

# Immune biomarkers in the spectrum of childhood noncommunicable diseases



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A biomarker is an accurately and reproducibly quantifiable biological characteristic that provides an objective measure of health status or disease. Benefits of biomarkers include identification of therapeutic targets, monitoring of clinical interventions, and development of personalized (or precision) medicine. Challenges to the use of biomarkers include optimizing sample collection, processing and storage, validation, and often the need for sophisticated laboratory and bioinformatics approaches. Biomarkers offer better understanding of disease processes and should benefit the early detection, treatment, and management of multiple noncommunicable diseases (NCDs). This review will consider the utility of biomarkers in patients with allergic and other immune-mediated diseases in childhood. Typically, biomarkers

are used currently to provide mechanistic insight or an objective measure of disease severity, with their future role in risk stratification/disease prediction speculative at best. There are many lessons to be learned from the biomarker strategies used for cancer in which biomarkers are in routine clinical use and industry-wide standardized approaches have been developed. Biomarker discovery and validation in children with disease lag behind those in adults; given the early onset and therefore potential lifelong effect of many NCDs, there should be more studies incorporating cohorts of children. Many pediatric biomarkers are at the discovery stage, with a long path to evaluation and clinical implementation. The ultimate challenge will be optimization of prevention strategies that can be implemented in children identified as being at risk of an NCD through the use of biomarkers. (*J Allergy Clin Immunol* 2016;137:1302-16.)

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Terms in boldface and italics are defined in the glossary on page 1303.

Noncommunicable diseases (NCDs) are one of the major global challenges of the 21st century.<sup>1</sup> NCDs have been termed a “slow-motion disaster”<sup>2</sup> and a global crisis<sup>3</sup> as their prevalence increases in all countries, in all income groups, and at all ages. NCDs, often called chronic diseases, are generally considered one of 4 main types: cardiovascular diseases, cancers, chronic respiratory diseases, and diabetes. Allergic disease has been suggested as a fifth group given its high prevalence and early onset,<sup>4</sup> but neurocognitive diseases, inflammatory bowel diseases (IBDs), and others are also important NCDs. Worldwide, 2 of every 3 deaths each year are attributable to NCDs, with one third of those who die being less than 60 years of age.<sup>5</sup> In all regions of the world, the prevalence of NCDs is increasing because of an aging population and the globalization of common risk factors.<sup>5</sup> The main risk factors for NCDs, accounting for two thirds of all cases, are tobacco use; foods high in saturated and trans-fats, salt, and sugar; physical inactivity; and alcohol consumption.<sup>6</sup> NCDs not only directly threaten health, they influence economic development to compound the effect on health. The World Economic Forum ranks NCDs as one of the top global threats to economic development: a 10% increase in mortality caused by NCDs reduces annual economic growth by 0.5%.<sup>7</sup>

A shared feature of all NCDs is chronic low-grade inflammation promoted by modern diets, environmental pollutants,

#### Abbreviations used

ASD: Autism spectrum disorder  
CD: Crohn disease  
CRP: C-reactive protein  
EMP: Endothelial microparticle  
EPC: Endothelial progenitor cell  
FoxP3: Forkhead box P3  
IBD: Inflammatory bowel disease  
miRNA: MicroRNA  
MS: Mass spectrometry  
NCD: Noncommunicable disease  
OIT: Oral immunotherapy  
sFasL: Soluble Fas ligand  
UC: Ulcerative colitis

microbial exposure, and psychological and biological (eg, oxidative or endoplasmic reticulum) stress.<sup>1,8</sup> This low-grade inflammation differs from classical inflammation, which occurs in response to a threat or injury and leads to tissue repair and restoration of the basal homeostatic state. The low-grade inflammation in patients with NCDs is chronic; without effective treatment, basal homeostasis cannot be restored,

and the damage might already be done.<sup>9</sup> Inflammatory pathways in NCDs are multifactorial and part of a metabolic cascade, including cellular oxidative stress and insulin resistance, which induces allostatic overload, dysmetabolism, and ultimately chronic disease.<sup>10</sup> Changes in the gut microbiome have emerged as one of the pathways leading to chronic low-grade inflammation.<sup>11</sup> Altered gut colonization and reduced microbiome diversity occur in response to both changed nutritional patterns and the built environment; a diverse microbiome is essential for normal immune development and regulation.<sup>12</sup> There is global interest in gut dysbiosis in multiple NCD settings, including beta-cell autoimmune disease,<sup>13</sup> atopic dermatitis,<sup>14</sup> and IBDs.<sup>15</sup> Despite the clear link between the gut microbiome and development of these diseases and obesity, cardiovascular disease, and metabolic disorders more generally, the causal relationship between alterations in the gut microbiome and ill health is likely to be complex. There can be multiple different pathways for different organisms; these pathways might or might not overlap in some of their stages. However, specific microbe-derived metabolites, such as short-chain fatty acids, have emerged as examples whereby cross-talk between the microbiome and host is achieved.

Although NCDs are most prominent in adulthood, development in early life influences predisposition to NCDs. This starts as

## GLOSSARY

**ADIPOKINES:** Cytokines secreted by adipose tissue.

**FLAGGRIN:** A filament-associated protein that binds to keratin fibers in epithelial cells and is essential for regulation of epidermal homeostasis.

**FLOW CYTOMETRY:** A technology that is used to analyze the physical and chemical characteristics of particles in a fluid as it passes through at least 1 laser. Cell components are fluorescently labeled and then excited by the laser to emit light at varying wavelengths. Flow cytometry allows for the characterization and separation of immune cells.

**IL-4:** A pleiotropic cytokine produced by activated T cells, this cytokine is a ligand for the IL-4 receptor that also binds to IL-13, which can contribute to many overlapping functions of this cytokine and IL-13.

**IL-5:** A major maturation and differentiation cytokine expressed by T<sub>H</sub>2 cells and eosinophils in mice and human subjects. IL-5 has been shown to play an instrumental role in eosinophilic inflammation in patients with allergic diseases.

**IL-10:** A cytokine produced primarily by monocytes and, to a lesser extent, by lymphocytes that has pleiotropic effects in immunoregulation and inflammation by limiting the immune response to pathogens and thereby preventing damage to the host.

**IL-12:** A cytokine produced by dendritic cells, macrophages, and human B-lymphoblastoid cells in response to antigen stimulation. IL-12 is involved in the differentiation of naive T cells into T<sub>H</sub>1 cells. It is known as a T cell-stimulating factor that can stimulate the growth and function of T cells. It stimulates the production of IFN- $\gamma$  and TNF- $\alpha$  from T cells and natural killer cells and reduces IL-4-mediated suppression of IFN- $\gamma$ .

**IL-13:** A cytokine produced primarily by T<sub>H</sub>2 cells that is involved in several stages of B-cell maturation and differentiation and is critical to the pathogenesis of allergen-induced asthma but operates through mechanisms independent of IgE and eosinophils.

**LPS:** An endotoxin found in the outer membrane of gram-negative bacteria that elicits a strong immune responses in animals.

**MASS CYTOMETRY:** A mass spectrometry technique that analyzes cell properties using antibodies tagged with rare earth elements.

**MASS SPECTROMETRY:** An analytic technique that separates ions by their mass.

**RAPAMYCIN (MECHANISTIC TARGET OF RAPAMYCIN):** A macrolide produced by the bacterium *Streptomyces hygroscopicus*, which has immunosuppressant functions in human subjects. It prevents activation of T and B cells by inhibiting IL-2 production.

**REGULATORY T (TREG):** A subset of T cells that control inflammation and induce tolerance by secreting anti-inflammatory cytokines. They are CD4<sup>+</sup>CD25<sup>+</sup> T cells under the control of the transcription factor FoxP3 that develop and emigrate from the thymus to perform their key role in immune homeostasis. Treg cells secrete immunosuppressive soluble factors, such as IL-9, IL-10, and TGF- $\beta$ .

**T<sub>H</sub>2 CELLS:** A distinct lineage of CD4<sup>+</sup> effector T cells that secrete IL-4, IL-5, IL-9, IL-13, and IL-17E/IL-25. These cells are required for humoral immunity and play an important role in coordinating the immune response to large extracellular pathogens.

**T<sub>H</sub>17 CELLS:** A subset of activated CD4<sup>+</sup> T cells that are responsive to IL-1R1 and IL-23R signaling. They are regulated by the IL-6/signal transducer and activator of transcription 3/retinoic acid-related orphan receptor  $\gamma$ t lineage control and produce the cytokines IL-17A, IL-17F, IL-17AF, IL-21, IL-22, IL-26 (human), GM-CSF, macrophage inflammatory protein 3 $\alpha$ , and TNF- $\alpha$ . T<sub>H</sub>17 cells act as a bridge between adaptive and innate immunity, where they promote neutrophil activation, immunity to pathogens, and inflammation.

**TNF- $\alpha$ :** Secreted primarily by macrophages, this cytokine's primary role is the regulation of immune cells. Moreover, it is involved in the regulation of a wide spectrum of biological processes, including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.

**TOLL-LIKE RECEPTOR (TLR):** A class of receptors expressed on macrophages and dendritic cells that recognize conserved microbial particles that can activate an immune response.

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early as pregnancy, when maternal body composition and diet influences the infant's risk of NCDs later in life.<sup>4</sup> Therefore NCDs should be studied by using a life-course approach, with overall risk depending on the sequential effects of the developmental timeline with different metabolic trajectories, age-dependent decrease in plasticity, and subsequent differential responses to subsequent risk factors.<sup>16</sup> Monitoring of these timelines and trajectories would not only benefit the understanding of disease processes but also enable risk stratification for disease intervention and even prevention. Biomarkers likely provide the necessary tool for this approach to be successful.

## WHAT ARE BIOMARKERS?

A biomarker (biological marker) is a quantifiable biological characteristic that provides an objective measure of health status or disease. For disease, biomarkers have the potential for use in risk stratification, early detection, identification of the treatment of choice and monitoring response to treatment, surveillance, and drug monitoring and development. Biomarkers are also used in other clinical scenarios, such as microbial identification and diagnostics. A biomarker can be a gene, molecule, or other biological characteristic as long as it can be measured accurately and reproducibly and is a valid indicator of the process or outcome being "marked." More detailed discussion of the various but overlapping definitions of biomarkers from the National Institutes of Health Biomarkers Definitions Working Group and the World Health Organization, among others, can be found elsewhere.<sup>17</sup>

Traditionally, biomarkers have been used as indicators of a specific disease state and range from cardiac troponins for myocardial infarction to genetic markers of cancer risk to stool calprotectin for IBDs. Many of these biomarkers were first identified during targeted studies, typically of isolated molecules within *in vitro* cellular systems and/or case-control comparison of clinical specimens. The "omics" era has led to unbiased discovery-based approaches to identify biomarkers, including panels (also called clusters or signatures) of biomarkers. Omics approaches, particularly proteomics, metabolomics, and cellomics, but less so transcriptomics and epigenomics, are used in 2 broad ways: to better understand biological processes and for clinical biomarker discovery. Although the latter is not intended to provide insight into disease processes, biological plausibility can help progress validation and implementation of any discovered biomarkers.

Biomarkers can also be used as surrogate or intermediate end points in translational and clinical research, where the challenge is to know what the relationship is between any measure and the relevant clinical end point.<sup>17</sup> The use of biomarkers in this way might be most useful during clinical trials and for lifestyle interventions, such as nutrition.<sup>18</sup> This is critical for those who work in the disease prevention arena so that the success or failure of an intervention can be considered before a clinical end point is achieved, which can take many years.

Among the omics approaches for biomarker discovery, proteomics is currently the most widely used. The proteome reflects splice variants and posttranslational modifications of proteins that dictate the structure, function, localization, maturation, and turnover of proteins, all of which change rapidly in response to environmental signals.<sup>19</sup> Sample types studied include serum, plasma, saliva, urine, exhaled breath, tissue biopsy specimens,

mucosal secretions, cerebrospinal fluid, and other biological specimens. The plasma proteome is of particular interest; not only is blood a routinely collected clinical sample, but plasma is postulated to reflect the sum of multiple site-specific proteomes containing representatives of the entire set of more than 300,000 estimated human polypeptide species resulting from splice variants and posttranslational modifications.<sup>20</sup>

An emergent biomarker family measurable in the circulation are the microRNAs (miRNAs). miRNAs are small endogenous noncoding RNAs with a critical posttranscriptional gene-regulatory role.<sup>21</sup> Aberrant miRNA expression is implicated in disease, and disease-specific miRNAs, tissue-specific miRNAs, or both have been identified; these are surprisingly stable and can be measured relatively easily. Most progress in the use of miRNAs as biomarkers has been made in cancer, where their utility has been considered for monitoring disease progression, outcomes, recurrence, and metastasis.<sup>22</sup>

Within the field of pediatrics, biomarker discovery remains dominated by targeted approaches. Here we will review what is known about pediatric biomarkers for NCDs and identify emergent areas of study, highlighting traditionally identified biomarkers, as well as those revealed with untargeted approaches. Given the common genetic factors that contribute to inflammatory diseases, as recently shown with pediatric autoimmune diseases,<sup>23</sup> the challenge is to identify downstream proteins, metabolites, miRNAs, and other molecules that offer insight in a disease-specific manner. The microbiome might also serve as a biomarker as deep sequencing and transcriptomics approaches become more readily usable.

## BIOMARKERS IN PATIENTS WITH NCDs IN CHILDHOOD

### Allergy

Allergic disorders, including atopic dermatitis, food allergy, allergic rhinitis, and asthma, affect more than 1 billion persons across the globe, and their prevalence is expected to quadruple by the 2050s.<sup>24</sup> Atopic dermatitis, which is typically accompanied by IgE sensitization to food, and food allergy are the earliest-onset NCDs and the most common chronic NCDs of childhood worldwide.<sup>25,26</sup> They are considered the first steps on the allergic march, whereby atopic dermatitis and food allergy in infancy progress to asthma and allergic rhinitis in later childhood and into adult life.<sup>27,28</sup> As for many other NCDs, the development and deployment of biomarkers for monitoring disease severity, classifying and clarifying disease subsets, and guiding treatment decisions is an active area of translational research. Given the early age of onset and the prospect of lifelong disease, there is also incredible value in predictive biomarkers usable around birth to identify children at risk of these diseases. Failing that, identifying those children with atopic dermatitis, food allergy, or both who will progress to asthma, rhinitis, or both would be of immense use.

### Atopic dermatitis and food allergy

Much of the biomarker work within atopic dermatitis relates to monitoring disease severity and clinical improvement. Many investigators report an association between disease activity and circulating factors and then speculate that these could act as disease markers or new therapeutic targets. These circulating factors include soluble adhesion molecules, such as intercellular

adhesion molecule 1<sup>29</sup>; the antibacterial peptide LL-37<sup>30</sup>; interleukins, such as IL-31<sup>31</sup> and IL-18<sup>32</sup>; and numerous chemokines, such as mucosa-associated epithelial chemokine (CCL28),<sup>33</sup> thymus and activation-regulated chemokine (CCL17),<sup>34</sup> and cutaneous T cell-attracting chemokine (CCL27).<sup>35</sup> However, there are few evaluation studies to progress these molecules to clinical use. Candidates are usually analyzed because of their role in disease pathogenesis and therefore are common to multiple allergic disorders, inflammatory disorders, or both. Although potentially suitable for disease monitoring,<sup>36</sup> they are less likely to offer the specificity required for predictive biomarkers. Detailed analysis of CCL17 also underlines one of the real challenges of biomarker discovery in children. Circulating CCL17 levels correlate inversely with age and therefore are much higher at 0 to 1 year of age than in older age groups,<sup>34</sup> highlighting the natural age-dependent variation in the abundance of proteins and other classes of molecules. This is a particular challenge for diagnostic and prognostic biomarkers, but for predictive biomarkers implemented as early in life as possible, this is unlikely to be a problem.

The utility of miRNAs for atopic dermatitis prognosis in children also has been explored.<sup>37</sup> Notably, both comorbidities and the sample type analyzed affect the findings. Serum miR-483-5p levels were increased in children with atopic dermatitis and associated with other atopic conditions, such as asthma or hay fever. For miR-203, urine levels were decreased but serum levels were increased in the children with atopic dermatitis.<sup>37</sup> There is also interest in plasma miRNA biomarkers in patients with eosinophilic esophagitis, with several differentially expressed in pediatric patients with eosinophilic esophagitis.<sup>38</sup> Some of these miRNAs mapped with glucocorticoid treatment and normalization of eosinophil histology and cell counts and therefore could offer a less invasive approach to monitor treatment efficacy.<sup>38</sup> There is much interest in miRNAs for better understanding disease pathogenesis and informing treatment decisions and use as biomarkers. To date, most studies have focused on profiling specific miRNAs in various allergic disease settings, where they have been found to have a role in T-cell, eosinophil, and epithelial function.<sup>39</sup> Hopefully, we will see progress to using these as biomarkers in multiple disease settings.

Studies with omics approaches for atopic dermatitis are only just being reported. Examples include a mass spectrometry (MS) study of serum to reveal changes in energy metabolism measurable as increased levels of carnitine, free fatty acids, and lactic acid, among others, in patients with atopic dermatitis<sup>40</sup> and changes in the glycoproteome (glycosylation patterns are influenced by physiologic status<sup>41</sup>) by using 2-dimensional electrophoresis, showing increased CD5L and decreased apolipoprotein E levels with atopic dermatitis.<sup>42</sup> Many of these are pilot discovery studies providing new mechanistic insight, such as a role for CD5L in protecting eosinophils from death by apoptosis,<sup>42</sup> but in the longer term offer a potential source of biomarkers.

For food allergy in particular, biomarkers for predicting good oral immunotherapy (OIT) outcomes would be highly beneficial, and to date, the focus has been on immunoglobulin and cytokine responses. It is well known that allergen-specific IgE and IgG<sub>4</sub> are modified by OIT, but IgG<sub>1</sub>, IgG<sub>3</sub>, and IgA levels also change, with poor responders having the smallest changes in these immunoglobulins.<sup>43</sup> High allergen-specific IgA levels at the

outset of OIT or high allergen-specific IgG<sub>1</sub> levels after the rush phase might be useful biomarkers to predict positive immunologic and clinical response to OIT.<sup>43</sup> Individualizing egg OIT based on an IgE-dependent dosing strategy and increasing maintenance dose individually based on the egg white IgE level have been reported to improve clinical egg tolerance.<sup>44</sup>

Predictive biomarkers for atopic dermatitis and food allergy are a highly desirable adjunct to allergic disease prevention strategies. There is a burgeoning literature focused on analysis of samples collected at birth or in early infancy, but much of this work is candidate driven, with omics discovery approaches only just emerging. Increased fecal calprotectin levels at 2 months of age have been associated with increased risk of atopic dermatitis and asthma/asthmatic bronchitis by 6 years of age.<sup>45</sup> This highlights the link between the microbiome, intestinal inflammation, and allergic disease and the potential utility of monitoring gut health for predictive biomarkers in multiple disease settings. Similarly, nutritional status could be monitored through biomarkers,<sup>46</sup> as could environmental exposures that effect disease manifestation.<sup>47</sup>

Candidate approaches have also been undertaken with umbilical cord blood. Circulating factors studied in this context include soluble Fas ligand (sFasL), levels of which were increased in umbilical cord blood of newborns with atopic dermatitis.<sup>48</sup> The biological relevance of sFasL might relate to keratinocyte apoptosis, and subjective severity of atopic dermatitis in infancy correlated positively with sFasL levels at birth.<sup>48</sup> Although neonatal sFasL levels were higher than maternal levels, a positive relationship between maternal and umbilical cord blood levels indicates the value of analyzing samples from mother and child for discovery of circulating biomarkers around the time of birth.

Levels of the circulating allergy-related chemokines CCL17 and CCL22 (macrophage-derived chemokine) at birth have also been associated with development of atopic dermatitis up to 3 years of age<sup>49</sup> and increased total IgE levels over the first 6 years of life,<sup>50</sup> respectively. Given the latter did not relate to specific IgE or clinical allergic disease, it is unlikely a suitable standalone marker but might serve well as part of panel.

Some other common observations that warrant further evaluation have emerged. The most notable of these relates to **regulatory T cells**, for which reduced numbers, function, and/or forkhead box P3 (FoxP3) expression at birth relate to atopic dermatitis, food allergy, or both in early childhood.<sup>51-53</sup> However, a consensus methodological approach is required if the evaluation of this or any other cell type is to have any value as a biomarker. Genetic biomarkers might also have predictive utility. As an example, a **filaggrin** loss-of-function mutation associated with skin barrier dysfunction was linked to food allergy in older children, especially after early evidence of food allergen sensitization and eczema.<sup>54</sup>

The prospect of omics approaches applied at or near birth is particularly promising. Vernix is usually abundant on the skin of the newborn and can be collected noninvasively. The protein composition of vernix reflects skin functional responses with various families of proteins found in the vernix, including cytoskeletal proteins, cell adhesion molecules, cell junction proteins, and transcription factors.<sup>55</sup> A liquid chromatography tandem MS analysis of vernix revealed a strong negative correlation between levels of polyubiquitin-C and calmodulin-like protein 5 and the development of atopic dermatitis at 2 years of age. These were suggested as candidate biomarkers for



identifying newborns predisposed to development of atopic dermatitis.<sup>55</sup>

Methodological and data analysis approaches pose the greatest immediate challenges to the clinical use of predictive biomarkers for atopic dermatitis and food allergy. However, the ultimate challenge is optimization of prevention strategies that can be implemented in children identified as being at risk through the use of biomarkers.

### Allergic rhinitis

There is little biomarker work within pediatric patients with allergic rhinitis. Recently, miR-146a expression in nasal mucosa and PBMCs has been identified as a promising biomarker for the pathogenesis and management of allergic rhinitis in children, including during sublingual immunotherapy.<sup>56</sup> miR-146a expression was decreased in PBMCs and nasal mucosa of children with allergic rhinitis and inversely correlated with disease severity. This miRNA is highly expressed in regulatory T cells,<sup>57</sup> and FoxP3 mRNA expression in PBMCs from children with atopic dermatitis was also significantly decreased. Both miR-146a and FoxP3, as well as serum *IL-10*, levels increased with sublingual immunotherapy.

### Asthma

Asthma, a chronic heterogeneous airway disease characterized by local inflammation and varying degrees of tissue remodeling, affects around 300 million persons of all ages, with increasing incidence.<sup>58</sup> It is the most common chronic disease of childhood. Along with other allergic disorders, asthma begins in conjunction with IgE sensitization to allergens, which most often occurs early in life. Recent years have seen much attention given to diagnosis and early intervention, with alteration of the natural history of the disease and elimination of acute asthma exacerbation as key goals.

Subphenotypes and associated pathogenetic mechanisms or endotypes of asthma are increasingly used to classify disease and better direct therapy. Single and combination biomarkers serve to identify these clusters, with the presence of eosinophilic T<sub>H</sub>2-type inflammation perhaps the most important feature. Distinctions based on this parameter guide inhaled corticosteroid treatment and biologic therapies.<sup>59-61</sup> Endobronchial tissue gene expression profiling revealed that asthmatic patients can be grouped as T<sub>H</sub>2-high, T<sub>H</sub>17-high, and T<sub>H</sub>2/T<sub>H</sub>17-low, and the clinical value of this should become apparent from follow-on studies.<sup>62</sup>

IgE and eosinophils have long-standing roles as biomarkers of allergic disease, and other single biomarkers that have emerged of value include nitric oxide and periostin. Serum levels of allergen-specific IgE guide identification of disease triggers and inform allergen avoidance strategies. Sputum eosinophil counts and ratios between peripheral blood eosinophils and neutrophils can cluster patients based on steroid responsiveness and are useful for identifying patients suitable for anti-*IL-4*, anti-*IL-5*, and anti-*IL-13* therapy.<sup>63-66</sup> Nitric oxide is produced by the bronchial epithelium through the action of inducible nitric oxide synthase in response to IL-4 and IL-13.<sup>67</sup> It can be measured as the fraction of exhaled nitric oxide, which is a biomarker for eosinophilic airway inflammation, highly corticosteroid sensitive, and relatively easily measured using point-of-care diagnostics.<sup>67-72</sup>

Periostin is an extracellular matrix protein secreted by airway epithelial cells and lung fibroblasts that is also induced by IL-4 and IL-13.<sup>73</sup> Its levels are increased in air-liquid interface-

cultured nasal and bronchial epithelial cells isolated from asthmatic children.<sup>74</sup> As a ligand for  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins, it supports adhesion and migration of epithelial cells and contributes to tissue remodeling.<sup>75</sup> As a biomarker of type 2 inflammation and remodeling in asthmatic patients, serum levels of periostin, especially in combination with fraction of exhaled nitric oxide and blood eosinophil numbers, might predict the efficacy of anti-IgE antibody treatment.<sup>76</sup> Serum periostin levels are also higher in asthmatic children<sup>77</sup> and correlate with the response to inhaled corticosteroids, at least in adults.<sup>78</sup>

Serum miRNA 21 has been suggested to be a novel biomarker for allergic inflammatory diseases because its levels were increased among children with asthma or eosinophilic esophagitis.<sup>79</sup> A profile of 10 miRNAs, including miR-638, has been associated with asthma in children aged 6 to 18 years and could distinguish those with severe asthma and more severe acute exacerbations.<sup>80</sup> More work is required to evaluate the disease-specific utility of these miRNAs.<sup>54</sup>

There is an ever-growing demand for noninvasive techniques and biomarkers associated with disease control, as well as prediction, identification, and management of acute asthma exacerbations. Nasal lavage, serum, saliva, exhaled breath condensate, induced sputum, and urine are some of the biological samples evaluated. Quite promising biomarker discovery approaches of exhaled breath include a range of MS approaches, including gas chromatography MS, selected ion flow tube MS, and ion mobility mass spectrometry, which allow detection of volatile organic compounds and the study of the metabolome.<sup>81,82</sup> Such techniques will also help reveal the underlying pathophysiology of acute asthma exacerbations.

There is little, if any, progress in biomarker discovery for predicting the development of asthma and other allergies. Early-life production of LPS-stimulated *TNF- $\alpha$* , but not IL-6, IL-10, or *IL-12*, by PBMCs from infants was associated with a higher risk for asthma.<sup>83</sup> Combined exhaled volatile organic compounds, PBMC inflammatory gene expression (TLR4, catalase, and *TNF- $\alpha$* ), and clinical information (Asthma Predictive Index) in preschool-aged children could predict asthma at 6 years of age.<sup>84</sup> Similarly, levels of inflammatory markers (IL-2, IL-4, IL-8, and IL-10) in exhaled breath condensate at 2 to 3 years of age were increased among children who were persistent wheezers up to 5 years of age compared with those who had never wheezed.<sup>85,86</sup> Further study is required to evaluate the efficacy of these as clinically useful biomarkers.

### Autoimmunity

Autoantibodies are longstanding diagnostic biomarkers of organ-specific and nonspecific autoimmune disorders and are part of diagnostic and classification criteria. With the ongoing discovery of novel autoantibodies, it has become evident that profiles of these biomarkers rather than single entities better distinguish disease phenotypes. The utility of autoantibody detection is not restricted to diagnosis but extends to prediction, prognosis, and prevention of autoimmunity. This plethora of autoantibodies and the numerous detection and analytic technologies now available dictate a need for standardization and harmonization of autoantibody nomenclature and testing. Requesting the appropriate test based on clinical features remains essential for optimal use of available biomarkers and requires close communication between the clinician and laboratory

specialist. Autoantibodies relevant to common autoimmune diseases in childhood are listed in Table 1.<sup>87-106</sup>

Metabolic profiling of children with type 1 and type 2 diabetes has attracted interest as a means of investigating disease pathogenesis and identifying predictive biomarkers.<sup>107</sup> These blood-based approaches, including from the umbilical cord, are all at the discovery stage. Progressing these to clinical implementation is an enormous challenge. For type 1 diabetes, a large prospective study of children recruited before 4.5 months of age is underway: the Environmental Determinants of Diabetes in the Young (TEDDY) study.<sup>108</sup> This study involves nearly 9000 high-risk children and will monitor development of islet autoimmunity and progression to type 1 diabetes to identify biomarkers of these. The metabolomic analysis of the samples collected is very carefully optimized.<sup>109</sup> This study highlights that very large prospective studies using multicenter and systems biology approaches are likely required to identify disease pathways that can be targeted for disease prevention.

Interest in the use of biomarkers for early diagnosis and therapeutic intervention in patients with rheumatoid arthritis has led to a number of studies investigating miRNA profiles in the joint and in peripheral blood. This has revealed shared profiles of overexpressed miRNAs, but it is the signature measurable in blood, including miR-24, miR-125a-5p, and miR-451, among others, that holds the most promise as biomarkers.<sup>110-115</sup> Recent work provided evidence for the use of 14-3-3 $\eta$  serum antibodies in patients with rheumatoid arthritis<sup>116,117</sup> and a diagnostic blood panel (including antinuclear antibodies, anti-Ro, anti-La, rheumatoid factor, and novel autoantibodies, such as anti-salivary gland protein 1, anti-carbonic anhydrase 6, and anti-parotid secretory protein) for Sjögren syndrome.<sup>118-120</sup> Levels of Dkk1, Wnt signaling pathway inhibitor 1 (DKK1), leptin, osteoprotegerin, osteopontin, and sclerostin are related to bone damage in patients with psoriatic arthritis.<sup>121</sup> Serum calprotectin has been proposed as a biomarker for anti-neutrophil cytoplasmic antibody-associated vasculitis and, more specifically, for indicating relapsers among patients treated with rituximab.<sup>122,123</sup>

Recently, activation of mechanistic target of rapamycin, a ubiquitous serine/threonine kinase with a role in numerous cell processes, including proliferation and survival, autophagy, and transcription, has been highlighted as a central pathway for the pathogenesis of systemic lupus erythematosus and other autoimmune diseases. Blockade of mechanistic target of rapamycin and follow-up of this biomarker holds promise in personalized treatment of patients with these diseases among others.<sup>124</sup> It is hoped that some of these biomarkers will improve diagnostic accuracy, but most remain at the discovery phase. These studies are almost exclusively in adults, and similar analyses should be pursued in pediatric populations.

## IBDs

IBDs are chronic inflammatory disorders of the gastrointestinal tract; chief among these are ulcerative colitis (UC) and Crohn disease (CD). IBDs arise from a complex interaction between genetics, the environment, the gut microbiota, and the mucosal immune response. Occurrence rates of CD and UC are generally reported to have increased for both adults and children.<sup>125</sup> The diagnosis of IBD entails a multistep strategy using clinical, endoscopic, and imaging modalities, but there has been a longstanding interest in the development of biomarkers to not only better facilitate diagnosis but also for monitoring treatment

efficacy, disease severity, and the risk of complications. Reliable surrogate markers to avoid repeated endoscopy and accurately report mucosal inflammation activity are particularly desirable. Circulating inflammatory markers, such as C-reactive protein (CRP), lack specificity for IBDs because they are also used in other clinical scenarios in which inflammation features, such as autoimmunity and infection. The neutrophil-derived fecal inflammatory markers calprotectin and lactoferrin are measured as indicators of the local inflammatory burden within the gastrointestinal tract and are already in clinical use. These biomarkers are useful for delineating IBDs from irritable bowel syndrome, management and monitoring of patients, and prediction of relapse, but their utility in monitoring mucosal healing requires refinement, and there is an agreed need for biomarker discovery strategies among gastroenterologists.<sup>126,127</sup>

Candidate biomarker approaches have been explored, for example, to show that measurement of a panel of antibodies to neutrophils, *Saccharomyces cerevisiae*, and *Escherichia coli* outer membrane porin correlates with disease activity and the location and outcome of disease in children/young adults aged 1 to 21 years. Measures were highly specific for CD, especially the subset of patients at increased risk of surgery.<sup>128</sup> Serum anti-glycoprotein 2 IgG and IgA might also be a novel marker of CD, especially in children.<sup>129</sup> More sophisticated “omics” technologies for biomarker discovery using blood, intestinal mucosa, and feces are also being pursued.<sup>130</sup> Alongside classical proteomic approaches, the use of imaging MS on histologic samples has been explored.<sup>131</sup> Imaging MS enables direct measurement of multiple individual species within a complex clinical sample through histology-directed mass spectral protein profiling.<sup>131</sup> Such an approach requires tissue samples obtained during endoscopy. Blood or fecal biomarkers remain the ideal, although the utility of exhaled breath condensate and exhaled nitric oxide for pediatric IBD has been considered.<sup>132</sup> Analysis of exhaled breath is a well-established tool for noninvasive assessment of lung disease, yet there were some features that allowed differentiation of samples from children with asthma versus those with IBDs; for example, pH was lower in patients with IBDs than in either asthmatic patients or control subjects.<sup>132</sup> A larger study that would incorporate omics approaches is planned by this group.

Recently, there has been a flurry of articles exploring miRNA signatures in patients with IBDs using blood, saliva, and colonic tissue to reveal differential expression profiles associated with UC versus CD. Importantly, these IBDs could be discriminated from other inflammatory disorders using machine learning techniques for expression profiling.<sup>133</sup> There is now much interest in evaluating these in large, independent, clinically well-characterized cohorts, and it would be good to see such studies extended to include pediatric populations.

## Other diseases

The role of the immune system and inflammation in metabolic and neurologic disorders is being increasingly recognized. For example, *Toll-like receptors* link inflammation and oxidative stress to hypertension, insulin resistance, and obesity and are postulated to have a role in fetal programming of these diseases.<sup>134</sup> Metabolic syndrome is a cluster of cardiometabolic features (ie, insulin resistance, central obesity, dyslipidemia, and hypertension) that impose increased risk for cardiovascular disease and type 2 diabetes mellitus. These features are increasing in prevalence secondary to the obesity epidemic. Lifestyle

**TABLE I.** Common autoimmune diseases of childhood and relevant autoantibodies

Autoimmune disease	Autoantibodies	Age	Comments	References
Type 1 diabetes	Anti-Insulin (IAA) Anti-glutamate decarboxylase (GAD) Anti-islet cell (ICA) Anti-insulinoma associated (IA-2) Anti-zinc transporter (ZnT8)	5-50 y 10-12 y 10.9 ± 3.9 y Adult	<ul style="list-style-type: none"> <li>IAAs are often the first specific detectable autoantibodies among children and are identified in ~50% of children with T1 diabetes.</li> <li>GADs usually appear &lt;5 y after appearance of anti-insulin autoantibodies.</li> <li>All autoantibodies can appear before onset of symptoms, and prevalence increases after disease manifestation.</li> </ul>	87-91
Celiac disease	Anti-gliadin Anti-endomysium (EmA)/tissue transglutaminase	9 mo to 12.3 y Adult 0.5-92 y		92-94
Autoimmune thyroiditis	Anti-thyroperoxidase Anti-thyroglobulin	1-19 y		95,96
Pediatric lupus	Antinuclear antibodies (ANA) Anti-double-stranded DNA Anti-Sm Anti-PCNA Anti-ribosomal P protein (RPP) Anti-nucleosome Anti-histone Anti-phospholipid Anti-SS-A (Ro) Anti-SS-B (La) Anti-U1-nRNP Anti-C1q	11.0 ± 3.6 y 4-18 y	<ul style="list-style-type: none"> <li>High levels of anti-RPP point to children with different autoimmune diseases or mixed connective tissue disease.</li> </ul>	97-99
Juvenile idiopathic arthritis	Rheumatoid factor (RF) ANA Anti-HMG Anti-CCP/CCA	0.5-11.4 y 15.59 ± 4.13 y	<ul style="list-style-type: none"> <li>RF is identified in ~15% of cases.</li> <li>Anti-cardiolipin is found in ~30% of cases in low titers and without association to clinical antiphospholipid syndrome.</li> <li>ANAs are identified in ~30% of cases.</li> <li>Anti-CCP/CCA levels are rarely positive.</li> </ul>	100-102
Juvenile dermatomyositis (JDM)	Antibodies against: P155 Mi-2α Mi-2β TIF1γ MDA5 NXP2 SAE1 Ku PM100 PM75 Jo-1 SRP PL-7 PL-12 EJ OJ Ro-52	<18 y 1-80 y	<ul style="list-style-type: none"> <li>Approximately 10% of children with JDM show myositis-specific autoantibodies.</li> <li>Children with anti-Jo-1 and/or anti-SRP tend to follow a chronic continuous disease course.</li> <li>Anti-SRPs are identified in children polymyositis with severe muscle weakness and thinning or atrophy and often are not responsive to single medication.</li> <li>Children with anti-Mi-2 typically have mild-to-moderate disease and respond well to treatment.</li> <li>Anti-p155 is the most common autoantibody found in children with JDM.</li> </ul>	103-105
Multiple sclerosis	Anti-myelin oligodendrocyte glycoprotein (MOG)	1.3-15.8 y	<ul style="list-style-type: none"> <li>Anti-aquaporin is used for exclusion of optical neuromyelitis.</li> </ul>	106

interventions implemented in early childhood are of interest to prevent the increase in cardiovascular disease and type 2 diabetes mellitus. The early detection of children at risk of the metabolic and cardiac derangements caused by obesity is critical, and readily and objectively quantifiable biomarkers would be ideal. Candidate biomarkers that have shown promise in the past include CRP, but it lacks the necessary precision. Various *adipokines*, especially leptin and adiponectin, have also been investigated as

biomarkers. Circulating adiponectin, but not leptin, tracked with health improvement and improved insulin sensitivity and loss of fat mass after lifestyle intervention in overweight and obese children, and therefore adiponectin was postulated as a good biomarker for monitoring the efficacy of such lifestyle interventions.<sup>135</sup>

Much of the biomarker work in children relates to obesity and cardiovascular risk, especially seeking markers of endothelial

**TABLE II.** Summary of immune biomarkers in childhood NCDs

Disease	Biomarker	Sample type	Age	Analysis approach	Proposed use	Reference
Atopic dermatitis	sICAM-1	Serum	10-16 y	Immunoassay	Disease monitoring	29
Food allergy	LL-37	Serum	6-15 y	Immunoassay	Disease monitoring	30
	IL-31	Serum	1-10 y	Immunoassay	Disease monitoring	31
	IL-18	Serum	0-6 y	Immunoassay	Disease monitoring	32
	CCL28	Serum	0-10 y	Immunoassay	Disease monitoring	33
	CCL17	Serum	0->16 y	Immunoassay	Disease monitoring	34
		Serum	Birth	Immunoassay	Risk monitoring/disease prediction	49
	CCL27	Serum	1-11 y	Immunoassay	Disease monitoring	35
	miR-203	Serum/urine	0.5-6 y	Global miRNA profiling and qRT-PCR	Disease monitoring and progression	37
	miR-483-5p	Serum	0-3 y	MS	Mechanistic insight/ biomarker discovery	40
	Carnitine					
	Free fatty acids					
	Lactic acid*	Plasma	2-7 y	Two-dimensional electrophoresis	Mechanistic insight/ biomarker discovery	42
	CD5L					
	Apolipoprotein E	Serum	5-12 y	Microarray	Response to OIT	43
	Allergen-specific IgA and IgG					
	Egg white IgE	Serum	1-16 y	ImmunoCAP	Response to OIT	44
	Calprotectin	Feces	2 mo	Immunoassay	Risk monitoring/disease prediction	45
	sFasL	Plasma	Birth	Immunoassay	Risk monitoring/disease prediction	48
	CCL22	Plasma	Birth	Multiplex immunoarray	Risk monitoring/disease prediction	50
	CD4 <sup>+</sup> regulatory T cells	Whole blood/PBMCs	Birth	Flow cytometry, qRT-PCR, DNA methylation	Risk monitoring/disease prediction	51-53
	Polyubiquitin C	Vernix	Birth	MS	Risk monitoring/disease prediction	55
	Calmodulin-like protein 5	Blood/saliva		DNA genotyping	Risk monitoring/disease prediction	54
	Filaggrin					
Allergic rhinitis	miR-146a	Nasal mucosa and PBMCs	4-14 y	qRT-PCR	Pathogenesis and management	56
	FoxP3	PBMCs				
Asthma	IL-10	Serum		Immunoassay		
	FENO/eosinophils	Exhaled air/blood	6-19 y	NIOX analyzer/ hematology analyzer	Disease phenotype/ treatment response	76
	FENO/eosinophils/ periostin	Exhaled air/blood	12-75 y	NIOX analyzer/ hematology analyzer/ immunoassay	Treatment efficacy	69
	Periostin	Airway biopsy specimens	14-85 y	Immunohistochemistry	Mechanistic insight/ biomarker discovery	73
		Epithelial cells	6-16 y	Cell culture/qRT-CR	Mechanistic insight/ biomarker discovery	74
		Serum	6-15 y	Immunoassay	Mechanistic insight/ biomarker discovery	77
	miR-21	Serum		qRT-PCR	Disease monitoring	79
	miR-638 and others	Blood	6-18 y	Global miRNA profiling	Mechanistic insight/ biomarker discovery	80
	TNF-α	LPS-stimulated PBMCs	Birth/3 mo	Cell culture/immunoassay	Risk monitoring	83
	Volatile organic compounds	EBC	2-4 y	MS	Risk monitoring	84
	TLR4, catalase, TNF-α	PBMCs		qRT-PCR		
	Clinical			Asthma Predictive Index		
	IL-2	EBC	2-3 y	Multiplex immunoarray	Risk monitoring	85
	IL-4					
	IL-8					
	IL-10					

(Continued)



TABLE II. (Continued)

Disease	Biomarker	Sample type	Age	Analysis approach	Proposed use	Reference
IBD	ANCA	Serum	1-21 y	Immunoassay	Increased risk of surgery	128
	ASCA					
Other diseases	Anti-OmpC Anti-glycoprotein 2	Serum	2-18 y	Immunoassay	Diagnosis	129
	Adiponectin	Blood	5-15 y	Radioimmunoassay	Lifestyle intervention for obesity	135
	EPCs	Blood	12-18 y	Flow cytometry	Monitoring endothelial dysfunction	138
	EMPs					
	miR-125-5p	Plasma	5-10 y	Global miRNA profiling/ qRT-PCR	Monitoring endothelial dysfunction	139
	miR-342-3p					
	miR-365-3p					
	CRP	Saliva	11 y	Multiplex immunoarray	Intervention and prevention Type 2 diabetes	142
	Insulin					
	Adiponectin					
	Bradykinin	Serum	6-17 y	MS	Mechanistic insight/ biomarker discovery	143
	Naringenin					
	L-thyronine*				Obesity	
	Citrate	Plasma	4-7 y	MS	Mechanistic insight/ biomarker discovery	144
	Succinate					
	Creatinine					
	Glutaric acid				ASD	
	3-Aminoisobutyric acid					
	p-Hydroxyphenyllactate*					

ANCA, Anti-neutrophil cytoplasmic antibody; ASCA, anti-*Saccharomyces cerevisiae* antibody; CCL, chemokine (C-C motif) like; CD5L, CD5 antigen-like; EBC, exhaled breath condensate; FENO, fraction of exhaled nitric oxide; OmpC, outer membrane protein C; qRT-PCR, Quantitative real-time PCR; sICAM-1, soluble intercellular adhesion molecule 1; TLR4, Toll-like receptor 4.

\*And others.

dysfunction, an early but reversible step in the development of atherosclerosis.<sup>136</sup> Interest in endothelial progenitor cells (EPCs) and endothelial microparticles (EMPs) is driven by their correlation with cardiovascular risk functions and parameters of endothelial dysfunction.<sup>137</sup> These are postulated to reflect the balance between damage (EMPs) and regeneration (EPCs) and can be measured in blood using *flow cytometry*. Because EPCs and EPMs independently predict microvascular endothelial dysfunction, they could provide a useful measure of the effectiveness of intervention programs in obese children. Fewer EPCs (reduced repair function) and more EPMs (greater damage) have been reported in obese children aged about 15 years who had impaired endothelial microvascular function, increased systolic blood pressure, and increased arterial stiffness.<sup>138</sup> As with many other biomarker strategies, one of the main challenges to progressing EPCs and EMPs to clinical use relates to standardization of sample-processing methods and choice of antigens for flow cytometric identification. Whether monitoring changes in EPCs and EMPs would be of use in younger children who physiologically adapt to increased blood flow demands remains to be seen. However, miR-125a-5p, miR-342-3p, and miR-365-3p are potential plasma biomarkers for endothelial dysfunction in obese children aged 5 to 10 years.<sup>139</sup>

Flow cytometry of circulating leukocytes, cytokine analysis in serum, and cytokines and miRNAs from PBMCs of obese versus nonobese children revealed altered immune cell frequency (decreased invariant natural killer T-cell counts), an inflammatory environment (increased LPS-stimulated IL-1 $\beta$  levels from PBMCs),

and regulation of metabolic gene expression (increased TNF- $\alpha$  and leptin levels and decreased adiponectin levels) in the obese group.<sup>140</sup> Much of this profile has been linked causally to the onset of metabolic disease in adults and therefore offers some insight to disease trajectory in children. Dysregulated miR-33a and miR-33b were deemed of particular interest as future biomarkers.<sup>140</sup> miRNAs are also of interest for their role in developmental, physiologic, and cognitive processes within the central nervous system.<sup>141</sup>

Although plasma is the preferred biological fluid for biomarker discovery in adults, there is probably greater value in exploring even less invasive samples for children. Saliva is of particular interest given the ease of collection. In a study of candidate biomarkers typical of obesity (CRP, insulin, leptin, and adiponectin) in the saliva of 10-year-old children, the biomarker profile was reminiscent of changes well documented in the circulation and was found to relate to insulin resistance and a systemic increase in proinflammatory cytokine levels.<sup>142</sup> Altered levels of salivary biomarkers, as measured by using a multiplex immunoassay approach, were determined in obese children to identify those at high risk of type 2 diabetes mellitus. Sample collection was highly standardized, including fasting, time of collection, rinsing of the mouth, amount expectorated, temperature and processing controls, centrifugation to remove cells and cellular debris, careful curation of samples, and total protein assays. There is interest in developing this approach for longitudinal studies related to development of the metabolism and other disorders in children and to monitor the success of interventions.

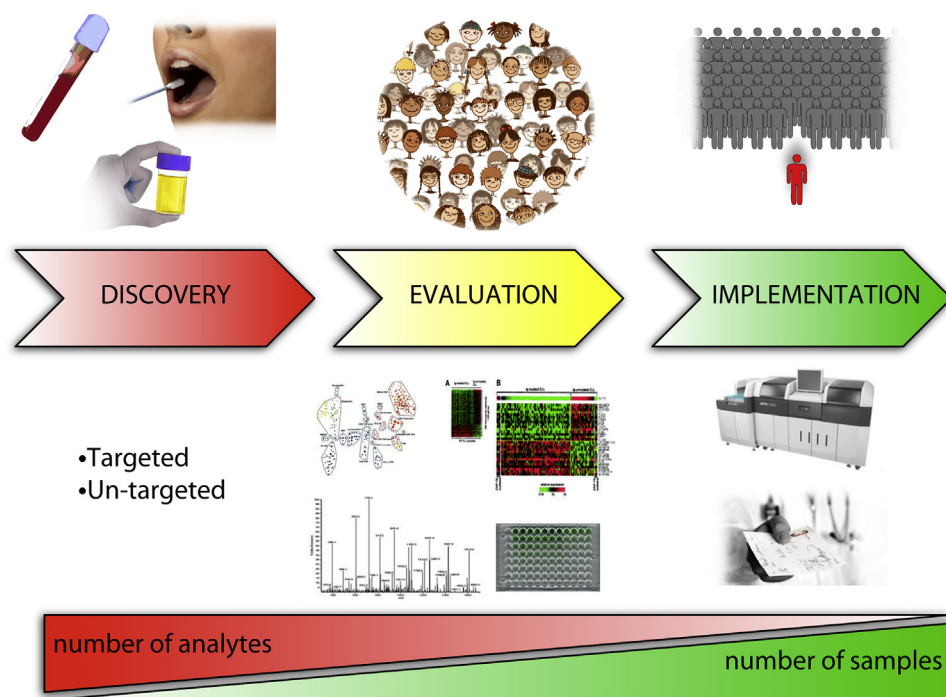


FIG 1. Pipeline from biomarker discovery to clinical implementation.

There are now studies emerging on metabolites as biomarkers in childhood. A biomarker discovery approach using untargeted global metabolite profiling with ultraperformance liquid chromatography/quadrupole orthogonal acceleration time-of-flight tandem micro-MS of serum from fasted 6- to 17-year-old children of varying body mass indexes identified 14 metabolites (eg, bradykinin, linoleic acid, L-thyronine, and naringenin) differentially expressed by body mass index.<sup>143</sup> These will be of use in future studies of the metabolic pathways altered with obesity that affect the risk of metabolic and other disorders to reveal novel therapeutic approaches and monitor interventions.

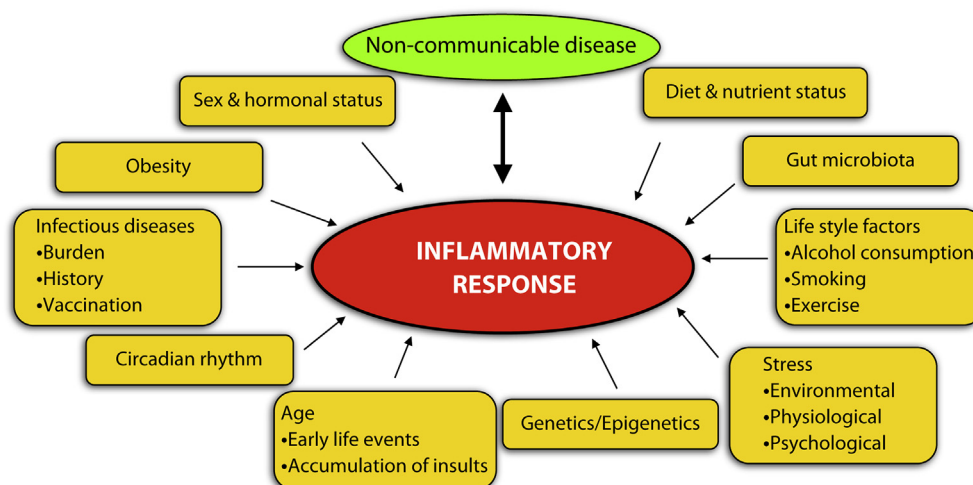
Similarly, a metabolomic approach has been used in autism spectrum disorder (ASD), with the long-term goal being to validate any discovered biomarkers in larger clinical studies and implement them for precision medicine. Blood plasma from 4- to 6-year-old children with ASD and age-matched control subjects was analyzed with a variety of MS methods, and multiple orthogonal analytic methods were used.<sup>144</sup> Some of the metabolites of interest had been associated previously with ASD (eg, creatinine<sup>145</sup>), and the study also revealed a variety of biomarkers associated with mitochondrial dysfunction (decreased citrate and increased succinate levels), which could relate to energy production, oxidative stress, or both. Combined with the increasing interest in immunometabolism,<sup>146</sup> there are likely to be data from global screens of energy pathways in relation to clinical phenotypes in the coming years. All of the biomarkers discussed in this section are summarized in Table II.

## Cancer

The focus of this review is immune-mediated NCDs of childhood. However, the use of biomarkers is most advanced for cancer, and therefore it is worthwhile considering the current

status in this field. Heterogeneity within cancer types has driven much of the need for precision medicine within this clinical specialty. The approaches used for biomarker discovery and validation are similar to those already discussed, ranging from traditionally identified single biomarkers to omics approaches. Breast cancer is an excellent example in which well-established single biomarkers (ie, estrogen receptor, progesterone receptor, and human epidermal growth factor 2) are used routinely for prognosis and to target therapy.<sup>147</sup> Efforts are now focused on further refining disease classification, especially molecular definitions to facilitate the use of rational therapies targeting specific molecular pathways.<sup>147</sup> Molecular profiling is already in use for breast cancer and other cancer types, such as metastatic colorectal cancer.<sup>148</sup>

Resurgent interest in cancer immunotherapies is accompanied by the need for complementary biomarkers. An example of this is the programmed cell death protein-1/programmed death-ligand 1 (PD-1/PD-L1) axis.<sup>149</sup> PD-L1 expression by cancer and immune cells is recognized to have a role in blocking anti-cancer immunity, and PD-L1–targeted therapies, such as an engineered humanized antibody, have been developed for treatment of melanoma, lung, kidney, bladder, and other cancers.<sup>150,151</sup> Emerging data indicate that this therapy is most effective in patients with tumors that highly express PD-L1,<sup>150</sup> which can be assessed immunohistochemically within the tumor biopsy specimen.<sup>152</sup> However, patients with PD-L1–low tumors also respond well to anti-PD-L1 therapy.<sup>149,151,152</sup> Despite early promise, the implementation of PD-L1 as a predictive biomarker remains problematic, but it does serve as a model for the general approach now being taken within the cancer field: parallel development of therapy and biomarker. Optimizing precision medicine approaches remains under debate but is likely an iterative process: because more subjects have their disease managed this way, the best timings and target populations will become apparent.



**FIG 2.** Factors that potentially modify measurable factors within human biological samples: many of these factors also modify the risk and phenotype of NCDs.

As for the other diseases discussed here, progress in implementation of diagnostic and prognostic biomarkers in pediatric cancer populations lags behind that in adults.<sup>153</sup> There are numerous studies identifying candidate biomarkers in small sample sets, but these require validation. Examples include surface-enhanced laser desorption/ionization time-of-flight MS to reveal platelet factor 4, connective tissue activating peptide III, and 2 fragments of complement C3a as potential circulating diagnostic biomarkers for acute lymphoblastic leukemia<sup>154</sup> and a variety of candidate biomarkers, such as osteopontin and metallothionein, reported by different groups for diagnosing various pediatric central nervous system tumors using either blood or cerebrospinal fluid.<sup>155</sup> One particular lesson to be learned from oncology is the effort made to offset the disappointing follow-on studies of promising new biomarkers typically related to the challenges of biomarker validation and implementation discussed herein.<sup>156</sup>

## BENEFITS AND CHALLENGES OF BIOMARKERS

Biomarkers must consistently and accurately predict a biological process or clinical outcome of interest. Most biomarkers in clinical use today have come about through the traditional experimental route outlined above. Today, these and untargeted (typically omics) approaches are used for biomarker discovery. It is very easy to speculate about the longer-term clinical utility of a novel potential biomarker, but the reality is a lengthy, painful, and expensive path from an exploratory to a qualified biomarker. Translation of a biomarker is a very slow process: discovery, validation/evaluation, clinical trial, and approval (Fig 1) can take more than a decade at vast cost. Biomarker discovery typically relies on targeted or untargeted quantification of multiple analytes in complex samples in a case-control fashion to provide a list of candidate biomarkers. The next stage requires validation of these candidate biomarkers in large and independent cohorts of patients to reveal clinical utility. This requires cost-effective and specific assays that might have to be developed from scratch; immunoassays are the standard because they readily lend themselves to translation, but immunoblotting and MS also provide valuable validation approaches. In particular, MS enables high-throughput

approaches that can overcome the bottleneck between discovery and verification.<sup>157</sup> Finally, clinical implementation requires development of an assay for use in clinical laboratories; health economics assessment, including identifying a willing manufacturer (ie, costs vs benefits); and approval by appropriate regulatory agents (eg, the US Food and Drug Administration).<sup>158</sup> Bringing together relevant partners can overcome the cost and intellectual property hurdles to biomarker qualification. An example is the Predictive Safety Testing Consortium, which relates to drug safety biomarkers and is a consortium between different pharmaceutical companies, academic institutions, and other partners under advisement of the US Food and Drug Administration, its European counterpart (the EMA), and the Japanese Pharmaceutical and Medical Devices Agency.<sup>159</sup>

The challenges facing the discovery, evaluation/validation, and clinical implementation of biomarkers are many and typically relate to sample choice and preparation, the instrumentation used to discover and validate biomarkers, and the downstream data and statistical analysis tools.

## Sample

A simple-to-collect sample, such as plasma, which is routinely used for a range of clinical diagnostics in hospitals and laboratories around the world, is an attractive option. However, global interrogation of the human plasma proteome is hindered by the most abundant plasma proteins, which constitute some 99% of the plasma proteome.<sup>20</sup> Successful biomarker discovery in plasma typically requires extensive sample fractionation and sophisticated analysis instrumentation, such as MS. One of the greatest challenges for clinical utility of biomarker analysis is sample heterogeneity caused by natural physiologic variation in health and disease (eg, sex, age, time of day, and lifestyle factors; Fig 2) and nonphysiologic factors (eg, specimen handling). All of these elements must be standardized. Biological variation also limits the usefulness of pooling individual biological specimens for biomarker discovery.<sup>160</sup>

## Equipment and data analysis

MS is the cornerstone of proteomics and metabolomics, providing high sensitivity, specificity, mass accuracy, and good

dynamic range, all while being relatively fast. High-throughput technologies enable rapid identification of candidate biomarkers, but many will require assay development *de novo* for the validation and implementation phases, although MS is already in clinical use. Advances in proteomics and metabolomics have allowed many investigators to rapidly and precisely measure the size and relative abundance of vast numbers of proteins or metabolites in complex mixtures, such as plasma, serum, saliva, and urine, with the goal of biomarker discovery. Translating these to clinical utility can prove difficult. Sophisticated computational approaches are required to distill all the information available into development of suitable assays and then an accurate classifier score validated in complex clinical cohorts.<sup>158</sup> Poor choice of approach at this stage can lead to failure through either selection of the wrong biomarkers at the discovery stage or disappointment at the validation stage. This has led to the development of computational pipelines that support discovery, validation, and implementation, including machine learning frameworks being developed to support clinical deployment.<sup>161</sup> Such pipelines have been developed in a number of areas, including cardiac transplantation, in which 5 biomarker candidates with biological relevant functions were identified and progressed to external validation.<sup>158</sup>

Multiparameter flow cytometry, either fluorescence based or, more recently, through rare element-labeled antibodies and *mass cytometry*, lends itself to biomarker discovery. However, this technology requires the use of antibodies or other labeling modalities against targets and therefore retains an element of targeted discovery. However, machine learning strategies can remove the investigator-biased constraints for identifying new cell subsets and other differentiating features. High dimensional mass cytometry enables measurement of some 40 features of an individual cell and has driven the need for single cell- and population-based computational strategies incorporating machine learning approaches to reveal multiparametric relationships.<sup>162</sup> There are an increasing number of computation tools to enable flow cytometry bioinformatics and support a range of activities related to data storage, retrieval, organization, and analysis for diagnosis and discovery.<sup>163</sup> At the validation stage, one of the greatest challenges is standardizing sample handling, reagents, instrument setup, and data analysis, but there are efforts toward standardization on many fronts, including the Human Immunology Project.<sup>164</sup>

miRNAs can be measured easily by using real-time quantitative PCR, which provides high-precision signal amplification and by using microarrays and next-generation sequencing platforms for high throughput to enhance the flow from discovery to validation to implementation. As with other technologies discussed herein, one of the major challenges is standardization of procedures at sample collection, storage, RNA isolation, miRNA quality, and quantity evaluation and preamplification, if required. There are then challenges at the analysis stage because all methods introduce some level of bias, and therefore normalization strategies are applied to raw expression data, and these are critical to meaningful data processing.<sup>165</sup> As for MS and flow cytometry, the development of sophisticated usable data analysis pipelines is an ongoing challenge.

## SUMMARY

The utility of biomarkers is 2-fold: to identify mechanistic pathways enabling better understanding of disease processes and

revealing novel therapeutic targets and to generate diagnostic or prognostic biomarkers that have clinical effect. The goal for the latter is an objective, accurate, and reproducible measurement that relates to the clinical scenario of interest. Such a biomarker must be accurate, sensitive, and specific; it helps if it is physiologically relevant to the disease or biological/pathologic state of interest; detection methods should be rapid, reliable, reproducible, and easily interpreted; and ideally, it should be measurable in a readily accessible biological fluid, such as plasma, urine, or saliva. The challenge is to translate a biomarker from the research environment to routine clinical use through the phases of discovery, validation/evaluation, and implementation, which can be an iterative process. Biomarker discovery for pediatric diseases lags behind that for adult diseases, but the epidemic of NCDs should catalyze such activity to provide much needed evidence-based approaches for risk stratification through the life course.

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