**Immune biomarkers in the spectrum of childhood non-communicable diseases**

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**Abstract**

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 non-communicable diseases (NCDs). This review will consider the utility of biomarkers in 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 where biomarkers are in routine clinical use and industry wide standardized approaches have been developed. Biomarker discovery and validation in childhood disease lags behind that for adults; given the early onset and therefore potential lifelong impact 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 optimisation of prevention strategies that can be implemented in children identified as being at risk of an NCD through the use of biomarkers.

Keywords: biomarkers; non-communicable diseases; children; inflammation; microRNA; blood; urine; breath; mass spectrometry

Abbreviations:

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| ANCA | anti-neutrophil cytoplasmic antibodies |
| Anti-OmpC | antibody to outer membrane porin of *E.coli* |
| ASCA | Anti-*Saccharomyces cerevisiae* antibodies |
| ASD | autism spectrum disorder |
| BMI | body mass index |
| CD | Crohn's disease |
| CRP | C reactive protein |
| DHEAS | dehydroepiandrosterone sulfate |
| EBC | exhaled breath condensate |
| EMA | European Medicines Agency |
| EMP | endothelial microparticle |
| EPC | endothelial progenitor cell |
| FDA | Food and Drug Administration |
| FeNO | fractional exhaled nitric oxide |
| IBD | inflammatory bowel disease |
| ICS | inhaled corticosteroid |
| miRNA | microRNA |
| MS | mass spectrometry |
| mTOR | mechanistic target of rapamycin |
| NCD | non-communicable disease |
| PBMC | peripheral blood mononuclear cells |
| PMDA | Japanese Pharmaceutical and Medical Devices Agency |
| qRT-PCR | quantitative real time polymerase chain reaction |
| UC | ulcerative colitis |

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**Introduction**

Non-communicable diseases (NCDs) are one of the major global challenges of the 21st century[1]. NCDs have been termed a "slow-motion disaster"[2] and a global crisis[3] as their prevalence increases in all countries, in all income groups, and at all ages. NCDs, often called chronic diseases, are generally considered as being one of four 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[4], but neurocognitive, inflammatory bowel and other diseases are also important NCDs. Worldwide, two out of every three deaths each year are attributable to NCDs with one third of those who die being under the age of 60 years[5]. In all regions of the world the prevalence of NCDs is rising due to an aging population and the globalization of common risk factors[5]. The main risk factors for NCDs, accounting for two-thirds of all cases, are: tobacco use; food high in saturated and trans fats, salt and sugar; physical inactivity; and alcohol consumption[6]. NCDs not only directly threaten health, they influence economic development to compound the impact on health. The World Economic Forum ranks NCDs as one of the top global threats to economic development: a 10% increase of mortality by NCDs reduces annual economic growth by 0.5%[7].

A shared feature of all NCDs is chronic low-grade inflammation promoted by modern diets, environmental pollutants, microbial exposure, and psychological and biological (e.g. oxidative or endoplasmic reticulum) stress[1, 8]. 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 NCDs is chronic; without effective treatment basal homeostasis cannot be restored and the damage may already be done[9]. Inflammatory pathways in NCDs are multi-factorial and part of a metabolic cascade, including cellular oxidative stress and insulin resistance, which induces allostatic overload, dysmetabolism, and ultimately chronic disease[10]. Changes in the gut microbiome have emerged as one of the pathways leading to chronic low-grade inflammation[11]. 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[12]. There is global interest in gut dysbiosis in multiple NCD settings including for example beta-cell autoimmune disease[13], atopic dermatitis[14], and inflammatory bowel disease[15]. 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 may be multiple different pathways for different organisms; these pathways may or may 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.

While NCDs are most prominent in adulthood, development in early life influences predisposition to NCDs. This starts as early as pregnancy, when maternal body composition and diet influences the infant's risk of NCDs later in life[4]. NCDs should therefore be studied using a life-course approach with overall risk depending on: the sequential effects of the developmental time-line with different metabolic trajectories, age-dependent decline in plasticity, and the subsequent differential responses to subsequent risk factors[30]. Monitoring of these time-lines and trajectories would not only benefit the understanding of disease processes but 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, identifying treatment of choice and monitoring response to treatment, in surveillance, and in 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 that come from the National Institutes of Health Biomarkers Definitions Working Group and the World Health Organization, among others, can be found elsewhere[31].

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 inflammatory bowel disease. 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 two broad ways: to better understand biological processes and for clinical biomarker discovery. While 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 endpoint[31]. The use of biomarkers in this way might be most useful during clinical trials and for lifestyle, such as nutritional, interventions[32]. This is critical for those who work in the disease prevention arena so that the success, or otherwise, of an intervention can be considered before a clinical endpoint is achieved as this may take many years.

Among the omics approaches for biomarker discovery, proteomics is currently the most widely used. The proteome reflects splice variants and post-translational modifications of proteins that dictate structure, function, localization, maturation and turnover of proteins, all of which change rapidly in response to environmental signals[33]. Sample types studied include serum, plasma, saliva, urine, exhaled breath, tissue biopsies, 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 post-translational modifications[34].

An emergent biomarker family measurable in the circulation is microRNA (miRNA). MicroRNAs are small endogenous non-coding RNAs with a critical post-transcriptional gene-regulatory role[35]. Aberrant miRNA expression is implicated in disease, and disease and/or tissue specific miRNAs have been identified and these are surprisingly stable and can be relatively easily measured. Most progress in the use of miRNAs as biomarkers is in cancer where their utility has been considered for monitoring disease progression, outcomes, recurrence and metastasis[36].

Within paediatrics, biomarker discovery remains dominated by targeted approaches. Here we will review what is known about paediatric 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, for example, with paediatric autoimmune diseases[37], 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 non-communicable diseases in childhood**

*Allergy*

Allergic disorders, including atopic dermatitis, food allergy, allergic rhinitis and asthma affect over one billion people across the globe and their prevalence is expected to quadruple by the 2050s[38]. Atopic dermatitis, typically accompanied by IgE sensitization to food, and food allergy are the earliest onset NCDs and the most common chronic NCDs of childhood worldwide[39, 40]. 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[41, 42]. 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 life long disease there is also incredible value in predictive biomarkers usable around birth to identify children at risk of developing these diseases. Failing that, identifying those children with atopic dermatitis and/or food allergy who will progress to asthma and/or rhinitis 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 ICAM-1[43], the anti-bacterial peptide LL-37[44], interleukins such as interleukin (IL)-31[45] and IL-18[46], and numerous chemokines such as mucosa-associated epithelial chemokine (MEC/CCL28)[47], thymus and activation-regulated chemokine (TARC/CCL17)[48], and cutaneous T cell attracting chemokine (CTACK/CCL27)[49]. However,

there are few evaluation studies to progress these molecules to clinical use. Candidates are usually analysed because of their role in disease pathogenesis so are common to multiple allergic and/or inflammatory disorders. While potentially suitable for disease monitoring[50], 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. CCL17 circulating levels correlate inversely with age so are much higher at 0 – 1 year than in older age groups[48] 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[51]. Notably, both co-morbidities and sample type analysed affects the findings. Serum levels of miR-483-5p were increased in children with atopic dermatitis and were associated with other atopic conditions such as asthma or hayfever. For miR-203, urine levels were decreased but serum levels were increased in the children with atopic dermatitis[51]. There is also interest in plasma miRNA biomarkers in eosinophilic esophagitis (EoE) with several differentially expressed in paediatric EoE patients[52]. Some of these miRNAs mapped with glucocorticoid treatment and normalisation of eosinophil histology and cell counts so could offer a less invasive approach to monitor treatment efficacy[52]. There is much interest in miRNAs for better understanding disease pathogenesis, for informing treatment decisions, and as biomarkers. Most studies to date have focussed 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[53]. Hopefully we will see progress to using these as biomarkers in multiple disease settings.

Studies using omics approaches for atopic dermatitis are only just being reported. Examples include: a mass spectrometry study of serum to reveal changes in energy metabolism measurable as increased carnitine, free fatty acids and lactic acid, amongst others, in atopic dermatitis[54]; and changes in the glycoproteome (glycosylation patterns are influenced by physiological status[55]) by two-dimensional electrophoresis showing increased CD5L and decreased Apolipoprotein E with atopic dermatitis[56]. Many of these are pilot discovery studies providing new mechanistic insight, such as a role for CD5L in protecting eosinophils from death by apoptosis[56], but in the longer term offer a potential source of biomarkers.

For food allergy in particular, biomarkers for predicting good outcomes to oral immunotherapy (OIT) 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 IgG4 are modified by OIT but IgG1, IgG3 and IgA also change, with poor responders showing the smallest changes in these immunoglobulins[57]. High allergen-specific IgA at outset of OIT or high allergen-specific IgG1 after rush phase might be useful biomarkers to predict positive immunological and clinical response to OIT[57]. Individualising egg OIT based on an IgE-dependent dosing strategy and increasing maintenance dose individually based on egg white IgE level has been reported to improve clinical egg tolerance[58].

Predictive biomarkers for atopic dermatitis and food allergy are a highly desirable adjunct to allergic disease prevention strategies. There is a burgeoning literature focussed 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. Raised fecal calprotectin at 2 months of age has been associated with increased risk of atopic dermatitis and asthma/asthmatic bronchitis by 6 years of age[59]. 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[60] as could environmental exposures that effect disease manifestation[61]. Candidate approaches have also been undertaken using umbilical cord blood. Circulating factors studied in this context include soluble Fas ligand (sFasL) which was elevated in umbilical cord blood of newborns who developed atopic dermatitis[62]. 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[62]. While neonatal levels of sFasL were higher than maternal levels, a positive relationship between maternal and umbilical cord blood levels indicates the value of analysing samples from mother and child for discovery of circulating biomarkers around the time of birth. Levels of the circulating allergy-related chemokines TARC/CCL17 and MDC/CCL22 at birth have also been associated with development of atopic dermatitis up to 3 years of age[63] and elevated total IgE over the first 6 years of life[64], 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 where reduced numbers,

function, and/or FoxP3 expression at birth relate to atopic dermatitis and/or food allergy in early childhood[65-67]. 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 following early evidence of food allergen sensitization and eczema[68].

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 non-invasively. The protein composition of vernix reflects skin functional responses with various families of proteins found in vernix including cytoskeletal proteins, cell adhesion molecules, cell junction proteins and transcription factors[69]. A liquid chromatography tandem mass spectrometry 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 dermaitis[69].

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 optimisation of prevention strategies that can be implemented in children identified as being at risk through use of biomarkers.

*Allergic rhinitis.* There is little biomarker work within pediatric allergic rhinitis. Recently, miR-146a expression in nasal mucosa and peripheral blood mononuclear cells (PBMCs) has been identified as a promising biomarker for pathogenesis and management of allergic rhinitis in children, including during sublingual immunotherapy (SLIT)[70]. miR-146a 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[71] and FoxP3 mRNA 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 SLIT.

*Asthma.* Asthma, a chronic heterogeneous airway disease characterized by local inflammation and varying degrees of tissue remodeling, affects around 300 million people of all ages with increasing incidence[72]. 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 exacerbations key goals.

Sub-phenotypes and associated pathogenetic mechanisms or endotypes of asthma are increasingly utilized to classify disease and better direct therapy. Single and combination biomarkers serve to identify these clusters with the presence of eosinophilic Th2 type inflammation perhaps the most important feature. Distinctions based on this parameter guide inhaled corticosteroid treatment (ICS) and biologic therapies[73-75]. Endobronchial tissue gene expression profiling revealed that asthmatic patients can be grouped as Th2-high, Th17-high and Th2/Th17-low and the clinical value of this should become apparent from follow on studies[76].

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 might 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[77-80]. Nitric oxide is produced by the bronchial epithelium through the action of inducible nitric oxide synthase in response to IL-4 and IL-13[81]. It can be measured as fractional exhaled nitric oxide (FeNO) which is a biomarker for eosinophilic airway inflammation, is highly corticosteroid sensitive, and is relatively easily measured using point-of-care diagnostics[81-86]. Periostin is an extracellular matrix protein secreted by airway epithelial cells and lung fibroblasts that is also induced by IL-4 and IL-13[87]. It is elevated in air-liquid interface cultured nasal and bronchial epithelial cells isolated from asthmatic children[88]. 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[89]. As a biomarker of type 2 inflammation and remodeling in asthma, serum levels of periostin, especially in combination with FeNO and blood eosinophil numbers, might predict the efficacy of anti-IgE antibody treatment[90]. Serum periostin levels are also higher in asthmatic children[91] and correlate with the response to inhaled corticosteroids, at least in adults[92].

Serum miRNA 21 has been suggested as a novel biomarker for allergic inflammatory diseases as it was elevated among children with asthma or eosinophilic esophagitis[93]. A profile of 10 miRNAs, including miR-638, has been associated with asthma in children aged 6 – 18 years and could distinguish those with severe asthma and with more severe acute exacerbations[94]. More work is required to evaluate the disease-specific utility of these miRNAs[68].

There is an ever-growing demand for non-invasive 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 mass spectrometry (MS) approaches including gas chromatography-MS (GC-MS), selected ion flow tube-MS (SIFT-MS), and ion mobility-mass spectrometry (IMS) which allow detection of volatile organic compounds and the study of the metabolome[95,96]. 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 tumor necrosis factor-α (TNFα) but not IL-6, IL-10 or IL-12 by PBMCs from infants was associated with a higher risk for developing asthma[97]. Combined exhaled volatile organic compounds, PBMC inflammatory gene expression (TLR4, catalase and TNFα), and clinical information (asthma predictive index) in preschool age children could predict asthma at 6 years of age[98]. Similarly, inflammatory markers (IL-2, IL-4, IL-8 and IL-10) in exhaled breath condensate at 2 – 3 years of age were elevated among children who were persistent wheezers up to 5 years of age compared to children who had never wheezed[99,100]. Further study is required to evaluate the efficacy of these as clinically useful biomarkers.

*Autoimmunity*

Autoantibodies are longstanding diagnostic biomarkers of organ-specific and non-specific 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 analysis 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 needs close communication between the clinician and the laboratory specialist. Autoantibodies relevant to common autoimmune diseases in childhood are listed in Table 1.

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[101]. 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[102]). This 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[103]. 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 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, amongst others, that holds the most promise as biomarkers[104-109]. Recent work provided evidence for the use of 14-3-3n serum antibodies in RA[110, 111], and a diagnostic blood panel (including ANA, anti-Ro, anti-La, rheumatoid factor, and novel SjS autoantibodies such as anti-salivary gland protein 1 (SP1), anti-carbonic anhydrase 6 (CA6) and anti-parotid secretory protein (PSP)) for Sjögren syndrome[112-114]. Levels of DKK1, leptin, osteoprotegerin, osteopontin, and scleostin are related to bone damage in psoriatic arthritis[115]. Serum calprotectin has been proposed as a biomarker for ANCA-associated vasculitis and more specifically for indicating relapsers among patients treated with rituximab[116, 117]. Recently, activation of mechanistic target of rapamycin (mTOR), 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 mTOR and follow-up of this biomarker holds promise in personalized treatment of patients suffering from these diseases among others[118]. 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.

*Inflammatory bowel diseases*

Inflammatory bowel diseases (IBDs) are chronic inflammatory disorders of the gastrointestinal tract; chief among these are ulcerative colitis (UC) and Crohn's disease (CD). IBDs arise from a complex interaction between genetics, 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[119]. The diagnosis of IBDs entails a multistep strategy using clinical, endoscopic and imaging modalities but there has been a longstanding interest in the development of biomarkers to better facilitate not only diagnosis but for monitoring treatment efficacy, disease severity, and the risk of complications. Reliable surrogate markers to avoid repeated endoscopy and to accurately report mucosal inflammation activity are particularly desirable. Circulating inflammatory markers such as CRP lack specificity for IBDs as they are also used in other clinical scenarios in which inflammation features such as autoimmunity and infection. The neutrophil-derived faecal 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 IBD from irritable bowel syndrome, for the 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 amongst gastroenterologists[120, 121].

Candidate biomarker approaches have been explored, for example, to show that measurement of a panel of antibodies to neutrophils, *Saccharomyces cerevisiae*, and *E.* coli outer membrane porin correlates with disease activity and the location and outcome of disease in children/young adults aged 1 – 21 years. Measures were highly specific for CD, especially the subset of patients at increased risk of surgery[122]. Serum anti-glycoprotein 2 (GP2) IgG and IgA might also be a novel marker of CD especially in children[123]. More sophisticated 'omics' technologies for biomarker discovery using blood, intestinal mucosa,

and feces are also being pursued[124]. Alongside classical proteomic approaches the use of imaging mass spectrometry on histological samples has been explored[125]. Imaging mass spectrometry enables direct measurement of multiple individual species within a complex clinical sample through histology-directed mass spectral protein profiling[125]. 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[126]. Analysis of exhaled breath is a well-established tool for non-invasive assessment of lung disease, yet there were some features that allowed differentiation of samples from children with asthma versus IBD, e.g. pH was lower with IBD than either asthma or controls[126]. A larger study that would incorporate omics approaches is planned by this group.

Recently there has been a flurry of papers exploring miRNA signatures in IBD 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[127]. 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 neurological 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[128]. Metabolic syndrome is a cluster of cardiometabolic features – insulin resistance, central obesity, dyslipidemia, and hypertension - that impose increased risk for cardiovascular disease (CVD) and type 2 diabetes mellitus. These features are increasing in prevalence secondary to the obesity epidemic. Life style interventions implemented in early childhood are of interest to prevent the rise in CVD and type 2 diabetes mellitus. The early detection of children at risk of the metabolic and cardiac derangements secondary to 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, improved insulin sensitivity

and loss of fat mass, after lifestyle intervention in overweight and obese children, so adiponectin was postulated as a good biomarker for monitoring the efficacy of such lifestyle interventions[129].

Much of the biomarker work in children relates to obesity and cardiovascular risk, especially seeking markers of endothelial dysfunction, an early but reversible step in the development of atherosclerosis[130]. Interest in endothelial progenitor cells (EPCs) and EMPs is driven by their correlation with cardiovascular risk functions and parameters of endothelial dysfunction[131]. They are postulated to reflect the balance between damage (EMPs) and regeneration (EPCs) and can be measured in blood using flow cytometry. As EPCs and EPMs independently predict microvascular endothelial dysfunction they could provide a useful measure of the effectiveness of intervention programmes in obese children. Fewer EPCs (reduced repair function) and more EPMs (greater damage) have been reported in obese children aged about 15 years old who had impaired endothelial microvascular function, raised systolic blood pressure, and increased arterial stiffness[132]. 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, 342-3p and 365-3p are potential plasma biomarkers for endothelial dysfunction in obese children aged 5 – 10 years[133].

Flow cytometry of circulating leukocytes, cytokine analysis in serum, and cytokines and miRNA from peripheral blood mononuclear cells in obese versus non-obese children revealed altered immune cell frequency (decreased invariant NKT cells), inflammatory environment (increased LPS-stimulated IL-1$\beta$ from PBMCs) and regulation of metabolic gene expression (increased TNF$\alpha$ and leptin, and decreased adiponectin) in the obese group[134]. Much of this profile has been linked causally to the onset of metabolic disease in adults so offers some insight to disease trajectory in children. Dysregulated miR-33a and 33b were deemed of particular interest as future biomarkers[134]. MicroRNAs are also of interest for their role in developmental, physiological and cognitive processes within the central nervous system[135].

While 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 systemic elevation of pro-inflammatory cytokines[136]. Altered levels of salivary biomarkers, measured using a multiplex immunoassay approach, were identified 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 metabolism and other disorders in children and to monitor the success of interventions.

There are now studies emerging on metabolites as biomarkers in childhood. A biomarker discovery approach using untargeted global metabolite profiling with ultra-performance liquid chromatography/quadruple orthogonal acceleration time of flight tandem micro mass spectrometry of fasted serum of 6 – 17 year old children of varying BMI identified 14 metabolites (e.g. bradykinin, linoleic acid, L-thyronine and naringenin) differentially expressed by BMI[137]. These will be of use in future studies of the metabolic pathways altered with obesity that impact on 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 – 6 year old children with ASD and age-matched controls was analysed with a variety of mass spectrometry methods and multiple orthogonal analytical methods were used[138]. Some of the metabolites of interest had been associated previously with ASD (e.g. creatinine[139]) and the study also revealed a variety of biomarkers associated with mitochondrial dysfunction (decreased citrate and increased succinate) which could relate to energy production and/or oxidative stress. Combined with the increasing interest in immunometabolism[140] there are likely to be data from global screens of energy pathways in relation to clinical phenotypes in the coming years.

*Cancer*

The focus of this review is immune-mediated NCDs of childhood. However, the use of biomarkers is most advanced for cancer so 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 where well established single biomarkers – estrogen receptor (OR), progesterone receptor (PR) and human epidermal growth factor 2 (HER2) – are used routinely for prognosis and to target therapy[141]. Efforts are now focussed on further refining disease classification, especially molecular definitions to facilitate the use of rational therapies targeting specific molecular pathways[141]. Molecular profiling is already in use for breast cancer and for other cancer types such as metastatic colorectal cancer[142].

Resurgent interest in cancer immunotherapies is accompanied by the need for complementary biomarkers. An example of this is the PD-1/PD-L1 axis[143]. PD-L1 expression by cancer and immune cells is recognised to have a role in blocking anti-cancer immunity and PD-L1 targeted therapies such as an engineered humanised antibody have been developed for treatment of melanoma, lung, kidney, bladder and other cancers[144,145]. Emerging data indicate that this therapy is most effective in patients with tumours that highly express PD-L1[144] which can be assessed immunohistochemically within the tumour biopsy[146]. However, patients with PD-L1 low tumours also respond well to anti-PD-L1 therapy[143, 145, 146]. 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. Optimising precision medicine approaches remains under debate but is likely an iterative process: as more individuals have their disease managed this way the best timings and target populations will become apparent.

As for the other diseases discussed here, progress in implementation of diagnostic and prognostic biomarkers in paediatric cancer populations lags behind that in adults[147]. 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 mass spectroscopy (SELDI-ToF-MS) to reveal platelet factor 4, connective tissue

activating peptide III and two fragments of complement C3a as potential circulating diagnostic biomarkers for acute lymphoblastic leukemia[148]; and a variety of candidate biomarkers such as osteopontin and metallothionen reported by different groups for diagnosing various pediatric central nervous system tumours using either blood or cerebrospinal fluid[149]. 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[150].

**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 exploratory to a qualified biomarker. Translation of a biomarker is a very slow process – discovery, validation/evaluation, clinical trial and approval (Figure 1) can take over 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 which might have to be developed from scratch; immunoassays are the standard as they readily lend themselves to translation but immunoblotting and mass spectrometry also provide valuable validation approaches. Mass spectrometry, in particular, enables high throughput approaches that can overcome the bottleneck between discovery and verification[151]. Finally, clinical implementation requires development of an assay for use in clinical laboratories, health economics assessment including identifying a willing manufacturer (i.e. costs versus benefits), and approval by appropriate regulatory agents (e.g. FDA)[152]. 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 FDA (U.S. Food and Drug Administration), its European

counterpart the EMA (European Medicines Agency), and PMDA (Japanese Pharmaceutical and Medical Devices Agency)[153].

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, 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 that constitute some 99% of the plasma proteome[34]. Successful biomarker discovery in plasma typically requires extensive sample fractionation and sophisticated analysis instrumentation such as mass spectrometry. One of the greatest challenges for clinical utility of biomarker analysis is sample heterogeneity due to natural physiological variation in health and disease (e.g. sex, age, time of day, lifestyle factors (Figure 2) and non-physiological factors (e.g. specimen handling). All of these elements must be standardized. Biological variation also limits the usefulness of pooling individual biological specimens for biomarker discovery[154].

*Equipment and data analysis.* Mass spectrometry 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 validation and implementation phases although mass spectrometry 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 to be difficult. Sophisticated computational approaches are required to distil all the information available into development of suitable assays and then an accurate classifier score validated in complex clinical cohorts[152]. 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[155]. Such pipelines have been developed in a number of areas including for cardiac transplantation where 5 biomarker candidates with biological relevant functions were identified and progressed to external validation[152].

Multi-parameter 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 to targets so 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 multi-parametric relationships[156]. There are an increasing number of computation tools to enable flow cytometry bioinformatics and support a range of activities related to data storage, retrieval, organisation and analysis for diagnosis and discovery[157]. At the validation stage one of the greatest challenges is standardizing sample handling, reagents, instrument set up, and data analysis but there are efforts towards standardization on many fronts including the Human Immunology Project[158].

MicroRNAs can be measured easily using real time quantitative PCR which provides high precision signal amplification, and by 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 pre-amplification if required. There are then challenges at the analysis stage as all methods introduce some level of bias so normalization strategies are applied to raw expression data and these are critical to meaningful data processing[159]. As for mass spectrometry and flow cytometry the development of sophisticated usable data analysis pipelines is an ongoing challenge.

**Summary**

The utility of biomarkers is two-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 impact. 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/pathological 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 paediatric diseases lags behind that for adult diseases but the epidemic of NCDs should catalyse such activity to provide the much needed evidence-based approaches for risk stratification through the life-course.

What do we know?

- The prevalence of NCDs is increasing in all countries, in all income groups, and at all ages due to the globalization of common risk factors (tobacco use; food high in saturated and trans fats, salt and sugar; physical inactivity; and alcohol consumption).
- A shared feature of all NCDs is chronic low-grade inflammation promoted by modern diets, environmental pollutants, microbial exposure, and stress; inflammatory pathways in NCDs are multi-factorial and part of a metabolic cascade.
- A biomarker can be a gene, molecule, or other biological characteristic as long as it can be measured accurately and reproducibly to provide an objective measure of health status or disease with potential for use in risk stratification, early detection, identifying treatment of choice and monitoring response to treatment, in surveillance, and in drug monitoring and development.
- Many biomarkers already in clinical use were first identified during targeted studies with the 'omics' era now leading to unbiased discovery-based approaches to identify biomarkers (including panels, clusters or signatures) to better understand biological processes and for clinical biomarker discovery.
- Translation of a biomarker is a very slow process – discovery, validation/evaluation, clinical trial and approval can take over a decade at vast cost.

What is still unknown?

- Sample types for biomarker discovery, validation and clinical implementation include serum, plasma, saliva, urine, exhaled breath, tissue biopsies, mucosal secretions, cerebrospinal fluid, and other biological specimens; natural physiological variation and non-physiological factors contribute to sample heterogeneity and sample collection, processing and storage must be optimised.
- One approach does not fit all: mass spectrometry, flow cytometry, microarrays and next generation sequencing offer high throughput approaches to biomarker discovery and validation - sample analysis tools need optimising for the clinical scenario of interest.
- Analysis of complex data sets is challenging and poor choice of analytical approach can lead to failure; computational pipelines that support discovery, validation, and implementation including machine learning frameworks are required to support clinical deployment.
- Biomarker discovery and validation in childhood disease lags behind that for adults; given the early onset and therefore potential lifelong impact of many NCDs there should be more studies incorporating cohorts of children.

**Table 1.** Common autoimmune diseases of childhood and relevant autoantibodies.

| Autoimmune Disease | Autoantibodies (Abs) | Age | Comments | References |
|---|---|---|---|---|
| Type 1 Diabetes | Anti-Insulin (IAA)<br>Anti-Glutamate decarboxylase (GAD)<br>Anti-Islet Cell (ICA)<br>Anti-Insulinoma associated (IA-2)<br>Anti-Zink-Transporter (ZnT8) | 5-50 years<br>10-12 years<br>10.9 ± 3.9 years<br>Adult | • IAA are often the first specific detectable Abs among children, identified in ~ 50% of T1 diabetic children<br>• GAD usually appear <5 years following appearance of anti-insulin Abs<br>• All Abs can appear before onset of symptoms, prevalence rises after disease manifestation | 160-164 |
| Coeliac disease | Anti-Gliadin<br>Anti-Endomysium (EmA)/Tissue Transglutamin | 9 month – 12.3 yrs<br>Adult<br>0.5-92 yrs | | 165-167 |
| Autoimmune thyroiditis | Anti-Thyroperoxidase<br>Anti-Thyroglobulin | 1-19 years | | 168, 169 |
| Pediatric Lupus | Antinuclear antibodies (ANA)<br>Anti-dsDNA<br>Anti-Sm<br>Anti-PCNA<br>Anti-Ribosomal P Protein (RPP)<br><br>Anti-Nucleosome<br>Anti-Histone<br>Anti-Phospholipid<br>Anti-SS-A (Ro)<br>Anti-SS-B (La)<br>Anti-U1-nRNP<br>Anti-C1q | 11.0 ± 3.6 years<br>4-18 years | • High levels of anti-RPP point to children with different autoimmune diseases, or mixed connective tissue disease. | 170-172 |
| Juvenile Idiopathic Arthritis | Rheumatoid Factor (RF)<br>ANA | 0.5-11.4 years | • RF is identified in ~ 15% of cases.<br>• Anti-Cardiolipin in ~ | 173-175 |

| | | | | |
|---|---|---|---|---|
| | Anti-HMG<br>anti-CCP/CCA | 15.59 ± 4.13 years | 30% of cases, in low titers and without association to clinical antiphospholipid syndrome<br>• ANA in ~ 30% of cases<br>• Anti-CCP/CCA are rarely positive | |
| Juvenile Dermatomyositis (JDM) | Antibodies against:<br>P155<br>Mi-2alpha<br>Mi-2beta<br>TIF1gamma<br>MDA5<br>NXP2<br>SAE1<br>Ku<br>PM100<br>PM75<br>Jo-1<br>SRP<br>PL-7<br>PL-12<br>EJ<br>OJ<br>Ro-52 | <18 years<br>1-80 years | • Approximately 10 % of children with JDM show myositis-specific autoantibodies<br>• Children with anti-Jo-1 and/or anti-SRP tend to follow a chronic continuous disease course.<br>• Anti-SRP are identified in polymyositis children with severe muscle weakness, and thinning or atrophy, and often are not responsive to single medication.<br>• Children with anti-Mi-2 typically have mild to moderate disease and respond well to treatment.<br>• Anti-p155 is the most common autoantibody found in children with JDM. | 176-178 |
| Multiple Sclerosis | Anti-Myelin Oligodendrocyte Glycoprotein (MOG) | 1.3 – 15.8 years | • Anti-Aquaporin for exclusion of optical neuromyelitis | 179 |

| Disease | Biomarker | Sample type | Age | Analysis approach | Proposed use | Reference |
|---|---|---|---|---|---|---|
| Atopic Dermatitis<br>Food Allergy | sICAM-1 | serum | 10 - 16 years | immunoassay | disease monitoring | 43 |
| | LL-37 | serum | 6 - 15 years | immunoassay | disease monitoring | 44 |
| | IL-31 | serum | 1 - 10 years | immunoassay | disease monitoring | 45 |
| | IL-18 | serum | 0 - 6 years | immunoassay | disease monitoring | 46 |
| | CCL28 | serum | 0 - 10 years | immunoassay | disease monitoring | 47 |
| | CCL17 | serum | 0 - >16 years | immunoassay | disease monitoring | 48 |
| | | serum | birth | immunoassay | risk monitoring/disease prediction | 63 |
| | CCL27 | serum | 1 - 11 years | immunoassay | disease monitoring | 49 |
| | miR-203<br>miR-483-5p | serum/urine | 0.5 - 6 years | global miRNA profiling & qRT-PCR | disease monitoring and progression | 51 |
| | carnitine<br>free fatty acids<br>lactic acid* | serum | 0 - 3 years | Mass spectrometry | mechanistic insight/biomarker discovery | 54 |
| | CD5L<br>apolipoprotein E | plasma | 2 - 7 years | 2D elcectrophoresis | mechanistic insight/ biomarker discovery | 56 |
| | allergen-specific IgA and IgG | serum | 5 - 12 years | DCP microarray | response to oral immunotherapy | 57 |
| | egg white IgE | serum | 1 - 16 years | ImmunoCAP | response to oral immunotherapy | 58 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | calprotectin | faeces | 2 months | immunoassay | risk monitoring/disease prediction | 59 |
| | soluble FasL | plasma | birth | immunoassay | risk monitoring/disease prediction | 62 |
| | CCL22 | plasma | birth | multiplex immunoarray | risk monitoring/disease prediction | 64 |
| | CD4+ regulatory T cells | whole blood/PBMCs | birth | flow cytometry, qRT-PCR, DNA methylation | risk monitoring/disease prediction | 65 - 67 |
| | polyubiquitin C<br>calmodulin-like protein 5 | vernix | birth | mass spectrometry | risk monitoring/disease prediction | 69 |
| | filaggrin | blood/saliva | | DNA genotyping | risk monitoring/disease prediction | 68 |
| Allergic Rhinitis | miR-146a | nasal mucosa & PBMCs | | qRT-PCR | | 70 |
| | FoxP3 | PBMCs | 4 - 14 years | | pathogenesis & management | |
| | IL-10 | serum | | immunoassay | | |
| Asthma | FeNO/eosinophils | exhaled air/blood | 6 - 19 years | NIOX® analyser/haematology analyser | disease phenotype/treatment response | 90 |
| | FeNO/eosinophils/periostin | exhaled air/blood | 12-75years | NIOX® analyser/haematology analyser/immunoassay | treatment efficacy | 83 |
| | periostin | Airway biopsies | 14 - 85 | immunohistochemistry | mechanistic insight/biomarker discovery | 87 |
| | | epithelial cells | 6 - 16 years | cell culture/qRT-CR | mechanistic insight/biomarker discovery | 88 |
| | | serum | 6 - 15 years | immunoassay | mechanistic insight/biomarker discovery | 91 |

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|---|---|---|---|---|---|---|
| | miR-21 | serum | | qRT-PCR | disease monitoring | 93 |
| | miR-638 & others | blood | 6 - 18 years | global miRNA profiling | mechanistic insight/biomarker discovery | 94 |
| | TNFα | LPS-stimulated PBMCs | birth/3 months | cell culture/immunoassay | risk monitoring | 97 |
| | volatile organic compounds | EBC | | mass spectrometry | | |
| | TLR4, catalase, TNFα | PBMCs | 2 - 4 years | qRT-PCR | risk monitoring | 98 |
| | clinical | | | athma predictive index | | |
| | IL-2<br>IL-4<br>IL-8<br>IL-10 | EBC | 2 - 3 years | multiplex immunoarray | risk monitoring | 99 |
| IBD | ANCA<br>ASCA<br>anti-OmpC | serum | 1 - 21 years | immunoassay | increased risk of surgery | 122 |
| | anti-glycoprotein 2 | serum | 2 -18 years | immunoassay | diagnosis | 123 |
| Other diseases | Adiponectin | blood | 5 - 15 years | radioimmunoassay | lifestyle intervention for obesity | 129 |
| | Endothelial progenitor cells<br>Endothelial microparticles | blood | 12 - 18 years | flow cytometry | monitoring endothelial dysfunction | 132 |
| | miR-125-5p<br>miR-342-3p | plasma | 5 - 10 years | global miRNA profiling/qRT-PCR | monitoring endothelial dysfunction | 133 |

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|---|---|---|---|---|---|
| miR-365-3p | | | | | |
| CRP<br>insulin<br>adiponectin | saliva | 11 years | multiplex immunoarray | intervention and prevention<br>type 2 diabetes | 136 |
| bradykinin<br>naringenin<br>L-thyronine* | serum | 6 - 17 years | mass spectrometry | mechanistic insight/biomarker discovery<br>obesity | 137 |
| citrate<br>succinate<br>creatinine<br>glutaric acid<br>3-aminoisobutyric acid<br>p-hydroxyphenyllactate* | plasma | 4 - 7 years | mass spectrometry | mechanistic insight/biomarker discovery<br>autism spectrum disorder | 138 |

Table 2. Summary of immune biomarkers in childhood non-communicable diseases.
*and others

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