymorphisms with altered NOS activity, increased IR, and the incidence of metabolic syndrome and T2DM. In contrast, the level of asymmetric dimethylarginine, an endogenous NOS inhibitor found in all cells and plasma, is increased, while NO production is decreased in insulin resistance. In addition, specific alternations in NO metabolism were found in T2DM (decreased neuronal nitric oxide synthase-nNOS protein expression in skeletal muscle) and obesity (reduced NO availability and reduced eNOS expression in skeletal muscle). Although rare, existing human studies confirm the role of the L-arginine/NO pathway in diabetes, showing that long-term oral L-arginine supplementation improves endothelial function, oxidative stress, and adipokine release in obese T2DM patients with insulin resistance [84]. Besides, an increased level of NO (measured indirectly) correlates with T1DM (saliva and plasma) [85,86] and T2DM (saliva) [86], while salivary NO level is lower in T2DM subjects with xerostomia [87]. Demonstrated reduced NO bioavailability in diabetes [88] could be influenced by another important player in diabetes pathogenesis that has biomarker potential, calcium. Indeed, the overall calcium metabolism was found to be disturbed in T1DM and T2DM, from decreased intestinal absorption to increased urinary excretion [89]. However, there is currently no consensus on the functional significance of plasma calcium alterations in diabetic patients. Interestingly, all three NOS isoforms are regulated by calcium, of which eNOS and nNOS directly via calcium-calmodulin [90], where an increase in calcium concentration is linked directly to NO production by eNOS [91]. However, this link is still controversial since other calcium-independent mechanisms are involved in regulating NOS activity: phosphorylation, availability of L-arginine, other reactive species, and oxidatively damaged biomolecules [90], many of which are disturbed in diabetes. Therefore, the synergistic effects of calcium-dependent and independent mechanisms likely impact altered NO homeostasis in diabetes. In addition to the NO and NO metabolites, hydrogen sulfide (H$_2$S) should be further investigated as a potential redox biomarker in diabetes since H$_2$S concentrations, similarly to NO, regulate a wide range of functions and are reduced in the plasma of patients with T2DM [92].

6. Lipofuscin as a novel redox biomarker of insulin-related metabolic diseases

Lipofuscin is a non-degradable cellular material composed of oxidized proteins, lipids, and metals stored in lysosomes, whose accumulation has been widely regarded as a hallmark of cellular aging [93]. The question has been posed whether lipofuscin can be repurposed as a biomarker of metabolic diseases that could predict the progression of obesity, metabolic syndrome, and diabetes. Nowadays, the contribution of pathological accumulation of lipofuscin is highlighted for many age-related diseases and complex conditions, which can also be regarded as metabolic diseases, including Alzheimer's and Parkinson's disease [94], denervation atrophy [95], inherited juvenile form of macular degeneration [96], lipid myopathy [97], chronic obstructive pulmonary disease [98], and Melanosis coli [99].

In the search for novel redox biomarkers reflecting insulin-related metabolic diseases, lipofuscin could be a promising and reliable candidate. Namely, lipofuscinogenesis, the formation of lipofuscin, occurs mainly in metabolically sensitive organs such as the brain, heart, liver, and adipose tissue (Fig. 4). The presence, subcellular distribution, and lipofuscin content vary greatly in tissue- and cell-specific manner. Besides, the amount of lipofuscin in specimens from individuals of the same age varies and could be linked to disease or diet variation, which are all associated with the rate of oxidative protein damage, mitochondrial functionality, proteasomal system, and lysosomal functionality [100].

The concept that the accumulation of lipofuscin in the cell is not only age-, but also metabolism-dependent is not new [100,101], given close relations between metabolic disorders and aging as well as the role of oxidative stress in metabolic syndrome [102,103]. Hyperinsulinemia is implicated in metabolic syndrome as a condition that precedes obesity and diabetes and is associated with pancreatic islet hyperplasia, and is thus recognized as an early indicator of metabolic dysfunction [104]. The association of hyperinsulinemia and insulin resistance to mitochondrial dysfunction [105] and iron levels through rapid and pronounced stimulation of iron uptake and loading by adipocytes [106–108] is well established. Furthermore, transferrin receptors colocalize with glucose transporters and insulin-like growth factor II receptors in the microsomal membranes of cultured adipocytes, suggesting that regulation of iron uptake by insulin occurs in parallel with its effects on glucose transport [109]. Indeed, obese people have increased iron levels [110,111] and become prone to IR and potentially harmful lipofuscinogenesis.

Hyperinsulinemia represents a risk factor for cardiovascular disease, neurodegeneration, diabetes, and cancer [112], while this type of dysfunction is a commonly observed feature in several animal models for obesity and diabetes [113,114]. Diabetes results in lipofuscin accumulation in various tissues and organs [115]. Since hyperinsulinemia is also part of those metabolic diseases [116,117], the molecular events linking insulin and lipofuscin accumulation warrant further investigation. Hyperinsulinemia drives an overproduction of hydrogen peroxide and increased iron uptake, creating a harmful milieu, which leads to oxidative damage. As a result, damaged organelles and macromolecules accumulate inside cells in the form of lipofuscin [100]. Since lipofuscin accumulates within lysosomes as an end product, it is assumed that it could not be removed [118,119] and could thus represent a hallmark redox biomarker of cellular aging [120–123].

Interestingly, some data shows that lipofuscin could be found in a soluble state in bodily fluids and quantified. Hegedus et al. [124] documented an increase of plasma lipofuscin levels in stored blood and quantified human plasma lipofuscin [125]. Recently, lipofuscin was measured in human plasma and saliva [126]. This data raises the question of the existence of extracellular lipofuscin and its origins. Extraneuronal lipofuscin was found in the brain samples, suggesting a possible physiological removal from the nerve cells to the capillary endothelium and its later elimination via phagocytosis by glia cells [127]. Indeed, recent findings have shown that lipofuscin could be exocytosed from the cardiomyocytes into circulation through capillary endothelium [128]. Hence, lipofuscin could be regarded as a promising redox biomarker of obesity and diabetes. The revival of non-invasive lipofuscin measurements in bodily fluids with accurate and reliable methods shows promise for monitoring insulin-related metabolic diseases [129].

7. The NRF2 redox pathway at the crossroads between the antioxidant defense and energy metabolism

The NRF2 pathway, a master regulator of cellular defense against oxidative and xenobiotic stresses, has emerged as a critical target of energy metabolism [130]. Nuclear factor (erythroid-derived 2)-like 2 (NRF2), a cap-n-collar basic leucine zipper transcription factor, serves to mount a cellular defense response against environmental and
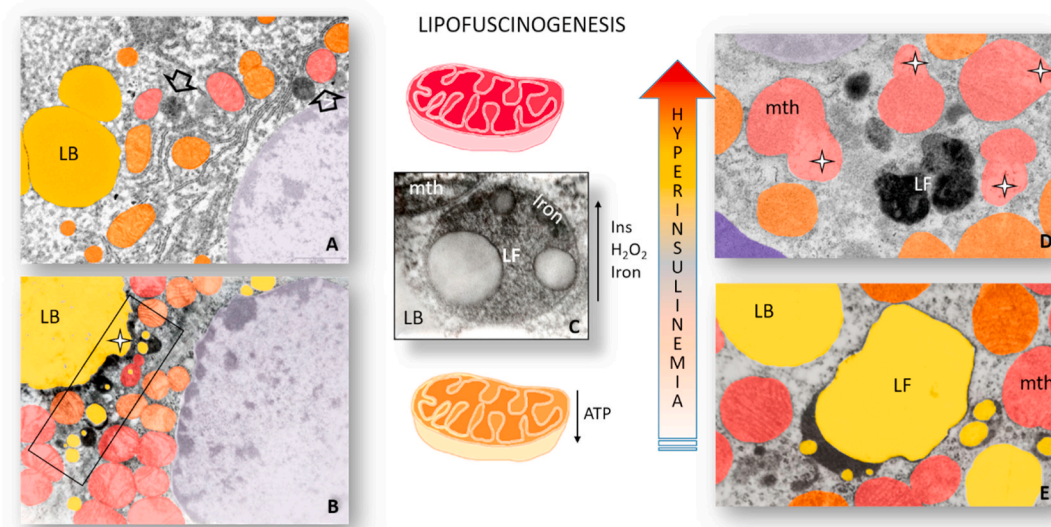
Fig. 4. Lipofuscinogenesis in obesity and type 2 diabetes. Lipofuscinogenesis in hepatocytes (A - arrow) and cardiomyocytes (D- LF) of obese mice and brown adipocytes of hyperinsulinemic rats (B -rectangle, E – LF) results in gradual lipofuscin accumulation (C). In hyperinsulinemia, a state preceding obesity and diabetes, excessive production of H₂O₂ and higher import of iron drive peroxidative damage of mitochondria (mth) and lipid bodies (LB) and lead to the formation of lipofuscin (LF) containing their undigested remnants and iron. White star – damaged, structurally altered lipid body (B) and mitochondria (D). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

endogenous stressors. Under conditions of lower stress, NRF2 is sequestered in the cytoplasm by associating with Kelch-like ECH-associated protein 1 (KEAP1) and Cullin 3 ubiquitin E3 ligase. Upon exposure to electrophilic and oxidative stresses, NRF2 becomes released from its cytosolic repressor KEAP1 and translocates to the nucleus, where it forms a complex with MAF and JUN proteins. This leads to NRF2 binding to antioxidant response elements (AREs) in numerous gene promoters and subsequent activation of diverse transcriptional programs, including activation of genes encoding phase 2 detoxification and antioxidant enzymes [131]. In addition, NRF2 regulates a large number of genes involved in gluconeogenesis and lipid metabolism as well as β-oxidation, mitochondrial biogenesis, and inflammation, processes which show robust diurnal variation (Fig. 5) [130,132–134]. Whilst there is strong evidence for the role of NRF2/KEAP1 pathway in

modulating adipogenesis, responses to high-fat diet (HFD), insulin sensitivity, and metabolic health, the direction of this control is conflicting in the literature. Previous research has shown that NRF2 inhibits adipogenic differentiation via activation of the aryl hydrocarbon receptor (AHR) pathway. Adipogenic stimulation of *Nrf2* knockout (KO) mouse embryonic fibroblasts (MEFs) led to increased intracellular lipid accumulation and elevated expression of peroxisome proliferator-activated receptor gamma and CCAAT/enhancer binding protein α (*Pparγ and Cebpα*, respectively), whilst delayed adipogenesis was seen in *Keap1* knockdown (KD) MEFs [135]. In line with these data, Xu et al. demonstrated that in MEFs isolated from mouse models of NRF2 activation, *Keap1*-KD, and leptin (ob/ob)-*Keap1*-KD, adipogenesis was suppressed, and adipogenic genes, such as *Pparγ*, *Cebpα*, and fatty acid-binding protein 4 (*Fabp4*) were decreased [136]. The same study
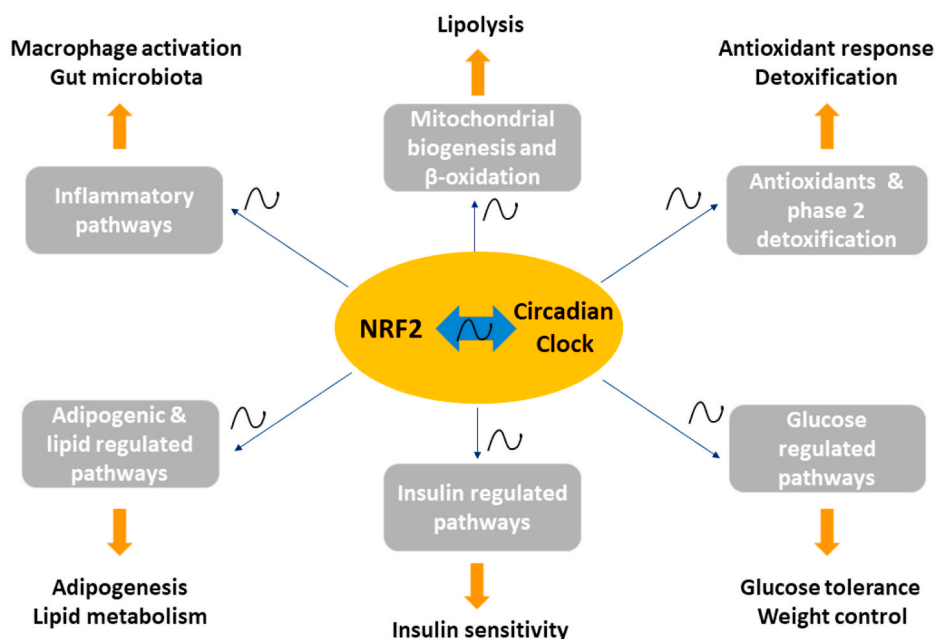

Fig. 5. Schematic diagram depicting the interconnected roles of NRF2 and circadian clock machinery in diverse transcriptional programs implicated in obesity, metabolic syndrome, and diabetes. Several different transcriptional programs are regulated by both NRF2 and the circadian molecular clock through positive and negative feedback control in various cell types and tissues. NRF2 and the molecular clock are also involved in the bidirectional regulation of each other. The target transcriptional programs display ~24h oscillations in various components and range from antioxidant/detoxification and inflammatory responses to pathways involved in mitochondrial biogenesis/β-oxidation, adipogenesis/lipogenesis, as well as insulin and glucose-regulated pathways. NRF2: nuclear factor (erythroid-derived 2)-like 2.

demonstrated that pharmacological NRF2 activation by sulforaphane (SFN) prevented adipogenesis and lipid accumulation [136]. Additional NRF2 inducers such as carnosic acid and carnosol originating from dried rosemary leaves were also shown to inhibit adipogenic differentiation by stimulating glutathione metabolism [137].

In contrast, other research has shown that *Nrf2* KO mice have lower amounts of adipose tissue and that mouse and human preadipocytes show suppressed adipocyte differentiation resulting from the down-regulation of *Pparγ* and *Cebpα* expression following *Nrf2* shRNAs whilst enhanced adipogenesis was seen with *Keap1* shRNAs [138]. In line with this work, another study demonstrated NRF2 as a positive regulator of adipogenesis using preadipocytes derived from white adipose tissue [139].

Further to its role in adipogenesis and adipose tissue maintenance, NRF2 also plays an important role in modulating obesity. CDDO-Im, a potent NRF2 activator, was shown to prevent body weight gain and WAT increase in response to HFD in wild type mice [140]. Similar findings were found using another NRF2 inducer, oltipraz, which prevented insulin resistance and weight gain in response to HFD [141]. Consistently, a number of other NRF2 inducers such as quercetin, curcumin, resveratrol, and chromium histidinate have all shown anti-obesity effects at least partially through NRF2 induction (reviewed in Ref. [142]).

Surprisingly, some *in vivo* animal studies show a complex role of NRF2 in influencing responses to HFD. For example, *Nrf2* KO mice were found to show blunted fat pad increases following HFD compared to wild-type mice, and this was independent of differences in food consumption, intestinal fat absorption, and physical activity [138]. Similarly, another study found that *Nrf2* deficiency protects against HFD-induced obesity through fibroblast growth factor 21 and improves glucose tolerance and insulin sensitivity [143]. On the other hand, *Keap1*-KD mice, which have increased NRF2 levels, also had lower body weights and fat masses than wild-type mice on HFD [136]. In line with this work, mice over-expressing NRF2 target, NAD(P)H quinone dehydrogenase 1 (*Nqo1*), conferred protection from diet-induced metabolic defects through the preservation of glucose homeostasis, insulin sensitivity, and lipid handling with improved physiological outcomes [144]. NQO1-overexpressing mice on HFD had lower adipose tissue macrophages and enhanced expression of lipogenic enzymes together with the reduction in circulating and hepatic lipids.

In contrast, some reports have shown that NRF2/KEAP1 pathway has no effects on body weight in response to HFD feeding; however, *Nrf2* KO mice were protected from HFD-induced hyperglycemia [145]. Whereas *Nrf2* KO mice were resistant to HFD-induced glucose intolerance, *Keap1* KD mice exhibited prolonged elevation of circulating glucose during a glucose tolerance test, even on a control diet. Indeed, some reports agree that whole-body *Nrf2* KO mice are more glucose-tolerant and insulin-sensitive than wild-type mice [143], whilst *Keap1* KD mice on a background of leptin deficiency (a genetic model of obesity) showed higher glucose concentration than control mice [136]. On the other hand, mice with myeloid-selective *Nrf2* deficiency were less sensitive to insulin, suggesting that NRF2 in myeloid cells acts negatively against the development of insulin resistance [146]. Interestingly, *Nrf2* deletion impairs glucose tolerance and exacerbates hyperglycemia in type 1 diabetic mice [147]. Indeed, NRF2 activation plays an important role in protection against autoreactive T-cell-induced T1DM using non-obese diabetic (NOD) mice, a polygenic model of human type 1 diabetes [148]. *Keap1* KD mice inhibited T-cell infiltration, ameliorated impairment of insulin secretion, and prevented the development of diabetes mellitus in NOD mice. This occurred through decreasing both plasma interferon-γ (IFN-γ) levels and IFN-γ-positive cell numbers in the pancreatic islets.

To resolve some of the conflicting data on the role of NRF2/KEAP1 pathway in obesity and metabolic health, studies have investigated the effect of *Nrf2* deletion in a tissue-specific manner. *Nrf2* deletion from adipocytes, but not hepatocytes, potentiated systemic metabolic dysfunction after long-term HFD-induced obesity in mice [149]. Mice with *Nrf2* loss in adipocytes showed a partially deteriorated glucose tolerance, higher fasting glucose levels, and higher levels of cholesterol and non-esterified fatty acids. In contrast, mice with *Nrf2* loss in hepatocytes had lower insulin levels and improved insulin sensitivity without changes to liver triglyceride accumulation. Furthermore, another study investigated the role of NRF2 in influencing the risk of metabolic syndrome by using a whole-body or adipocyte-specific *Nrf2* KO mice on a leptin-deficient ob/ob background, a model of extreme positive energy balance [150]. On ob/ob background, loss of *Nrf2*, globally or in adipocytes, showed reduced WAT mass but led to a more severe metabolic syndrome with aggravated insulin resistance, hyperglycemia, and hypertriglyceridemia. These new studies have revealed that NRF2/KEAP1 pathway has differential effects on weight control and metabolic health in a cell-type-specific manner.

Pharmacological NRF2 activation has shown more consistent outcomes regarding their protective effects on metabolic health. An NRF2 inducer SFN reversed disease signature in diabetic livers and attenuated exaggerated glucose production and glucose intolerance. Importantly, SFN reduced fasting blood glucose and HbA1c in obese patients with dysregulated T2DM [132]. Another NRF2 inducer, oltipraz, improved glucose metabolism and prevented insulin signaling impairments in mice on HFD [141]. Similarly, curcumin improved insulin sensitivity through the regulation of muscular mitochondrial redox balance; ellagic acid protected against HFD-induced metabolic syndrome and NRF2 agonist Dh404 prevented insulin resistance (reviewed in Ref. [142]). Although pharmacological NRF2 activation has shown promising results in several animal and human studies, the synthetic NRF2 inducers have caused adverse cardiac events and gastrointestinal toxicities in clinical trials [151]. Alternative supplementation with glucoraphanin, a stable precursor of SFN, attenuated weight gain, increased energy expenditure, and core body temperature led to improved glucose tolerance and insulin sensitivity in HFD-fed wild-type mice. This occurred via activating insulin-stimulated Akt phosphorylation in the liver, muscle, and WAT and preventing uncoupling protein 1 protein reduction in WAT. Glucoraphanin was also able to reduce circulatory levels of lipopolysaccharide, attenuate inflammatory signaling pathways such as JNK and extracellular signal-regulated kinase (ERK), and inflammation-activated M1-like macrophage accumulation in liver and adipose tissue. Finally, glucoraphanin was able to reduce hepatic elevated lipogenic gene expression, lipid peroxidation levels of MDA, and expression of NADPH oxidase subunits [151].

Overall, whilst some of the discrepancies in the above studies may result from the use of different cell types (e.g., primary *vs.* immortalized, MEFs *vs.* adipocytes), mouse genetic backgrounds as well as the length or types of HFD (milk fat *vs.* lard), it is important to note that most studies do not specify the gender or age of animal models used. Moreover, responses to HFD as well as various outputs of energy metabolism show profound time-of-day circadian differences, which is another important confounding factor to take into consideration. Therefore, future studies are urgently needed to decipher the precise temporal and cell-type-specific role of NRF2 and its target molecular mechanisms in regulating adipose tissue homeostasis and responses to HFD and determine how gender and age modulate this regulation.

8. Energy metabolism and redox regulation as a function of ~24h circadian rhythms

Circadian rhythms govern ~24h rhythms in daily human and mammalian physiology, metabolism, and behavior both in health and disease, including the daily rhythms in feeding activity, hormone production, and glucose metabolism [152]. The circadian timing coordinates temporal variation in biochemical processes with changes in nutrient intake in order to optimize energy balance and maintain metabolic homeostasis [153]. The circadian timing system consists of a central brain clock in the anterior hypothalamic suprachiasmatic nucleus (SCN) and various peripheral tissue clocks, which drive

positive/negative feedback loops in transcription/translation and metabolite abundance [154]. The central clock regulates food intake, energy expenditure, whole-body insulin sensitivity, and glucose homeostasis, whilst the local peripheral clocks also contribute to this regulation and fine-tune these processes. Some examples include the peripheral clock in the gut, which regulates glucose absorption, peripheral clocks in muscle, adipose tissue, and liver, which regulate local insulin sensitivity, and the peripheral clock in the pancreas which regulates insulin secretion [155–157].

Misalignment between circadian timing system and daily rhythms of sleep-wake behavior or food intake as a result of genetic, environmental, or behavioral circadian disruptions is associated with metabolic disorders such as obesity, insulin resistance, and diabetes [158]. Indeed, disrupted circadian rhythms of body temperature, heart rate, and fasting blood glucose are seen in both prediabetes and T2DM [159]. Moreover, emerging work has shown that chrono-based nutritional interventions such as time-restricted feeding can rescue obesity and metabolic syndrome in mice lacking a genetic circadian clock [156,157].

Current high-throughput technologies have allowed for the dynamic monitoring of diverse cellular processes over ~24h daily cycle, ranging from gene and protein expression to metabolite abundance, thus discovering potential circadian biomarkers. Indeed, ~40% of the genome is under circadian control across different mammalian tissues [160]. For example, circadian metabolomes have been reported for human serum, saliva, breath, and urine, as well as tissues from several species in different disease conditions and following genetic disruption [161]. Circadian proteomics of plasma proteins from human participants undergoing simulated night shift work showed 24h time-of-day patterns in 573 of 1129 proteins analyzed and revealed that circadian misalignment altered multiple proteins known to regulate glucose homeostasis and/or energy metabolism, with implications for altered metabolic physiology [162]. Social jetlag, a measure of disruption of the circadian system, has been associated with increased fasting glucose levels and a higher risk of overweight and metabolically obese status in a study with ~792 Brazilian participants [163]. Elevation of circulating FFA levels is associated with insulin resistance and diabetes risk [164]. FFA levels in healthy individuals show strong circadian variation, and sleep restriction relative to normal sleep led to its increased levels in nocturnal and early morning hours. This was associated with prolonged secretion of nocturnal growth hormone levels and early morning noradrenaline levels [164]. A recent genome-wide association study of reported day-time sleepiness analyzing 452,071 individuals from the UK Biobank has identified 42 genetic loci that clustered into specific biological subtypes and shared genetic links with several chronic diseases, including diabetes [165].

Previous work has characterized ~24h time-of-day variation and the effects of sleep deprivation on urinary metabolite profiles in healthy male participants under highly controlled environmental conditions by ^{1}H NMR spectroscopy [166]. Significant changes were observed with respect to both time-of-day and sleep deprivation, indicating that sampling time and sleep deprivation are highly relevant when identifying biomarkers in metabolic profiling studies. Further work by the same group using targeted liquid chromatography/mass spectrometry metabolomics identified 130 plasma metabolites every 2h over 24h, and showed that average metabolite concentrations were significantly altered by increased body mass (90 of 130) and T2DM (56 of 130). Moreover, ~40% of plasma metabolites showed daily rhythms, highlighting the importance of controlling the time-of-day for diagnosis and biomarker discovery [167]. In addition, a complementary study identified using untargeted and targeted liquid chromatography (LC)/MS metabolomics that acute sleep deprivation reduced amplitude of rhythms of >50% of rhythmic plasma metabolites identified [168]. The altered metabolite rhythms in overweight and T2DM individuals is consistent with previous work showing that individuals with T2DM have lower night-time serum melatonin levels and an increased risk of co-morbid sleep disturbances compared with healthy individuals [169].

Moreover, the effects of sleep restriction and loss were also examined using RNAseq profiling of WAT transcriptome between day and night. This has shown that sleep restriction blunts the normal daily variation in WAT transcriptome and that sleep deprivation led to further dampening [170]. This resulted in uncoupling of the clock-controlled regulated pathways of carbohydrate and lipid metabolism from the adipose tissue clock, leading to increased carbohydrate turnover and impaired glucose homeostasis [170]. In line with this work, human mature adipocytes show altered circadian rhythms in obese patients with age [171]. Similarly, a further study investigated whether the expression of clock genes in peripheral blood cells is impaired in T2DM and whether inflammatory markers are associated with circadian clock gene expression in T2DM patients. This study revealed that a number of core clock genes was decreased in leukocytes of T2DM patients, which was correlated with elevated levels of plasma inflammatory markers such as interleukin 6 and tumor necrosis factor α [172].

Circadian rhythms are highly interconnected to redox homeostasis, and this is demonstrated to be a bidirectional process as many metabolites and products of oxidative metabolism, as well as components of redox pathways, can feedback and influence circadian timing [173–177]. Recently we and others demonstrated that circadian molecular machinery regulates endogenous rhythms in NRF2 protein and transcript levels in several metabolic tissues, including the liver, lung, pancreas, and heart [158,178–180]. Moreover, we have shown that target antioxidant genes oscillate in tissues and cells in an NRF2-dependent manner [179], which is coupled to the daily oscillatory changes in cellular and tissue ROS levels [175]. In addition, we and others have observed redox oscillations in protein carbonylation marker and glutathione redox states in mouse tissues as well as human cells and bodily fluids [179,181–184].

A number of additional potential redox biomarkers have also been shown to be under circadian control. Circadian variation in lipid peroxidation marker MDA is observed in mouse erythrocytes [185] as well as in healthy human volunteers. Circadian control of plasma lipid peroxides (MDA) and serum ascorbic acid and uric acid levels were reported [186]. Night shift workers have lower levels of antioxidant defense components and higher levels of TBARS when compared to day workers [187]. Plasma TBARS concentrations are increased in the fasting state in obese men [188] whilst bariatric surgery reduced both TBARS and protein carbonyl concentrations in obese individuals [189]. Furthermore, the measurement of ~24h urinary excretion of 8-oxo-G oxidative damage marker suggests that obesity in men is also associated with increased oxidative damage to RNA [190]. The removal of oxidative damage to DNA/RNA such as 8-oxoguanine via the DNA base excision repair pathway is also under the circadian influence [191]. Altogether, these findings urge further work to examine the circadian rhythmicity of diverse redox biomarkers and antioxidant defense components in human research and animal models of metabolic diseases as well as test the potential of chrono-nutritional interventions in human clinical trials.

9. Cancer as a metabolic disease: deciphering the intriguing relationship with obesity, metabolic syndrome, and diabetes through redox biomarkers

The cumulative burden of neoplastic and metabolic diseases is hard to grasp, but current predictions do not seem optimistic [192,193]. Cancer is predicted to pose a higher threat to human health than cardiovascular diseases by 2030 [194]. Whilst obesity and diabetes take on epidemic proportions, evidence supporting the association between metabolic disorders and cancer has also accumulated. Prospective observational studies and meta-analyses show that obesity increases incidence [195,196] and mortality [197] for many cancers. Likewise, there is substantial evidence linking diabetes and cancer [198], although the association is more complicated to dissect, primarily due to the confounding influence of obesity on both diabetes and cancer development. In general, the association of site-specific cancers with obesity

further increases in diabetes, including colorectal, pancreatic, liver, gall bladder, kidney, breast, and endometrial [199,200]. The relationship becomes even more complicated when different antidiabetic and anti-neoplastic treatments are considered [201,202].

In light of the abundant epidemiological data and critical research highlighting metabolic reprogramming and redox regulation as two inseparable phenomena in cancer, the view has gradually shifted towards contemplation of cancer as a redox-metabolic disease [203–206]. Thus, obesity, metabolic syndrome, and diabetes could be regarded as potential driving forces for cancer development, acting through systemic and local influences on the tumor microenvironment [207,208]. Within this framework, it is not surprising that cancer-associated adipocytes emerged as critical players in cancer progression, especially for site-specific cancers strongly associated with obesity, such as colorectal [209], breast [210], and prostate [211].

Obesity-associated circulating levels of glucose, free fatty acids, insulin, insulin-like growth factor 1 as well as adipose tissue inflammation and dysregulated cytokine and adipokine signaling are some of the mechanisms that link obesity and cancer and are extensively reviewed elsewhere [212,213]. More recently, research focus has shifted towards metabolic cross-talk as one of the integral aspects of cancer-adipose tissue two-way communication [214,215]. Several exciting studies, including our own, showed not only that metabolic support provided by adipocytes is instrumental for cancer progression and metastasis but that it is redox-sensitive [216–218] and obesity-mediated [219–221] (Fig. 6). Thus, disturbed redox regulation in cancer-adipose tissue cross-talk could provide one of the missing links between metabolic diseases and cancer.

Great efforts were invested in identifying redox biomarkers that could predict cancer risk, stage of progression, and therapeutic outcome. Most commonly analyzed redox biomarkers are oxidation and glycation products of nucleic acids, proteins, and lipids in plasma, serum, urine, and saliva [222]. Results from prospective, case-control, and nested case-control studies provide valuable information about the potential of these biomarkers to serve as clinical parameters but often yield inconclusive or contradictory results. A crucial consideration is their usefulness in individuals with concomitant metabolic and neoplastic diseases since similar parameters are often used to differentiate obesity, metabolic syndrome, diabetes, and several types of cancer.

One of the most studied lipid peroxidation products, MDA, was found to be consistently elevated in plasma/serum of patients with breast [223], prostate [224,225], and colon cancer [226]. Among markers of nucleic acid oxidation products, serum level of 8-oxo-dG was found to be inversely associated with lymph node metastasis in breast cancer patients [227], while urinary levels were inversely associated with longer survival in colon cancer patients [228]. Measurement of advanced glycation products also showed predictive potential in several types of cancer. A prospective nested case-control study showed an association between plasma Nε-carboxymethyl-lysine and the risk of prostate cancer [229], while Nε-carboxymethyl-lysine content in breast cancer tissue was associated with better prognosis with tamoxifen treatments and worse prognosis with chemotherapy [230]. Urinary levels of isoprostanes were generally associated with the cancer risk and stage of progression for breast [231], prostate [232,233], and colon cancer [234], although a recent large follow-up study found no association with these malignancies [235].

One of the challenges in the biomarker research field is that previously found associations are often lost when larger sample sizes are considered. For example, a case-control study found a direct association of urinary levels of 15-F2t-isoprostane, and an inverse association of 8-oxo-dG with breast cancer risk [231]. Two years later, the same group found no difference in urinary 15-F2t-isoprostane and 8-oxo-dG between breast cancer patients and controls in a large population-based case-control study [236]. Another issue concerns the uniformity of study cohorts, especially regarding confounding factors such as obesity. This is nicely illustrated in an example where a large prospective nested
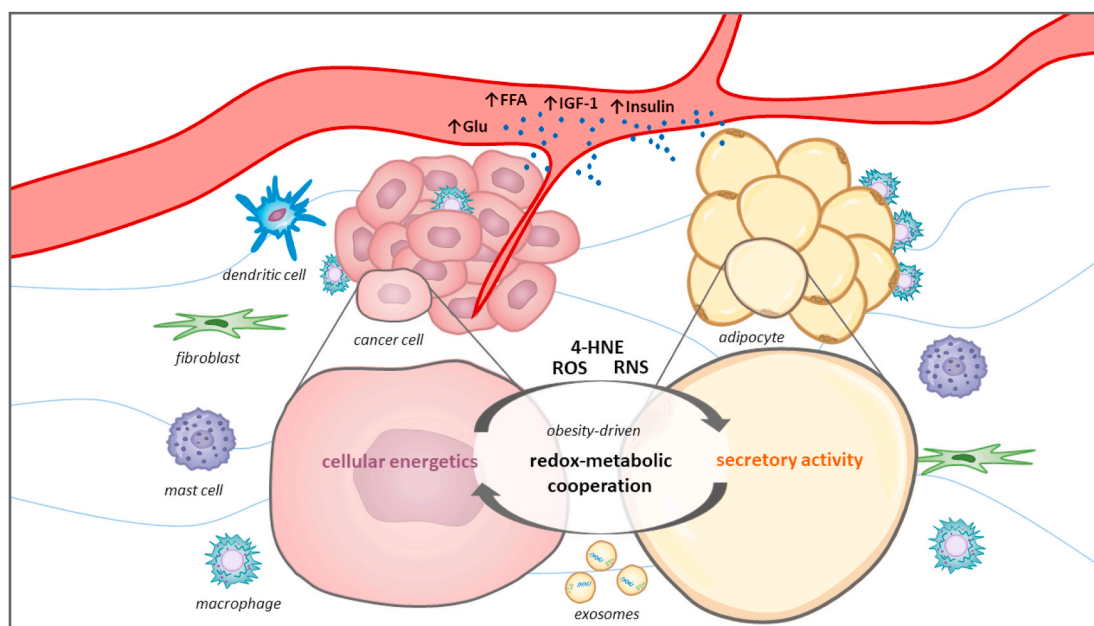

Fig. 6. Schematic representation of obesity-driven cancer-adipose tissue cross-talk. Cancer cells exist within an intricate tumor milieu comprised of stromal constituents, namely extracellular matrix components and numerous stromal cell types, including fibroblasts, adipocytes, mast cells, dendritic cells, immune cells, and vascular endothelial cells, among others. Obesity can shape the tumor microenvironment through systemic and local influences acting on both cancer cells and adipocytes, thus (re)framing their cross-talk and (re)directing cancer proliferation, metastasis, and therapeutic resistance. Recently, a concept of metabolic cooperation was introduced to describe the nutritional and metabolic support provided by adipocytes through the supply of substrates for carbohydrate and lipid metabolism. Moreover, this cooperation seems to be supported by redox regulatory mechanisms and may include redox synchronization between cancer cells and adipocytes. Redox-metabolic profiling of cancer and adipose tissue could be instrumental in advancing personalized therapeutic approaches, especially for individuals with concomitant metabolic diseases such as obesity, metabolic syndrome, and diabetes. Glu: glucose; FFA: free fatty acids; IGF-1: insulin-like growth factor 1; 4-HNE: 4-hydroxynonenal; ROS: reactive oxygen species; RNS: reactive nitrogen species.

case-control study found no differences in urinary levels of 15-F2t-iso-prostane and 2,3-dinor-5,6-dihydro-15-F2t-IsoP between breast cancer patients and controls, but further analysis according to BMI revealed that high levels of isoprostanes increase the cancer risk for obese and decrease the cancer risk for normal-weight women [237]. Moreover, the same redox biomarkers may be indicative of some but not other types of cancer; such is the case with plasma protein carbonyls. Several studies found an increase in breast cancer risk in women with higher levels of plasma protein carbonyls [238,239], while a large randomized, placebo-controlled trial found no association with a prostate cancer risk [240].

We are beginning to appreciate the importance of complex metabolic diseases in shaping the tumor microenvironment, while the accompanying research of underlying redox regulatory mechanisms is truly in its infancy. Nevertheless, results from recent comprehensive transcriptomic, proteomic, and metabolomic studies emphasize the enormous potential of individual profiling for cancer diagnostic and prognostic advancements, highlighting that study of redox-metabolic networks is a fertile ground for the search for biomarkers that will guide our approach in personalized therapy designs. More extensive population-based prospective and nested control-case studies employing multivariate analysis are needed to draw definite conclusions.

10. Conclusions and future perspectives

"All right, said the Cat; and this time it vanished quite slowly, beginning with the end of the tail, and ending with the grin, which remained some time after the rest of it had gone".

Lewis Carroll (Alice in Wonderland)

This quote illustrates the duality of present perspectives when we talk about redox biomarkers in complex disease conditions such as those related to obesity, metabolic syndrome, and diabetes. Currently, we are beginning to understand the redox bases of metabolic diseases but are encountering numerous challenges in validating redox biomarkers. These include overlapping of metabolic conditions, differential clinical presentation and progression, the involvement of a wide range of organs and organ systems, limitations in the availability of human biological samples, and the absence of reference values of redox biomarkers in healthy subjects, including diurnal variations. On the other hand, after decades of testing redox biomarkers in plasma/serum, blood cells, urine, saliva, and other bodily fluids and available biological material, some redox biomarkers are approaching the fulfillment of strict criteria [2]. The development of various redox methodologies and less invasive approaches in recent years is encouraging. In addition to measuring markers of oxidative damage to biomolecules and alterations in antioxidant defense components, direct electrochemical measurement of reactive species (NO, H_2O_2, and even H_2S) has become a real possibility today. The selection of representative sets of biomarkers for specific metabolic diseases and their standardization is one of the first steps warranting further research. Rigorous evaluation of redox proteomes, lipidomes, metabolomes, and transcriptomes will be of great help in deciphering the complex redox-metabolic landscapes. This should be preceded by the validation of redox biomarkers in healthy subjects, taking into account the influence of lifestyle (diet, physical activity, sleep, work patterns) and diurnal fluctuations in metabolites influenced by circadian rhythms. The development of new methodologies for determining redox biomarkers in tissue biopsies, such as adipose tissue, could eliminate some of the challenges related to the standardization of redox biomarkers in obesity, metabolic syndrome, and diabetes. Emerging advances in redox biology, both research-wise and methodologically, allow us to be optimistic about the future of redox disease biomarkers in clinical medicine.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

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