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Review Article

Emerging Noninvasive Biomolecular Checkpoints for Healthy Microbiomes

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Dysbiosis commonly impacts otherwise healthy individuals over the age of 50 due to seemingly innocuous changes in diet, hydration, or physical activity. Noninvasive biomarkers are emerging as checkpoints for immune health, energy-yielding metabolization, and oxidative stress during the aging process. These biomarkers work individually and collectively to detect dysbiosis, injuries, and infections, providing opportunities for earlier interventions with greater certainty of a positive long-term outcome. Measurements of C-reactive protein (CRP), white blood cells (WBCs), and neutrophil elastase are reliable biomarkers of persistent immune response. The absence of urinary bikunin is an accurate measure of immune system recovery during injuries, surgeries, and infections. Oxidative stress by-products of 4-hydroxynonenal (HNE), such as HNE–albumin adduct, allow assessing immune exhaustion and poor cell oxygenation. Overwhelming the immune system reduces the ability of monocytes (CD14) to transform into macrophages and impairs the energy-yielding metabolism signaling of adipokines, lowering the ability to improve cardiorespiratory fitness (CRF) or achieve significant weight loss. Bacterial endotoxins in urine are reliable indicators of ongoing infections and dysbiosis of the gut. Efficient gut microbiome health is predicted by dietary metabolites spilled into urine such as β-hydroxybutyrate, 2-methylbutyrate, 1,5-anhydroglucitol, enterolactone, enterodiol, carboxy-4-methyl-5-propyl-2-furanpropanoic acid, p-cresol, hydroxytyrosol, ethyl glucuronide, and F2-isoprostane and with blood markers like ferritin, homocysteine, and total cysteine. Vitamins B12 and D and folate are also key biomarkers that can monitor nutrient absorption during the aging process.

Keywords: adiponectin receptor; health aging; infectious disease; innate immunity; macrocytic anemia; metabolic syndrome (metS); microbiome; nutrition; vitamin deficiencies

1. Introduction

Dysbiosis is a microbiome etiology whereby the commensalism and mutualistic bacteria needed for healthy digestion are overwhelmed by pathogenic bacteria causing onset of chronic inflammation and oxidative stress (Figure 1) [1–7]. Dysbiosis leads to dysregulation of energy-yielding metabolization and occurs with nutritional imbalances in carbohydrates, protein, and fats as well as by antibiotic usage [2, 8–12]. The gastrointestinal system digests complex biomolecules from food cells after commensal bacteria metabolize food cell wall membranes, which then releases carbohydrate, peptides, lipids and nutrients to the host's enterocytes lining the intestines and then into blood circulation (Figure 1) [13, 14]. Intestinal enterocytes have tight intracellular junctions preventing intact bacteria from crossing into the blood stream and allow intercellular digestion pathogenic bacteria into glycopeptides and endotoxins [13, 15]. During dysbiosis, enterocytes will release more endotoxins into blood circulation, causing a systemic activation of inflammasome stress responses [16, 17]. Prolonged systemic inflammasome stress reduces the immune system's ability to respond to localized injuries or infections and will eventually lead to immune exhaustion [18]. Nutritional interventions for dysbiosis reduce endotoxins and oxidative stress, thereby improving immune health [2, 8–11].

Chronic inflammatory stress during aging is often associated with metabolic syndrome (metS) and underlying

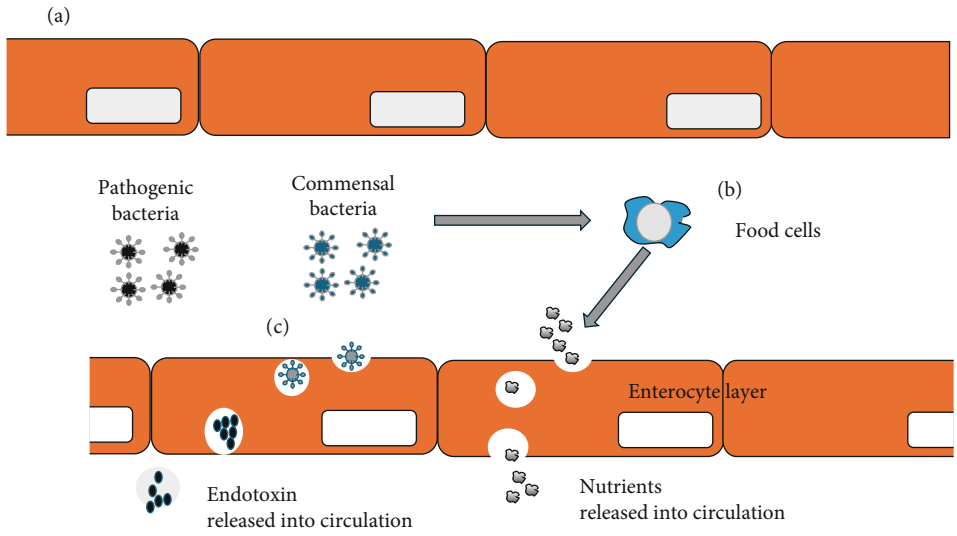

FIGURE 1: Gut metabolism of bacteria and nutrients. (a) The enterocyte layer of the gastrointestinal system prevents bacteria from crossing into the blood and invading the body. (b) Enterocytes digest complex biomolecules from food cells after commensal bacterial digestion and release carbohydrate, peptides, lipids and nutrients into blood circulation. (c) The enterocyte layer also digests excessive bacteria during dysbiosis and releases endotoxins and glycopeptides into blood circulation causing systemic oxidative stress and inflammation to tissues. Systemic endotoxins reduce the immune system's ability to response to specific injury and infection sites.

insulin resistance, which are well known to lead to higher morbidity and mortality risks associated with chronic diseases [19, 20]. Insulin resistance reduces the energy balance by impairing the body's ability to metabolize fatty acids and carbohydrates, which in turn increases obesity but also diminishes the immune response [21]. Diminished energy balance and immunity at age 60 and over are characterized by obesity, reduced mobility, poor fitness, loss of cognitive function, and poor gut microbiome [22, 23]. Persistent inflammatory responses damage tissues, leading to diabetes, autoimmune disorders, and epigenetic cellular changes in tissues [22, 24]. Healthy aging has been defined as increased life expectancy and quality of life, as measured by improvements in chronic inflammatory levels [25, 26]. Fitness is also an important healthy aging practice that improves chronic inflammatory levels [27, 28]. Poor nutrition is now well understood to alter gut health and impair energy-yielding metabolism, diabetes, and insulin resistance during aging [22, 23].

Diagnostics such as hemoglobin A1c (hbA1C) are examples of biomarkers that have been successfully used to promote healthy aging by monitoring the development of diabetes [29]. This biomarker measures the 30-day average of hyperglycemia, allowing a convenient quarterly check for improvements. The predictive value of biomarkers for health screening is greatly magnified when a noninvasive sample like saliva or urine allows repetitive measurement [30]. Calculation of biomarkers in saliva and urine requires accounting for specimen specific gravity [31]. Other common diagnostic biomarkers and risk factors such as lipidemia, hypertension, obesity, and the gut microbiome have failed to predict damaging impacts during aging early enough for interventions before such irreversible damage occurs [19, 20, 32]. Cardiorespiratory fitness (CRF) levels upon recovery after a defined exercise challenge are better measured by using digital tools and fitness equipment than by a biomarker [33, 34]. Measuring respiratory function by CRF is important for managing recovery from respiratory infections and injuries [33, 35]. Step and heartbeat monitors are common digital tools used for motivating fitness. However, they offer poor diagnostic accuracy for determining overall fitness level (receiver operating characteristic area under the curve [ROC AUC score] of 0.50–0.60) in nonelderly populations (age < 60). The system biology of the interaction between the microbiome and human nutrition is an emerging topic [22, 23]. New biomarkers are emerging as a means for gauging a person's unique nutrigenomic profile as aging progresses due to ever-increasing sensitivity of new bioanalytical methods [36, 37]. Noninvasive biomarkers are evaluated herein for their ability to predict improved immune response, metabolic energy, and oxidative stress levels to provide a scientific foundation for developing an in vitro diagnostic (IVD) panel for healthy aging.

2. Healthy Aging Diagnostics

2.1. Immune Health

2.1.1. Innate Immunity Response. Healthy aging requires a fully functionating innate immune system to completely resolve managing injuries and infections [19, 38]. The impact of innate immune system response to injury, infection, and dysbiosis is shown in Figures 2a, 2b, and 2c. Pathogenic bacteria are commonly acquired during aging as upper respiratory tract infections, urinary tract infection, or in wounds and injuries, as well as entering the gastrointestinal system in food [37]. These microbes can progress to bacteremia or bacteriuria with $> 10^4$ colony-forming units (CFUs)/milliliter [37]. Pathogenic bacteria may overcome the commensal bacteria of the host by producing virulence factors which increase pathogenicity and survival against

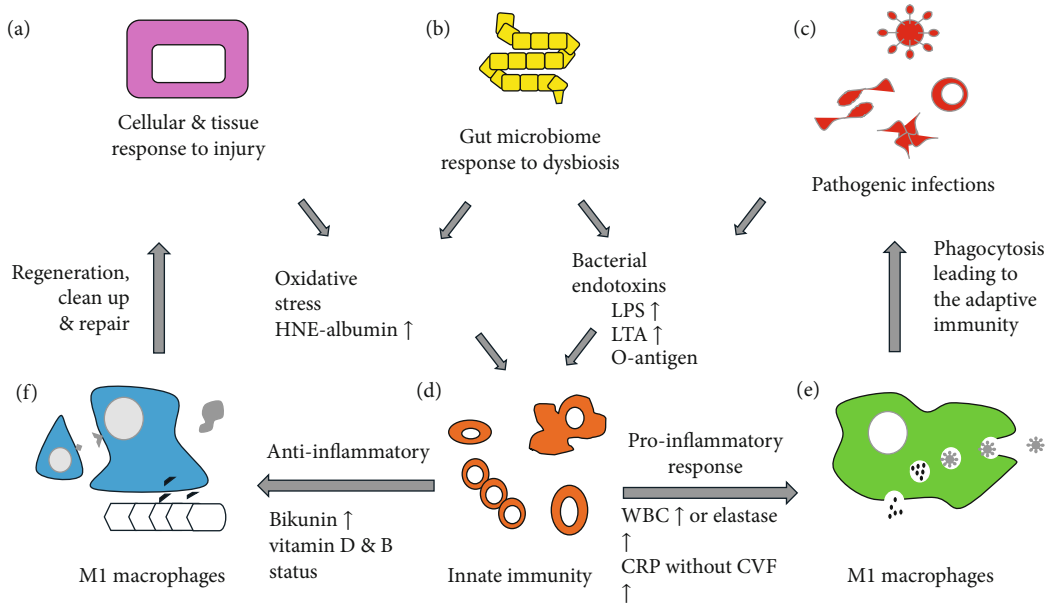

FIGURE 2: Innate immune response to injury, dysbiosis, and infection. (a) Epithelial cell, endothelial cell, smooth muscle, fibroblast, platelets, and other cells produce oxidative stress when tissues and organs are damaged during injuries, infections, and chronic disease. Oxidative stress triggers the innate immunity to release kallikrein, thrombin, plasmin, factors VII and X, and trypsin serine proteases to cause swelling, clotting, vascular dilation, and tissue remodeling. (b) The gut microbiome releases bacteria endotoxin into blood causing additional oxidative stress reflecting the balance of pathogenic and symbiotic microbiota during dysbiosis problems brought on by infectious illnesses, certain diets, or the prolonged use of antibiotics or other bacteria-destroying medications. (c) Microbes, parasites, and viruses which enter the body during infections through the respiratory, urinary tracts, gut, and wounds generate and release endotoxins into blood which causes additional oxidative stress and reflects cellular damage due to infection. (d) Neutrophilic innate immune cells migrate to inflamed tissues under oxidative stress and initiate a proinflammatory response to infections or tissue injury. Red blood cells respond by increasing oxygenation and initiating coagulation to repair hypoxic tissue under oxidative stress. Monocytes shed the C-terminal fragment of adiponectin receptor (AdipoR CTF) in response to oxidative stress to transform into M0 macrophages (CD14+/CD63+/TACE+) to initiate the adaptive immune response. (e) Neutrophilic innate immune cells release elastase to initiate pathogen destruction (phagocytosis). M1 macrophages (CD14+/CD63+/TACE+/CD206+ [mannose receptor]) initiate adaptive immunity for antigen presentation by responding to interferon gamma (IFN-γ). (f) Increased elastase releases the serine protease inhibitor, bikunin, to initiate the anti-inflammatory cascade. M2 macrophages (CD14+/CD63+/TACE+/CD220+ [insulin receptor]) initiate extracellular membrane remodeling, cellular regeneration, and clean-up of apoptotic cells by responding to IL4 and IL-13.

the host's immune response and antibiotics [17]. Bacteria cell lysis releases lipopolysaccharide (LPS), lipoteichoic acid (LTA), and O-antigen cell wall components during infections and dysbiosis which act as endotoxins, causing oxidative stress. Trace levels of endotoxin are now measurable by new technologies allowing earlier assessments of nutritional interventions for dysbiosis, injuries, and infection (Table 1) [2, 7, 37].

Chronic inflammation signifies a persistent innate immune cell response which is typical of chronic diseases of aging [20]. The innate immune system is driven by white blood cell (WBC), primarily composed of 60%–70% granulocytes (polymorphonuclear leukocytes), which include neutrophils, eosinophils, and basophils Figure 2d [38]. Granulocytes generate pro- and anti-inflammatory signals to fight off viruses, bacteria, fungi, or parasites and to repair injuries in tissues and organs. Neutrophils are the most common granulocytes, created in the bone marrow and typically circulating in the bloodstream for 6–10 h prior to self-destructing after one burst of activity. Injured cells and tissue, gut microbiome dysbiosis, and infectious pathogens all lead to endotoxins and oxidative stress in the circulatory system, which are now measurable by new high-sensitivity bioanalytical methods [37, 39–42]. Elevation in the number of gram − and + bacteria beyond $>10^4$ CFU/mL in a blood drop, urine, or saliva sample indicates an active pathogenic infection while $>10^2$ CFU/mL a residual infection (Table 1) [37, 39–42]. Endotoxins (LPS, LTA, and O-antigens) are released from the gut and infections to the bloodstream for elimination into urine [2, 7, 37]. Notably, pathogen levels in the gut and during infections are being impacted by modern methods of food production and nutrition [43, 44].

The pro- and anti-inflammatory response of the innate immune system to endotoxins and oxidative stress is shown in Figures 2d, 2e, and 2f. Elevation of WBC and elastase in blood, urine, or saliva is a reliable indication of an active innate immune response generating a proinflammatory response [19, 40, 45–48]. Complete cell counts of leukocytes (CD45+), granulocytes (CD45+CD15+), and monocytes (CD45+CD14+) using microscopy, flow cytometry, or immunocytochemistry (ICC) demonstrate elevation in the number of leukocytes (CD45+) in a blood drop (> 12,500/μL), urine (> 10/μL), or saliva [30] sample (> 230/μL) and therefore indicate an active innate immune response (see

TABLE 1: Noninvasive biomarkers for healthy aging.

Biomarkers	Biofluid	Process	Conditions detected
Immune health			
Bacterial endotoxins	Blood drop, saliva, urine	Phagocytosis	Infections, dysbiosis, and poor nutrition [2, 7, 37, 39–44]
WBC	Blood drop, saliva, urine	Active innate immunity	Proinflammatory response and CRF [19, 35, 40, 45–49]
Neutrophilic elastase	Blood, urine	Active innate immune response	Immune stress due to cytokines, INFγ, and chemokines [19, 40, 45–48, 50, 51]
CRP	Blood drop, saliva	Proinflammatory	Proinflammatory response autoimmunity and infection [38, 52–60]
Bikunin	Urine	Anti-inflammatory	Anti-inflammatory response healing during infections and injury [19, 38]
Vitamin D	Blood drop, urine	Antioxidant capacity	Vitamin D deficiency and immune responsiveness [8, 61–69]
Active Vit B12 and folate	Blood drop, saliva	Microbiome health	Immune response and good gut bacterial growth [66–87]
Energy-yielding metabolism			
β-Hydroxybutyrate	Urine	Metabolic efficiency	Good ketogenesis with fatty acid uptake, β-oxidation, and lipogenesis [88–90]
Adiponectin and leptin	Blood drop	Poor metabolism	Poor adipose health for signaling of oxidization of fatty acids (obesity) [19, 20, 51, 88, 91]
Autoantibodies to AdipoR CTF	Blood drop	Poor metabolism	Deactivation of the AMPK response for fatty acid uptake, β-oxidation, and lipogenesis [19, 20]
HbA1c	Blood drop	Poor metabolism	Prediabetic hyperglycemia and damaging advanced glycation end products [52, 90]
2-Methylbutyrate	Urine	Dysbiosis	Unhealthy nutrition with carbohydrate or protein overload causing fermentation stress of gut bacteria [10]
1,5-Anhydroglucitol	Urine	Dysbiosis	Hyperglycemia, overdoing high-carbohydrate diet (HCD) [92]
Enterolactone and enterodiol	Urine	Microbiome health	Sufficient fiber diet for good gut bacterial growth [93, 94]
Ferratin	Urine, saliva	Dysbiosis	High-protein diet causing oxidative stress and bacterial fermentation [10, 95, 96]
Carboxy-4-methyl-5-propyl-2-furanpropanoic acid	Urine	Microbiome health	Good fish protein and 3nFA diet for reduced oxidative stress [95, 97]
p-Cresol	Urine	Dysbiosis	Overdoing soy leading to cytotoxic microbiome imbalance [95]
Hydroxytyrosol and ethyl glucuronide	Urine	Dysbiosis	Excessive alcohol consumption leading to cytotoxic microbiome imbalance [98, 99]
F2-Isoprostane	Urine	Microbiome health	Good natural antioxidative diet levels including fruits, vegetables, cereals, and nuts [100–102]
Homocysteine/total cysT	Urine	Poor metabolism	Insufficient protein diet, unhealthy lean weight, and need of protein intake [10, 95, 96]
Oxidative stress			
Ferratin and transferrin hemoglobin	Blood, saliva	Cell senescence and poor oxygenation	Macrocytic age-related anemia [74, 75, 103–106]
Active Vit B12 and folate	Blood drop, saliva	RBC oxygenations	Macrocytic age-related anemia [74, 107, 108]
HNE–albumin ratio to albumin	Blood, saliva, urine	Oxidative stress, poor oxygenation	Overwhelmed immune system, tissue necrosis, vascular thrombus, and fibrosis damage to poor oxygenation [109–112]

Table 1) [19, 40, 45–48]. Human neutrophilic elastase is a well-established marker of neutrophilic exposure. A release above a threshold of 10 neutrophils/μL is significant in any biofluid (see Table 1) [39, 50, 51]. Neutrophils are motile, entering into the intestinal spaces of tissues upon vascular permeation due to proinflammatory signaling of swelling, clotting, vascular dilation, and tissue remodeling, allowing phagocytosis of pathogenic bacteria [7].

WBC counts are strongly correlated to CRF during aging [113, 114]. The National Health and Nutrition Examination Survey has demonstrated WBC counts as a measure of risk of chronic disease, infections, cancers, morbidity, and mortality [33, 49]. A person's physical fitness does improve personal WBC counts, cardiovascular risk, and respiratory function [33]. Other inflammatory biomarkers associated with elevated WBC such as cytokines, chemokines, and growth factors are also predictive of healthy aging in many cases [88, 115]. Fitness interventions complemented with restriction diets can lead to greater body mass index (BMI) loss and lower WBC counts within 1 year, with neutrophilic leukocytes and platelets most impacted. Other inflammatory markers showed no statistically significant changes upon intervention, such as C-reactive protein (CRP), interleukins (IL-6, IL-8), tumor necrosis factor-α (TNFα), c-peptide (inactive insulin form), and T cell markers of adaptive immunity. Additionally, monocyte chemoattractant protein (MCP-1) and IL-18 biomarkers were statistically significantly activated neutrophilic leukocytes during autoimmune disease.

Continuous chronic inflammation increases oxidative stress and leads to excessive protease activity where receptors, like the adiponectin receptor (AdipoR), are deactivated and immune exhaustion is likely [19, 51]. Chronic inflammation can be reduced with a combination of nutrition and fitness [52, 53]. High-sensitivity CRP is a blood biomarker that has been used to monitor improvements in chronic inflammation (2–10 mg/L) in cardiovascular disease [54–56]. Values above 10 mg/mL reflect true clinical inflammation due to infection, injury, or autoimmune disease [38]. Saliva CRP measurements are only slightly reflective of blood value due to diurnal variation and elevate from 238.5 ± 94.78 to 1519.5 ± 660.4 pg/mL during inflammation [57]. Values of CRP only improve with significant weight loss and improved CRF [33, 58]. Notably, many common anti-inflammatory medications, such as aspirin and nutritional supplements, such as vitamins C and E, omega-3 fatty acids, and zinc, lower CRP levels without corresponding changes in the WBC counts [54, 59, 60].

2.1.2. Bikunin Response. Bikunin in urine is a reliable indicator of infection and/or recovery from injuries [19]. Bikunin is a serine protease inhibitor released by neutrophilic elastase to suppress proinflammatory serine proteases [19, 38]. As a drug named ulinastatin, bikunin is well established as protecting the body during acute circulatory failure, sepsis, ischemic injury, cardiac arrest, traumatic brain injury, pancreatitis, and many conditions from immune-mediated apoptosis [19, 116–119]. Bikunin is rapidly eliminated from the body during infection or injury so that the immune system can continue to repair tissue damage and eliminate pathogens (Table 1) [19]. Urinary bikunin is a well-studied biomarker for chronic inflammation and more sensitive than CRP, persisting until the immune system returns to normal status [19, 38]. Bikunin correlates strongly with WBC counts and is formed by neutrophilic elastase whereas CRP is produced by the liver in a delayed mechanism to activate C3 complement and cause opsonization. Bikunin reflects worsening inflammation in patients > age 50 with metS, diabetes, and chronic diseases in a range from 2.0 to 7.5 mg/L. These patients are generally overweight (32% with BMI 25–30) or obese (52% with BMI > 30). Bikunin correlates with lower risk of comorbidities when conventional diagnostics fail to predict death and complications. Bikunin values above 7.5 mg/mL are diagnostic of clinical injuries and systemic infections, upper respiratory infections, urinary tract infections, cardiovascular tissue injury, glomerulus nephritis, and pancreatitis [38].

Bikunin as a serine protease inhibitor prevents prolonged proteolysis and is an anti-inflammatory response that protects cells and tissues from immune-mediated apoptosis leading to irreversible cellular changes and autoimmune disease (Figure 2e) [120]. Prolonged neutrophilic exposure by elevated WBC counts causes tissue damage by constant activation of immune-mediated apoptosis through proinflammatory signaling caused by constant release of inflammatory proteases (neutrophilic elastase, cathepsin G, proteinase 3, and granzyme B) leading to shedding of cytokines (lL-1, IL-6, IL-8, IL-10, and TNFα), interferon gamma (IFN-γ), chemokines (MCP-1, CXCL9, CCL11, and CXCL), and growth factors [19, 20, 38, 51]. Prolonged elevation of bikunin indicates a proinflammatory state where immune cells are causing damage to tissues and inducing poor wound healing [19, 20, 38]. Bikunin inhibits the release of cytokines, chemokines, and growth factors in the Janus kinase and signal transducer and activators of transcription (Jak-STAT) pathway, increasing protein kinase B (Akt) and phosphatidylinositol 3-kinase (PI3K) phosphorylation by blocking the potassium large conductance calcium-activated channel (KCNMA) [19, 116–119]. Bikunin also inhibits urokinase activation of mitogen-activated protein-extracellular signal–regulated kinase (MAPK/ERK) by plasmin, protease active receptor (PAR) activation of protein kinase C by trypsin, and granzyme B activation of caspase 8. It further blocks the extrinsic death factor receptor to help prevent immune-mediated apoptosis and promote tissue generation [19]. Bikunin also inhibits epithelial cell, endothelial cell, smooth muscle, fibroblast, and platelet release of serine proteases (kallikrein, thrombin, plasmin, trypsin, and factors VII and X) from causing additional swelling, clotting, and vascular dilation [19, 38].

2.1.3. Macrophage Response. The macrophage response of the innate immune system is triggered by endotoxins and oxidative stress and an imbalance caused by injury, dysbiosis, and infection (Figures 2d, 2e, and 2f). Monocytes (CD45+CD14+) are the third most common type of innate immune cells and account for 2%–10% of leucocytes in the blood and are essential to immune health, with elevation of > 800/μL indicative of an active innate immune response

(Table 1) [19, 20, 38, 51]. Monocytes transform into macrophages as they migrate into body tissues for phagocytosis by first differentiating into M0 macrophages (CD45+CD14 +CD63+) due to the release of neutrophil protease and the presence of oxidative stress in tissue [51]. M0 macrophages become proinflammatory M1 macrophages (CD45+CD14 +CD63+TACE+CD206+) in response to IFNγ and bacterial endotoxins (LPS, LTA, and O-antigen) to activate phagocytosis [51]. Monocytes become insulin resistant during the aging process, becoming less responsive to endotoxins and reactive oxidative species (ROS) [51, 121–123]. M0 macrophages influence the classical and nonclassical processes of adaptive immunity and antigen presentation. Monocytes can perform phagocytosis using intermediary opsonizing proteins such as antibodies or complements that coat the pathogen, as well as by binding to the microbe directly via pattern-recognition receptors that recognize specific pathogens. Monocytes are also capable of killing infected host cells via antibody-dependent cell-mediated cytotoxicity. This process occurs with M0 macrophages differentiated into M2 anti-inflammatory macrophages (CD45+CD14+CD63 +CD220+) in response to the presence of IL-4 and IL-13 and cells killed by phagocytosis during repair of tissue.

Vitamin D has a direct endocrine function and regulatory impact on immune health, improving macrophage response, lowering WBC counts, and decreasing immune cell death [8, 61, 62]. Vitamin D improves the total antioxidant capacity and reduces microbiota-generated oxidative stress and production of ROS while increasing glutathione (GSH). Measuring the need for vitamin D supplementation to promote immune health is independent of the effects of common anti-inflammatory medication, and its deficiency is measured by a blood value of < 20 ng/mL (Table 1) [63]. Notably, concentrations of CRP are lowered to 2.02 mg/dL (1.95–2.08) from 2.60 mg/dL (2.41–2.82) after treatment for vitamin D deficiency [64]. Vitamin D deficiency is also measurable in saliva, with values of 17.4 ± 8.0 ng/dL improved to 20.8 ± 6.3 ng/dL upon supplementation [65].

2.2. Energy-Yielding Metabolism

2.2.1. Metabolic Response. Poor energy-yielding metabolism can be attributed to obesity and metS with a commensurate increased risk of morbidity and mortality [124, 125]. The underlying insulin resistance observed with metS and obesity impacts the energy metabolism of the body and impairs CRF by limiting the ability of the body to oxidize fatty acids and restore energy [19]. Progression to the onset of diabetes is diagnosed by an HbA1c level greater than $> 6.5\%$ or by a fasting blood glucose or oral glucose tolerance test. Continued hyperglycemia leads to advanced glycation end products attaching to proteins which in turn degrades cell function and destroys elastin and collagen in skin and tissue [19]. Treatments to reduce hyperglycemia help to reduce weight but do not necessarily address the underlying insulin resistance or progression of chronic diseases [19]. Insulin resistance can be measured by understanding the compartment model of pancreatic secretion of insulin, its utilization, and metabolization [19]. It is not sufficient to just measure pancreatic insulin secretion to determine insulin resistance. Fasting to determine glucose and insulin values over time must be considered [19]. Blood and urine levels of β-hydroxybutyrate provide a measure of metabolic efficiency for carbohydrate and fat burning under lower lipid peroxidation levels stress during caloric restriction diets without a fasting collection (Table 1) [89]. Insulin resistance impairs the ability of the body to respond to oxidized fatty acids and hyperglycemia, thereby limiting the ability to heal injuries and fight infections [19, 51, 126]. A reduced aerobic fat burning ability increases oxidative stress and occurs with impaired adipose tissue [19]. The health of the adipose can be measured by adipokine, adiponectin, and leptin levels as risk factors for an inability to tolerate a hypercaloric high-fat diet and a significant risk of developing metS and diabetes (Table 1) [19, 127]. Further, while modest elastin/collagen preservation with topical antioxidants can somewhat improve skin health, the underlying metabolic stress is not resolved and further degradation can be expected [128].

2.2.2. Adipokine Response. Adiponectin and leptin are adipokines which provide a measure of adipose health for optimal energy-yielding metabolism [19, 51, 88, 91]. Leptin predicts adipose health and appetite control through the Sh2b1 neuroreceptor directly linked to brown fat transformation which regulates body weight and insulin resistance and which could be impacted by artificial sweeteners [129]. Adiponectin is an insulin-sensitizing hormone released from adipose tissue that explains how cells are activated to oxidize fatty acids and store glycogen for future energy [19]. Adiponectin secretion from the adipose is suppressed during obesity and diabetes, offering a metabolic pathway explanation for insulin resistance [19, 51]. Adiponectin's mechanism of action is not through insulin's activation of Akt but rather through the adiponectin activation of AMP-activated protein kinase (AMPK), which stimulates glucose uptake and lipid oxidation to produce energy [19, 91]. Activation of AMPK reduces oxidative stress in the tissues and organs and increases oxidization of fatty acids in liver and muscle controlling whole-body glucose homeostasis [130].

Adiponectin signaling of AMPK is deactivated by proteolytic shedding of the C-terminal fragment from the adiponectin receptor (AdipoR CTF) (Figure 2e,f) [51]. AdipoR CTF shedding correlates with an increased inflammasome measured by bikunin [20]. In obesity/age models, proteolytic shedding of AdipoR CTF correlates with an impaired energy-yielding metabolism and increases with obesity, age, and poor diet leading to Type 1 and 2 diabetes [19, 51]. Pancreatic damage was observed with increased neutrophil infiltration, leading to AdipoR CTF shedding and the presence of helper T cells and antigen activation presentation [51]. Antibodies to AdipoR CTF can be measured in humans and increase with aging, obesity, and diabetes [19, 20]. A healthy immune system is characterized by continuous shedding of AdipoR CTF [20, 51]. A lack of AdipoR CTF shedding reflects immune system exhaustion and a generally impaired energy-yielding metabolism, increasing the risk of morbidity and mortality in chronic diseases [19, 20]. A prolonged inflammasome leads to loss of proteostasis

or excessive protease activity without inhibition, which in turn leads to complete shedding of AdipoR CTF and hyperglycemia with impaired monocyte immune response [51].

Deactivation of the AMPK response by AdipoR CTF shedding occurs naturally in monocytes transforming into macrophages (M0, M1, and M2) for fighting microbial pathogens and repairing injuries [51, 131]. Loss of AMPK signaling reduces the ability to oxidize fatty acids and increases immune cell sensitivity to oxidative stress [51, 131]. Shedding of AdipoR CTF occurs with the shedding of cytokines like TNFα, IL-6, and IL-12 by the TNFα convertase enzyme (TACE) during the proinflammatory response initiated by any neutrophil protease [51]. Immune cells lacking AdipoR CTF are also unable to bind to an insulin degradation enzyme, which correlates with high intracellular insulin levels characteristic of cellular insulin resistance [51]. Oxidative stress exceeding the active inflammasome response (loss of proteostasis) indicates exhaustion of AdipoR CTF shedding and limits the ability of the immune system to fight infection and make repairs [51]. Bikunin regulates proteostasis to maintain the energy-yielding metabolism of AMPK by inhibiting PAR activation and activating phosphorylation of Akt/PI3 by blocking KCNMA [19, 51].

2.2.3. Nutritional Response. Carbohydrate and protein fermentation by gut bacteria produces short-chain fatty acids like 2-methylbutyrate in blood and urine indicative of dysbiosis (Table 1) [10]. Hyperglycemia produces urinary 1,5-anhydroglucitol indicative of an excessive carbohydrate diet (Table 1) [92]. The quantity and source of dietary proteins regulate the production of cytotoxic metabolites by gut microbiota, which alter the rectal mucosa of the host [10]. Amino acids, glycans, and other metabolites generated and digested by gut bacteria create environmental controls that are cytotoxic or promoting of bacterial species [8, 132–134]. Ferritin is indicative of excessive protein intake, while homocysteine and total cysteine predict unhealthy lean weight and need for protein intake (Table 1) [10, 95, 96]. Protein intake in a hypercaloric diet has been linked to metS, obesity, KD, LD, and DM [95, 135, 136].

Measuring nutritional status can improve the microbiota to promote an optimized metabolism [2, 6, 7]. A restricted Mediterranean-inspired diet in the PREDIMED-Plus Trial clearly demonstrated improved glucose metabolism–related parameters (fasting glucose, HbA1c, and insulin resistance) in a diet high in n-3 fatty acids (n-3 FAs) [88, 90]. Weight loss in metS occurred after 12 months and correlated with β-hydroxybutyrate and phospholipids shifting toward n-3 FAs from n-6 fatty acids with improved lipogenesis [88, 90]. The best sources of n-3 FAs are fish followed by red meat, linseed oil, and canola oil [95, 97]. Fish diet and n-3 FAs are measured by urinary carboxy-4-methyl-5-propyl-2-furanpropanoic acid and have shown beneficial effects in glycemic control and insulin sensitivity [95, 97]. The intake of fish increases plasma and erythrocyte n-3 FAs, allowing eicosapentaenoic acid and docosahexaenoic acid concentrations to reduce oxidative stress [137]. Dietary intake of palmitate and oleate also has a broad impact on systemic and tissue lipid profiles in humans [138].

Tolerance to hypercaloric western diets which are heavy in simple carbohydrates, protein, fat, and preservative varies greatly between person and race [139]. Metabolic adaptation in lipogenic transcription factors for fatty acid uptake, β-oxidation, and lipogenesis alters the α-ketoglutarate pathway to remove excess acetyl-CoA carboxylase from the cell mitochondria in the mammalian rapamycin (mTOR) pathway, reducing a person's ability for weight loss and ketogenesis [90]. Mitochondria are the oxidative energy reaction centers in cells that have their own DNA and can produce an excess of ROS, which can then induce programed cell death (apoptosis) and reduce mitochondrial efficiency further, which already diminishes during aging. Intermittent fasting by spacing caloric loads of carbohydrates and fat food reduces oxidative stress [89]. Reducing intake of foods with toxic metabolites reduces mitochondrial stress. For example, urinary excretion of cytotoxic p-cresol is increased in an excessive soy protein diet [95]. Excessive alcohol consumption of 0.2–0.1 g (2-1 drinks)/day increases oxidative stress, which can be measured by urinary hydroxytyrosol and ethyl glucuronide levels [98, 99].

Dietary interventions with indigestible carbohydrates such as whole grain and rye wheat fibers have not yielded improved energy metabolism but did increase commensal gut microbes and reduce lipidemia [128]. A sufficient fiber diet can be measured by urinary enterolactone and enterodiol lignan metabolites [93, 94]. Antioxidant foods including fruits, vegetables, cereals, and nuts include natural antioxidants such as vitamin C, vitamin E, flavanols, anthocyanins, quercetins, and polyphenols at subcytotoxic levels that induce robust cellular in vivo signaling [100–102, 140]. Antioxidant vitamins such as C, D, and E and omega-3 fatty acids have been associated with reduced oxidation, stress, and inflammation [59, 141, 142]. Vitamin C supplementation causes a reduction in inflammation (CRP and IL-6) improved energy-yielding metabolism by blood glucose, triglycerides, and hbA1C after 8 weeks of treatment [143–145]. Concentrations of n-3 and n-6 fatty acids in saliva are reflective of food intake and not chronic oxidative stress [146]. Vegetarian diets with natural vitamin E (< or 100 IU/day resveratrol) improve WBC counts and CRP and may have antithrombotic effects. However, supplemented vitamin E requires levels in the cytotoxic range (400 IU/day α-tocopherol) to decrease lipid oxidative stress [147, 148]. Metabolites of F2-isoprostanes measure the effects of an antioxidative diet [100–102]. The effects of ingesting tomatoes, plantains, grapes, legumes, nuts, and citrus are detected by urinary glycerates, stachydrine, hypaphorine, tryptophan, and proline derivatives [92, 136, 149]. A lack of plant-based diet and overdoing protein intake can be detected by urinary gamma glutamyl peptides due to overwhelming of GSH homeostasis [95].

Precision nutrition (nutrigenomics) is gaining interest as a means to address nutrient and vitamin deficiency on a personalized basis by measuring specific biomarkers [150]. Vitamin B deficiency (B1, 2, 3, 5, 6, and 12, folate, and biotin) is well studied and known to increase with age, metS, and an inadequate diet [70–72]. Gut absorption of vitamin B does not decline with age but rather the cell metabolism

and signaling diminish as well [73–80]. Vitamin Bs are known to regulate bacterial growth and are strongly associated with improved immune cell response and an optimized gut microbiome [151–153]. Markers of gut microbiome health are important to judge the idiosyncratic and highly individualized response to vitamin and nutrient supplements [6, 154]. Treatments with vitamins B and D boast immune health claims against various pathogens, reducing inflammation and oxidative stress and improving metabolic processes [61, 66–69]. Supplementation for vitamin B and D deficiency is known to reduce the risk or severity of cardiovascular disease, stroke, sepsis, dementia, and other conditions [81, 82, 152].

Testing for vitamin B12 and folate deficiency is a well-accepted component of the optimization of an energy-yielding metabolism (Table 1) [72]. Vitamin B deficiency can be measured by immunoassay for the holotranscobalamin complex (active B12) that promotes the uptake of cobalamin by all cells via specific receptors, including leukocytes, neutrophils, and monocytes [83–85]. Active B12 is normally at 200–900 pg/mL in serum, while values of 30–200 and < 30g/mL indicate borderline and clinical deficiency [65, 66, 83–87]. Folic acid deficiency leads to vitamin B deficiency at < 2 ng/mL in serum, with > 4 ng/mL considered normal. Endogenous folate is abundant in saliva due to diet intake of 0.32 pmol/ng saliva, but systemic deficiency can be measured by immunoreactive folate-binding proteins [155]. Active B12 and folic acid–binding protein are found in saliva, but normal and deficiency ranges are yet to be established (Table 1) [155, 156].

2.3. Oxidative Stress

2.3.1. Cell Senescence Response. Cell senescence and autophagy during aging can be predicted by impaired immune health and increased oxidative stress biomarker levels (Table 1 and Figure 2d,f) [25, 157, 158]. Tissue survival and regeneration rely on oxygenation for thriving cellular health [159]. Oxygenation is a key factor in tissue health, and management of oxygenation (blood oxygen) declines in pulmonary and vascular diseases associated with aging (Figure 2d) [160]. Vascular thrombus and fibrosis damage impair oxygenation, a self-perpetuating cycle leading to cardiac stress and tissue necrosis, which can be detected by standard coagulation and cardiac panels [55, 103]. Pathological lack of oxygenation (hypoxia) or low partial pressure of oxygen (hypoxemia) leads to tissue necrosis and loss of autophagy in all tissues, including the brain and heart. Necrosis and fibrosis markers specific to vascular and neurological systems are important indicators of ongoing damage [118, 161–164].

Oxidative carbonyl species impact the cellular function of the mTOR pathway implicated in many age-related disorders [51, 90, 165]. Poor injury recovery is characterized by poor monocyte polarization into M1/M2 macrophages and lack of clean-up of cellular materials damaged by reactive oxygen species and resulting oxidative stress [51]. Oxidative adducts of lipids induce monocytes to produce proinflammatory M1 macrophages through the liver X receptor and to activate clearing microbial pathogens and wounds [109, 148]. Oxidative stress is therefore indicative of poor immune health and persistent biomolecular and cell damage [19, 146]. Increased oxidation of lipids and proteins has been implicated in cardiovascular comorbidities, hypertension, and autoimmune disease. Thus, poor aging is correlated to cellular signaling in the NF-kappa B and Jak-STAT pathways which regulate the death Fas receptor (APO-1) for the natural programmed cell death (apoptosis) need for recycling of cellular materials (autophagy) in order to eliminate by-products of oxidative stress [165, 166]. Further, neutrophilic infiltration causes activation of the Jak-STAT-2,4 pathways through TACE proteolysis and leads to this immune-mediated apoptosis through cytokine and growth factor release [51]. However, continuous proinflammatory stress prevents the signaling switch to the Jak-STAT-1,3 pathway and JNK stress-activated protein kinases needed for tissue regeneration and regulation of senescence through repression of p38 MAP kinase and expression of tumor protein p53, the "guardian of the genome" [167]. The Jak-STAT-1,3 pathway also activates PIWI-dependent nuclear receptor complex formation in this process. The PIWI nuclear receptor binds to piRNAs with a length of ~26–31 nucleotides for directing DNA methylation in epigenetic gene control of cellular differentiation during tissue remodeling [168]. Tissue self-renewal and regeneration are initiated by tissue pluripotent stem cells which rely heavily on growth factor activation of specific stem cell factors, hormone receptors, and hormones. Impaired tissue self-renewal occurs with diminished hormonal response cell responses in aging leading to incorrect cellular differentiation and loss of tissue function [169].

2.3.2. 4-Hydroxynonenal (HNE) Response. Persistent chronic inflammation without repair is characterized and measurable by oxidative stress biomarkers such as HNE [7, 19, 70, 170]. Oxidative stress pathways including superoxide anion, singlet oxygen, hydroxyl radical, hydrogen peroxide, peroxynitrite anion, and nitric oxide all lead to protein, enzyme, lipid, DNA, and cellular damage, inactivation, and programmed death (apoptosis) [70, 171]. Oxidative stress, including hypoxia, results in nitrosative stress, endoplasmic reticulum stress, mitochondrial dysfunction, and carbonylic stress. Nitrosative and oxidative stress are measured by and associated with ROS. Every individual has a highly variable and often dynamic ability to tolerate unhealthy oxidative stress levels. Measuring unhealthy oxidative stress can be inferred through ROS levels but more accurately assessed through fragments, adducts, or metabolites of tissue, cellular, or nucleic damage. Malondialdehyde (MDA) and HNE are aldehydes that result from lipid peroxidation of polyunsaturated fatty acids [70]. Many biomarkers of oxidative stress such as MDA, HNE, 8-hydroxy-2′-deoxyguanosine (OHdG), and nitrotyrosine are not stable in biological fluids and are poor markers [7, 19, 70, 170]. The formation of HNE is the end of the chain of reaction of all ROS and nitrosative species. Reactive HNE reacts with proteins, lipids, and glycoconjugate to form stable measurable adducts, allowing the quantitation of a 30-day average level of oxidative stress in an organism [110, 172].

Protein adducts of HNE for serum proteins such as albumin allow measurement of systemic oxidative stress, whereas cell-specific proteins measure specific stress to tissue (Table 1) [109–112]. The attachment of HNE to proteins occurs immediately to form an adduct which can be detected by immunoassays. Adducts to proteins are a stable method for assessment of overall oxidative stress [31, 172, 173]. Adducts of HNE to human serum albumin (HNE–albumin) have been shown highly reproducible within a patient, reflecting a 30-day average oxidative stress correlating with nitrotyrosine, OHdG, and MDA [174]. Albumin has a fairly constant protein presence in blood at 3.4–5.4 g/L, saliva at 0.1–2 mg/L, and urine at 0.5–80 mg/L [19, 31]. HNE–albumin in urine is 0.59 ± 0.09 and 2.0 ± 0.5 mg/dL for low and high inflammation, respectively (Table 1) [31]. Systemic oxidative stress (> 1.5 mg/mL HNE–albumin) and chronic inflammation (2.0–7.5 mg/L bikunin) are observed in 69% of adults over 50 without diabetes or cardiovascular disease and 82% of adults with diabetes in the absence of known infections or recent surgeries [31]. The ratio of HNE to albumin is typically s 1:1–1:5 or ~1.5–2.2 nmol of adduct [31]. Oxidative stress is measured relative to the total amount of albumin in the sample (HNE–albumin/albumin) [31]. Measuring oxidative stress adds to the assessment of chronic inflammation by determining if the immune system is addressing oxidative stress factors or is in fact overwhelmed [19, 31, 51]. Antioxidant supplementation like vitamins D and C decreases carbonylic reactive species like MDA and HNE [104, 175, 176]. Vitamin C also decreases oxidative stress by interaction with GSH and paraoxonase-1 [104, 105, 175, 176].

The kidney is a highly sensitive oxygen sensor and mediates red blood cell (RBC) production in response to hypoxia due to release of erythropoietin and activation of the hypoxia inducible factor 1 to increase blood oxygen-carrying capacity production [177, 178]. Slight hypoxia, such as caused by higher altitudes (up to 8000 ft, 2500 m), increases the blood's oxygen-carrying capacity [178, 179]. Slight hypoxia, when combined with good metabolic health, results in increased blood hemoglobin (Hgb) and delays the onset of replicative senescence in neurological cultured cells and extends lifespan in animal models [180, 181]. However, unhealthy blood oxygen saturation (< 90%) caused by low-perfusion conditions or high altitudes above 14,000 ft/4300 M closely simulates poor aging [182, 183]. The brain requires an ongoing and stable oxygen supply to support its underlying functions. The role of oxygen in the functional activity of the brain is important in the relationship of healthy aging and its potential pathologies. Clinical signs and symptoms of poor oxygenation are fatigue, dyspnea on exertion, vertigo, palpitation, low blood pressure, pallor, and headaches resulting from diminished delivery of oxygen to tissues. Age-related reduction of mental quality of life is caused by fatigue, headaches, weight loss, impaired balance, mood changes, muscle loss, neuropathy, dementia, weakness, leukopenia, neuropsychiatric behavior, and gastrointestinal issues [72].

2.3.3. Hgb Response. Markers of anemia seek to address the inability of RBCs to transport oxygen [74, 75, 105]. Age-related anemia is estimated to impact 900 million people globally over the age of 65 [85, 184]. Improving detection and treatment of age-related anemia improves mental quality of life and plays an important role in the effectiveness of aging care [71, 72]. The primary diagnostic method of general anemia is low Hgb concentration or hematocrit value in blood [103]. Anemia pathophysiology is categorized as being due to RBC efficiency, accelerated RBC destruction, and impaired RBC production and judged by the size and shape of the RBC as distinguished by cytology and hematology. Age-related anemia due to accelerated RBC destruction causing Hgb deficiency can be due to autoimmune disease, toxins, infections, cancers, or medicines and can be detected by Hgb in blood. Age-related pernicious macrocytic anemia is typically associated with impaired vitamin and nutrient absorption [74, 107, 108]. Abnormal RBC response to oxidative stress damage (ferroptosis) has been used to explain impairment [86]. Macrocytic anemias are commonly due to related vitamin B deficiency, hypothyroidism, testosterone deficiency (40–50-year men), or medication such as metformin, aspirin, and nitrous oxide. Monitoring of these hormones is typically performed in an endocrinology assessment of macrocytic anemia [185]. Active B12 and folate measurements are useful. Additionally, biomarkers identified for chronic alcohol consumption, malnutrition, vegetarian diets, and genetic factors further contribute to age-related macrocytic anemia (Table 1).

Vitamin and nutrient supplements with Vit B, iron, zinc, and iodine are a safe, well-accepted treatment for resolving anemia due to Hgb deficiency and improving nutritional status [104, 106]. Treatments can be monitored by blood immunoassays and iron panels [103]. Hgb deficiency is likely when the serum ferritin level is less than 50 ng per milliliter (112.35 pmol per liter). Serum ferritin values greater than or equal to 100 ng per milliliter (224.70 pmol per liter) generally exclude iron deficiency anemia (Table 1). Transferrin is also measured to estimate the total iron-binding capacity. Lower transferrin with higher ferritin indicates a lower total iron-binding capacity and could indicate increased oxidative stress to RBCs. The normal range for transferrin in serum is 215–380 mg/dL. A predictable amount of transferrin is present per Hgb found. Anemia has also been detected in saliva, where the mean level of salivary ferritin in subjects with iron deficiency was significantly higher at 139.37 ± 47.90 μg/dL when compared to the levels in nonanemic subjects at 94.18 ± 62.90 μg/dL [186–188]. Transferrin is typically < 4.0 mg/L in saliva and correlates with the amount of Hgb in saliva, which normally ranges from 0.045 to 2 mg/dL Hgb [45, 189]. The measurement of transferrin and ferritin in saliva for anemia requires accounting for bleeding of the gums by additionally measuring and ratioing to Hgb [31].

3. Challenges and Prospects

Progressing noninvasive biomarker panels to widespread clinical practice will require new technologies enabling one-touch and rapid measurements utilizing novel point of care systems [37]. Analytical validation of autocalibration, standardization, high reliability, and error-free use of these systems must be proven capable when administered by

untrained users. Noninvasive urine or saliva analysis must correct for sample variation caused by changes in sample concentration. Blood finger-prick samples of ~1 μL must correct for collection variations in hematocrit values. Additional clinical validation studies will be needed with larger populations to set normal reference ranges, define quantitation requirements, and establish relationships needed for promoting healthy gut microbiomes [30]. Each biomarker will have to undergo independent validation before the integration of multiplexed panel results can be applied to machine learning analysis of multimodal data and further validated. Outcome studies using precise nutritional interventions lasting only 3–6 months will have to demonstrate an improvement of dysbiosis to support widespread clinical adoption.

4. Conclusion

Healthy aging biomarkers are showing value for establishing trends in normal immune health, normal energy-yielding metabolism, and oxidative stress. Chronic inflammation and oxidative stress leading to dysbiosis, tissue damage, and infections can be monitored before signs of clinical pathology become evident. Biomarkers of diet, vitamins, and nutrition related to improved digestive health and energy conversion can potentially keep oxidative stress from exceeding the inflammasome response and allow continued immune system health. Biomarkers of tissue oxygenation health relate directly to mobility and mental quality of life, with mobility itself preventing an increase in the by-products of dysfunction, cellular senescence, and impaired regeneration. Maintaining healthy gut microbiomes can be aided by early monitoring of biomarkers that enable adherence to a personalized nutritional plan and when used in combination with broad coverage of key aging factors.

Nomenclature

Active B12	holotranscobalamin complex
AdipoR	adiponectin receptor
AdipoR CTF	C-terminal fragment from the AdipoR
AGE	advanced glycation end products
Akt	protein kinase B
AMPK	AMP-activated protein kinase
BMI	body mass index
CD	cluster of differentiation
CFU	colony-forming units
CRF	cardiorespiratory fitness
CRP	C-reactive protein
CXC	cysteine-X-cysteine
CCL	CXC ligands
n-3 FAs	n-3 fatty acids
GSH	glutathione
hbA1C	hemoglobin A1c
Hgb	hemoglobin
HNE	4-hydroxynonenal
HNE–albumin	adducts of HNE to human serum albumin
IFN-γ	interferon gamma
IL	interleukin
IVD	in vitro diagnostics
Jak-STAT	Janus kinase and signal transducer and activators of transcription
KCNMA	potassium large conductance calcium-activated channel
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAP	mitogen-activated protein
MAPK/ERK	kinase-extracellular signal–regulated kinase
MCP	monocyte chemoattractant protein
MDA	malondialdehyde
metS	metabolic syndrome
mTOR	mammalian target of rapamycin
OHdG	hydroxy-2′-deoxyguanosine
PAR	protease active receptor
PI3K	phosphatidylinositol 3-kinase
RBC	red blood cell
ROC AUC score	receiver operating characteristic area under the curve
ROS	reactive oxidative stress
TACE	TNFα convertase enzyme
TNFα	tumor necrosis factor-α
WBC	white blood cell

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Disclosure

The authors have conducted this biomarker research independently in accordance with a policy on objectivity in research.

Conflicts of Interest

The authors declare no conflicts of interest.

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