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Immune response biomarkers in human and veterinary research

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ABSTRACT

Biomarkers are increasingly utilised in biological research and clinical practice for diagnosis of disease, monitoring of therapeutic prognosis, or as end points in clinical studies. Cytokines are small molecules that orchestrate immune responses and as such have great potential as biomarkers for both human and veterinary fields. Given the ease of sampling in the blood, and their high prevalence in clinical applications we will focus on protein detection as an area for biomarker discovery. This is facilitated by new technological developments such as digital ELISA that have led to significant increases in sensitivity. Two highly relevant examples include type I interferons, namely IFNα, that is now directly quantifiable by digital ELISA from biological samples. The application of this approach to the study of the unique bat interferon response may reveal novel findings with applications in both human and veterinary research. As a second example we will describe the use of CXCL10 as a disease biomarker in Tuberculosis, highlighting findings from human and mouse studies that should be considered in veterinary research. In summary, we describe how cytokines may be applied as novel biomarkers and illustrate two key examples where human and veterinary research may complement each other in line with the One Health objectives.

1. Introduction – the clinical potential of protein based biomarkers

Biomarkers are a broad category of medical indicators that can be measured objectively to determine different medical states or responses to an intervention. Therefore, they can be used for basic diagnosis, therapeutic prognosis, or as end points in clinical studies. Examining how biomarkers are used in research and the clinic in human and veterinary fields can be informative for both fields. Biomarkers may consist of nucleic acids, proteins, metabolites, and even images as long as they can be recorded and measured consistently and objectively. With the huge progress in genomics since the successful sequencing of the human genome, much attention has focused on the potential use of genetic biomarkers. However, despite dramatic reductions in sequencing costs and increases in capacity, the clinical potential of this approach still remains largely unmet with only 55 unique pharmacogenomic biomarkers (many of these biomarkers have multiple indications so the overall number is higher) currently approved by the Food and Drug Administration (FDA) for human treatment decisions [1]. The majority of these genetic markers are single nucleotide polymorphisms (SNPs), although more recently Next Generation Sequencing (NGS) techniques are being successfully applied in particular for cancer mutation detection.

In contrast to the use of genetic markers, an estimated 325 proteins can be reliably measured in human plasma by conventional clinical chemistry or Enzyme-Linked ImmunoSorbent Assay (ELISA), with concentrations ranging between 10^{-3}M and 10^{-12}M [2]. Of these proteins an estimated 175 are currently approved by the FDA as clinical biomarkers highlighting the advanced maturity of these technologies and also potentially their greater clinical relevance as compared to genetic biomarkers [3]. In addition, there is an estimated 4000 secreted proteins that are present in the circulation at concentrations less than 10^{-12}M, offering huge potential for new biomarker discoveries. Novel technologies such as digital ELISA and Mass Spectometry based approaches now have the ability to unlock this potential for both human and veterinary research.

2. Cytokines and Chemokines as immune response biomarkers

Blood and plasma-borne biomarkers are not only desirable for their accessibility, but also because they offer the potential for serial monitoring. Unlike SNPs which are static, protein responses are dynamic and can therefore potentially reflect disease status or even responses to therapeutic intervention. Plasma carries information from most organs

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and tissues [3,4], therefore, by analysing plasma protein composition, we can potentially capture the variation occurring in a wide range of human and animal tissues due to different disease states. There is a large dynamic concentration range for plasma proteins [4], allowing their classification into three main categories: high abundance plasma proteins such as albumin (35–50 mg/ml), immunoglobulin proteins (5–18 mg/ml) and tissue origin proteins or cytokines, with the latter being at the lower concentration (0–5 pg/ml).

Of particular interest for immunological studies are cytokines, which are small signalling molecules that orchestrate immune responses by enabling cell to cell communication and recruitment of immune cells to infection sites [5]. Cytokines can be further subdivided into interleukins (IL), interferons (IFN), chemokines and tumor necrosis factors (TNF), which may promote either pro-inflammatory or anti-inflammatory responses. They have the capacity to stimulate and modulate the immune system and are therefore great indicators of normal immunological processes, pathological processes or responses to treatment. Thus, cytokines have become highly relevant immune response biomarkers, and they are currently being used in a wide range of clinical situations. Relevant examples include the potential diagnostic value of IL-17 for both rheumatoid and psoriatic arthritis (24.3 ± 9.6 pg/ml in the inflammatory joint) [6,7]; elevated levels of IL-1β, IL-6 and TNFα that are associated with severity and progression of chronic liver disease (31.4 ± 3.0 pg/ml, 23.0 ± 4.0 pg/ml and 21.5 ± 3.9 pg/ml, respectively, in cirrhotic liver) [8]; and a cytokine signature based on IL-18, TNFα and IL-4 has been proposed as a predictive biomarker for Glatiramer acetate responsiveness for the treatment of multiple sclerosis [9]. These examples illustrate how cytokines have become invaluable biomarkers for diagnosing disease, for measuring progression/severity of disease states and predicting and monitoring the effects of therapeutic interventions. This is not only true for human immunology, but numerous examples can also be found in veterinary medicine. A good illustration of this is the study carried out by Bertuglia and colleagues, in which the kinetics of pro-inflammatory cytokines such as TNFα were associated with equine osteoarthritis (mean of ~100 pg/ml in synovial fluid and ~35 pg/ml in serum) [10]. Furthermore, changes in IFNα, IL-8 and TNFα have been described during classical swine fever infection (for example, peak between 3 and 8 days post-infection of IFNα reaching levels up to 1100 pg/ml) [11] and particular cytokine profiles have been associated with natural African trypanosome infections in cattle (mean of ~280 pg/ml of IL-10 in infected cattle *versus* ~150 pg/ml in uninfected animals, as well as ~5 pg/ml of TNFα and ~2.8 pg/ml in infected and uninfected animals, respectively) [12]. Overall, cytokine biomarkers hold great potential to monitor health and disease states both in the field of human and animal medicine.

3. Type I interferons – key immune response cytokines

The very first cytokine identified was the description of a soluble factor that protected cells from viral infection made by Isaacs and Lindenmann in 1957 [13,14], thus initiating this field of research. Despite this discovery now more than 60 years ago, the direct measurement of type I IFN protein in biological samples has remained challenging. Type I IFN mRNA is usually present at only trace levels in peripheral blood mononuclear cells (PBMCs) from healthy individuals, and current ELISAs have proved either insensitive or unreliable for detection of the protein leading to the development of a wide variety of proxy assays of type I IFN signalling [15–19]. Such low levels of circulating IFN protein likely reflect the high biological potency of type I IFN, with most cell types expressing a type I IFN receptor (IFNAR). Indeed, in addition to their role in anti-viral protection, it has become clear that inappropriate or excessive exposure to IFN can have major detrimental effects [20], so that a balance must be struck between the ability to fight infection *versus* the risk of inflammatory disease. This latter point is well illustrated by the very strong correlation of enhanced type I IFN signalling in systemic lupus erythematosus (SLE), juvenile-onset dermatomyositis (JDM) and type I interferons [21]. Furthermore, in humans multiple species of type I IFN exist, with this heterogeneity arising from distinct encoding genes, including 13 functional α genes and one β gene situated syntenically on human chromosome 9p. Differences in IFN-subtype production, activity site, and profile allows for a subtlety in immune function that is presently poorly understood.

Despite its key role in many diseases, the study of type I IFN protein levels in biological samples has been challenging until recently. Conventional sandwich ELISA is the most widely used method for detection of IFNα protein. However, despite being specific, simple and rapid, type I IFN ELISA assays present important limitations, in particular insufficient sensitivity. In addition, the measurement of all IFNα subtypes requires the use of multiple assays each with their own detection capacity and sensitivity. The limit of detection (LOD) of classical ELISA is within the picomolar range, which is insufficient to detect IFNα protein in biological samples. As previously mentioned, this fact possibly reflects the biological potency of type I IFN, thereby highlighting the lack of tools to monitor levels of these cytokines in infection and autoimmunity. To overcome this limitation, several biological proxy assays have been developed to quantify type I IFN by measuring induced gene expression or functional activity [15–19,22,23]. Nonetheless, it is important to emphasize that these assays do not provide a direct measurement of the IFNα protein.

However, the recent development of digital ELISA, in particular Single molecule immunoassay (Simoa) [24], represents a significant advance over previous approaches and has now made possible the direct quantification of cytokines such as type I IFNs [25]. Simoa utilizes paramagnetic beads, coupled to specific antibodies, that are isolated in femtolitre-sized nanowells specifically designed to isolate a single bead in a high throughput manner. This innovative step reduces the non-specific background fluorescent signal allowing the specific signal to be measured in a digital fashion. The results are both significant increases in sensitivity and increased confidence in low measurements as the previous non-specific technical noise has been greatly reduced. The application of this approach to the quantification of IFNα protein at attomolar concentrations has allowed the description of this protein in human plasma, cerebrospinal fluid (CSF), and cellular lysates for the first time in diverse disease cohorts ranging from autoimmunity to viral infection (median 1.6 fg/ml, interquartile range [IQR] 0.95–4.6 fg/ml in plasma from healthy individuals, median 20 fg/ml, IQR 0.60–234 fg/ml in plasma from SLE patients and median 56 fg/ml, IQR 14–120 fg/ml in plasma from JDM patients. IFNα protein could also be detected in CSF from patients with central nervous system infection; median 4,174.2 fg/ml IQR 2,437.4-11,173 fg/ml) [25]. Therefore, this novel approach may also now be applied to veterinary research questions if specific antibodies are available for the particular species of interest.

4. The unique IFNα response of bats

An area of research where this approach could provide novel insight for both human and animal fields is the immune response of bats to viral infection. Bats are recognised as a potential reservoir for many viruses such as coronaviruses, filoviruses, lyssaviruses, and henipaviruses that can cause severe disease in humans but not in bat species [26]. Likely as a result of their close co-evolutionary history with viruses, which enables them to live in a symbiotic relationship, bats are considered an important source of future viral pandemic outbreaks. To better understand this unique scenario, a recent fascinating study sequenced the IFNα locus of the Australian black flying fox, *Pteropus Alecto* [27]. This study identified in bats a highly contracted type I IFN family consisting of only 10 IFNs that includes three functional IFNα loci. A comparison of the sizes of the IFNα locus in different animals revealed that it ranged from 25 kb in fish to 1 Mb in pig, with a trend toward increasing size through evolution, with the notable exceptions of smaller sizes in chickens and bats (30 kb and 250 kb respectively)

[27]. Consistent with the increase in the genomic size of the IFN locus, gene duplication also occurred in the vertebrate type I IFN family, from an initial four type I IFNs in fish to 42 in pigs. Specifically, for IFNα species, bats are again the notable exception having the fewest (three) of the different animals studied, which have between 7–18 IFNα loci. The bat IFNα species were highly similar; sharing 93–96% amino acid sequences, and were relatively similar to human IFNα proteins with 79–85% amino acid similarity including predicted signal peptides and conserved binding domains for IFNαR1 and IFNαR2.

In terms of a better understanding of anti-viral immunity, of particular interest in bats was the observation that the three IFN-α genes (but not IFNβ) were constitutively expressed in healthy tissue of two different bat species. This resulted in the induction of a subset of IFN-stimulated genes associated with antiviral activity and resistance to DNA damage. Furthermore, upon Poly IC stimulation (TLR3 agonist) IFNα transcript levels did not change, in contrast with IFNβ levels, which showed a strong induction. This suggests a distinct division of function between different type I interferon species that has been challenging to ascertain so far in humans or other mammals. Due to the lack of available tools, the levels of IFNα protein have not been quantified in bats, but it has been hypothesised that much of the IFNα mRNA may remain un-translated, providing a source of transcripts for rapid translation when required [26]. Therefore, the quantification of IFNα at the protein level may help our understanding of how bats constitutively restrict viruses, without necessarily inhibiting their replication. Crucially the bat interferon response appears to avoid much of the negative inflammatory signalling that is associated with IFN responses in humans. Recent *in vitro* analysis suggests that the Poly IC induced response in bats may not trigger the inflammatory TNFα pathway [28]. The authors presented evidence that this may occur through a unique repressor (c-Rel) binding motif in the TNFα promoter that was present in 3 species of bats examined, but not in other mammals. As such, a better understanding of the structure and function of these unique IFNα species and their signalling pathways may lead to the development of novel cytokine based anti-viral therapies. IFNα has been widely used as a treatment for chronic viral hepatitis with relative success; roughly 50% of patients fail to respond (currently this treatment has been replaced by more potent and effective direct acting antivirals). Type I IFNs, specifically IFNβ, are also used as a therapy for Multiple Sclerosis [29,30], and anti-IFNα and anti-IFNAR antibodies have been proposed for treating Lupus and other automimmune diseases with mixed results [31–34]. Therefore, insights and new findings from the unique bat interferon response could have wide ranging implications for many human disease areas.

5. A case study: cytokine biomarkers in human and bovine tuberculosis

5.1. Diagnosis of human and bovine tuberculosis

It is estimated that a quarter of the worldwide population is latently infected with *Mycobacterium tuberculosis* (*M. tb*), the causative agent of human tuberculosis (TB), 5–10% of whom will develop active TB disease [35]. Pulmonary TB is the most common manifestation of the disease, but other body sites can also be affected in cases of extra-pulmonary TB. According to the *WHO Global Tuberculosis Report 2017* three main tests currently exist for diagnosing TB disease: smear microscopy, culture methods and PCR molecular tests, for example the Xpert MTB/RIF assay (Cepheid), which amplifies genomic DNA from the bacteria. All three tests are based on testing of sputum samples, which can be challenging to obtain, particularly in children and HIV-infected individuals. Thus, there is an urgent, unmet clinical need for a blood-based biomarker test to diagnose patients with active TB disease. Currently, there are two main assays to identify whether an individual is infected or not with *M. tb*, however, such tests do not permit discrimination between latent infection and active TB disease, in particular in endemic areas. These are the Mantoux tuberculin skin test (TST) and interferon-gamma release assays (IGRA). In TST, tuberculin purified protein derivative (PPD) is injected intradermally, whereas for IGRA tests, blood is incubated *ex vivo* with specific TB antigens (eg ESAT-6, CFP-10 and TB7.7). If the tested individual is infected with *M. tb*, an immune response will be elicited and detected, either in the form of a skin reaction in the former or in the detection of secreted IFNγ by antigen-specific T cells in the latter.

Nonetheless, humans are not the only species susceptible to tuberculosis infection. *Mycobacterium bovis* (*M. bovis*) is the main causative agent of bovine tuberculosis, which targets a wide range of wild and domestic animals [36]. Of particular interest is *M. bovis* infection of cattle, for productivity and economic reasons, as well as for potential zoonosis and impact on public health [37]. Early diagnosis of infected animals is essential to limit transmission and spread of the disease with control measures typically involving a test and slaughter strategy. This situation is further complicated by the fact that *M. bovis* may have several animal reservoirs in different geographical settings, such as badgers, deer, possums and buffaloes. Currently, the absence of both effective diagnosis tests for multiple species and vaccines makes containment of the disease extremely difficult.

The tools employed to diagnose bovine tuberculosis can be summarised into two main categories; detection of T-cell specific immune responses by TST or IGRA, as well as serological tests for detection of specific antibodies [37]. Although currently available tests are widely used and play a key role in the management of tuberculosis disease, they still present important limitations. The specificity of the TST is compromised by potential cross-reaction with environmental mycobacteria and with vaccination by the bacilli Calmette-Guérin (BCG) [38–41], which was implemented almost 100 years ago and still remains the only clinically approved TB vaccine [42]. Furthermore, for humans, we are still lacking the ability to identify people at greater risk of developing active TB disease. Thus, important challenges remain in the field of TB diagnostics and there is an urgent need for the development of novel, highly sensitive blood-based tests. Without them, meeting the WHO goal of TB eradication by 2030 will not be possible.

5.2. CXCL10 - an induced cytokine biomarker for diagnosis of Tuberculosis

In the last fifteen years, whole blood-based tests based on measuring IFNγ have become essential for diagnosis of *M. tb*. Currently, two commercial IGRAs are available: the QuantiFERON-TB Gold In-Tube test (Qiagen) and the T-SPOT TB test (Oxford Immunotec). The antigens of choice have the potential to elicit specific T cell responses, based on IFNγ as the biomarker readout. The QuantiFERON test uses conventional ELISA to measure IFNγ with a positive cut-off value of 0.35 IU/ml or 14 pg/ml. The T-SPOT TB test is based on IFNγ Enzyme-Linked ImmunoSpot (ELISPOT); less than 10 spots in the Nil Control and more than 20 spots in the positive control must be obtained for the test to be valid. The test result is considered positive if the number of spots obtained with *M. tb* antigen stimulation with the number of nil spots subtracted is higher than 8. Research has focused on the identification of novel biomarkers with the aim of providing increased diagnostic accuracy. Ruhwald and colleagues reviewed 22 clinical studies in which the pro-inflammatory cytokine IFN-inducible protein (IP) 10/CXC chemokine ligand (CXCL)10 was investigated as a potential surrogate biomarker to IFNγ in the context of *M. tb* infection (the following levels – mean [range] - of IFNγ and CXCL10 were obtained upon *M.tb* antigen stimulation: 5 [< 5-12] pg/ml and 91 [37–464] pg/ml of IFNγ in controls and TB patients, respectively, and 40 [20–159] pg/ml and 1025 [497–2080] pg/ml of CXCL10) [43]. IFNγ induces a broad transcriptional programme, which includes expression of CXCL10. Thus, it presents a biological amplification of the IFNγ signal, potentially increasing both robustness and sensitivity of T cell response measurements. This cytokine is produced in large quantities in patients with active TB as compared to controls [44] and its use as a biomarker for *M.*

tb infection could help to overcome some important limitations of IGRAs, such as testing in young children or immunocompromised individuals [39,45,46]. This monokine-based reporter has been shown to be both sensitive and accurate in studying antigen-specific T cell responses. However, despite being highly promising, CXCL10 detection assays still require standardisation and validation before they become routinely used in clinical practice, including sample processing, technological platforms and clinical cut-off values for positivity.

Similarly, IGRA tests also exist for *M. bovis*, with BOVIGAM (ThermoFischer) being the gold standard. They follow the same principle as the *M. tb* tests, that is, *ex vivo* whole blood antigen stimulation followed by detection of cell-mediated immune responses through the release of IFNγ. Despite being widely used, both sensitivity and specificity are limited and tests based on other approaches such as serology (Enferplex TB, Enfer Scientific) have been shown in some studies to give better diagnostic performance [47]. Thus, the potential of using CXCL10 as a biomarker for *M. bovis* in order to increase the sensitivity of conventional IGRAs arises as an attractive alternative. Although Waters and colleagues performed a study that did not show any advantages of using CXCL10 over IFNγ for diagnosis of *M. bovis* [48], more recent findings support the idea of this cytokine being a sensitive and valuable biomarker for *M. bovis* infection, both in cattle and buffaloes [49,50]. Currently, alternative single biomarkers as well as combination signatures are being intensively researched to improve efficacy of TB diagnostic tests. Amongst them, not only CXCL10 but also other cytokines such as IL-2, IL-10 and TNFα are being explored [51–53].

An additional complication in the use of CXCL10 as TB or other disease biomarkers relates to its previously described post-translational modifications in human and mouse studies. *In vitro* studies have shown that DPP4-mediated N-terminal truncation of the pro-inflammatory chemokine CXCL10 leads to the generation of an antagonist form [54,55]. Moreover, recent *in vivo* work performed in mice has demonstrated that this truncation alters lymphocyte migration and limits infiltration of the tumor parenchyma, a phenomenon that could be reversed using the DPP4 inhibitor sitagliptin [56]. Human studies utilising immunoassays that discriminate the full length agonist form of CXCL10 (referred to as $CXCL10_{1\text{-}77}$, or long CXCL10) from the NH_2-truncated form generated by DPP4 cleavage (referred to as $CXCL10_{3\text{-}77}$, or short CXCL10) have shown that elevated levels of short CXCL10 associated with increased DPP4 activity [57], both being negative predictors for viral clearance in chronic and acute hepatitis C (HCV) patients (mean plasma $CXCL10_{3\text{-}77}$ concentration of chronic HCV patients was 300 pg/ml *versus* 70 pg/ml in individuals with a sustained virological response) [55,57–59]. Final *in vivo* validation was shown through the inhibition of DPP4 activity by sitagliptin intervention in both healthy donors and chronically infected HCV patients that resulted in preservation of the long agonist form of CXCL10. Therefore, biomarker studies that rely on CXCL10 either in human or veterinary studies should consider these post-translationally modified isoforms.

6. Conclusions and remaining challenges

Assessing health and disease through the quantification of cytokines present in plasma is particularly attractive due to the ease, safety and feasibility of the method, as well as to the fact that plasma can reflect the state of an animal or human body at a particular time point. Therefore, this approach has been used for decades, although the progress made during the last years has been surprisingly modest. Possible reasons behind this slow advancement are the intrinsic complexity of biological systems and inherent inter-individual variability. Additional complications to using cytokines as biomarkers lie in the fact that these molecules have a short half-life in circulation and are often found at very low concentrations – such as IFNα concentrations in the fg/ml range in different autoimmune conditions. Thus, highly sensitive methods are required. Specifically, being able to associate single cytokine measurements to particular diseases with absolute certainty is extremely challenging. Moreover, accounting for non-disease variation, such as naturally occurring variability among different individuals, is essential when trying to define a reference interval to establish the boundaries of health. With the aim of improving the signal-to-noise in the context of disease diagnosis, two main strategies have been defined [3]. One involves the utilisation of more standardised approaches for studying induced immune approaches [60]; and a second complementary approach comprises the use of multiple cytokine markers detected with multiplex assays instead of a single analyte for the identification of more robust signatures [61]. In addition, studies that will define the genetic and environmental contributions to such naturally occurring variability to provide reference range values will help to better identify real biological signals from background noise [62].

Aside from the purely scientific considerations, there are further limitations when assessing the potential use of cytokines as biomarkers [3]. These include the lack of a shared central database such as exists for genetic studies, as well as basic systematic knowledge of the genetic impact on cytokine variability. Furthermore, protein measurements remain expensive meaning that large reagent and manufacturing innovations will be required if these assays are to become part of common clinical practice worldwide. Last but not least, other commercial considerations, including intellectual property and regulatory approval play a key role in making cytokine biomarker tests available.

Cytokines are key players in homeostasis and immune responses and thus hold great potential for becoming biomarkers of disease and response to treatment. Despite current limitations, numerous successful examples exist, both in human and veterinary medicine, of cytokine biomarkers that help diagnosis and monitoring of a wide range of diseases. Large efforts are being taken to overcome the prevailing challenges to benefit from the full potential of cytokines for novel biomarker discovery and implementation.

Conflict of interest

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