Metabolomics – A novel window into inflammatory disease

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Abstract

Inflammation is an important component of normal responses to infection and injury. However, chronic activation of the immune system, perhaps due to aberrant responses to normal stimuli, can lead to the establishment of a chronic inflammatory state. Such inflammatory conditions are often debilitating, and are associated with a number of important co-morbidities including cardiovascular disease. Resting non-proliferative tissues have distinctive metabolic activities and requirements, which differ considerably from those in infiltrating immune cells, which are undergoing proliferation and differentiation. Immune responses in tissues may therefore be modulated by the relative abundance of substrates in the inflamed site. In turn immune cell activity can feed back and affect metabolic behaviour of the tissues, as most clearly demonstrated in cachexia - the loss of cellular mass driven by tumour necrosis factor-alpha (TNF-α) a key mediator of the inflammatory response. Here we discuss the potential for metabolomic analysis to clarify the interactions between inflammation and metabolic changes underlying many diseases. We suggest that an increased understanding of the interaction between inflammation and cellular metabolism, energy substrate use, tissue breakdown markers, the microbiome and drug metabolites, may provide novel insight into the regulation of inflammatory diseases.

Metabolism and inflammation

Inflammation is a normal and important response to infection and injury. The cardinal features of inflammation - swelling, redness, stiffness and increased temperature - are outward indicators of significant local changes in metabolism. Increasing blood flow in turn affects local nutrient supply and in particular oxygenation of the tissues while infiltrating activated immune cells bring additional metabolic stressors that must be met. Furthermore, inflammation is associated with the rapid influx, differentiation and proliferation of leukocytes. Infiltrating cells have distinct metabolic requirements and, together with the increased cell number, result in a significant alteration in the metabolic profile of the inflamed tissues. In turn, the environment of the tissues may alter the activity, behaviour or differentiation of the invading cells. The activity of macrophages and neutrophils in both clearance of infection and tissue repair is particularly significant due to the production of cytokines and cytotoxic molecules, including reactive oxygen species and reactive nitrogen species, consuming considerable oxygen, adenosine 5'-triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the process. Reactive species,
while essential for killing invading organisms, also put considerable stress on surrounding and distal tissues via loss of protective metabolites including reduced glutathione (GSH). In rheumatoid arthritis patients blood levels of GSH decline by 50% and this is associated with a 3-fold increase in lipid peroxides [1]. A similar picture is seen in healthy ageing and this is enhanced in individuals receiving medical attention (Figure 1) suggesting that the process of “inflammaging” may interact with pathological developments to promote changes in metabolism [2].

It is interesting to note that mechanisms of metabolic and immune control co-evolved, originating in single fat body organ as still seen in Drosophila melanogaster [3]. This association persists in higher organisms, where lymph nodes are embedded in perinodal adipose tissue that may influence immune responses [4]. In humans adipose tissue is well infiltrated with macrophages, and the production of inflammatory cytokines by both adipocytes and macrophages contributes to systemic inflammation [5]. This link between inflammation and metabolism is well demonstrated in cachexia, where the loss of cellular mass is driven by tumour necrosis factor-alpha (TNF-α) a mediator of the inflammatory response [5,6]. Under normal circumstances acute inflammatory triggers are cleared or repaired rapidly, with subsequent homeostatic return. However, there are a number of chronic inflammatory diseases where aberrant immune activation results in a persistent inflammatory state. The metabolic consequences of chronic inflammation extend beyond the local site of disease, driving important co-morbidities including accelerated atherosclerosis and cardiovascular disease. Understanding of the metabolic aspects may therefore be key to fully characterising inflammatory disease, but given the complexity of the interlinking metabolic pathways in different, organs, tissues and cells a systems biology approach, metabolomics, is needed to assess and interpret these metabolic changes.

**Principles of metabolomics**

Metabolomics is a novel systems approach that can be used to dissect the local and systemic metabolic consequences of inflammation. Just as genomics studies the genetic basis of phenotype, and transcriptomics and proteomics study the products of these genes, metabolomics seeks to understand the downstream effects caused by the action of these proteins and enzymes in the context of energy and metabolite consumption and regulation. A hypothesis-forming approach, it is driven by the non-discriminant analysis of the low molecular weight metabolite component of target samples. The potential of metabolomics has shown promise in the prediction and diagnosis of diseases including ulcerative colitis, rheumatoid arthritis, multiple sclerosis (MS) among others [7-9].

Metabolomic analysis starts with the acquisition of metabolite data from samples. There are a number of variations of techniques for this purpose, although the most commonly used are nuclear magnetic resonance (NMR) spectroscopy, or mass spectrometry (MS). Samples may be derived from any sample suitably processed to be cell-free, including urines, plasma, cerebrospinal fluid, faecal extracts and synovial fluid [7-9], and pH stable through addition of buffers. Alternatively cell extracts may be performed into any suitable solvent, such as methanol with chloroform extraction to remove protein components. Consistent processing and buffering is key to the success of metabolomic analysis due to the potential confounding...
factors that may result from variations in pH during acquisition – including shifted peaks and chemical reaction resulting in loss or gain of metabolites. Acquisition of samples results in spectra containing a number of peaks, representing proton resonance in $^1$H NMR or mass/charge (m/z) ratios in mass spectrometry. In practise however the goal of analysis is the same – using differences in peaks, and therefore metabolites, to describe the differences between sample groups. The large size of the produced datasets, and often numerous samples, requires the use of multivariate-type analyses to simplify and distil meaning from the resulting data.

Statistical methods for the analysis of metabolomic data may be broadly grouped into directed and undirected approaches [10]. In undirected analysis such as PCA, the goal is to describe the maximum variation in the dataset itself without providing information to the model about the class groupings. The output is a series of orthogonal principal components, describing the maximum to minimum variation in the data in turn through weighted peaks. If the source of largest variation in the data is the difference between control and test samples, principal components analysis (PCA) alone is sufficient to differentiate class groupings. However, in more variable samples, as is often the case in biological sourced materials, other confounding or random data may mask the true differences in the samples. Here directed analysis techniques may be more useful. Partial least squares regression (PLS) may be used to describe variation in one dataset by reference to another – for example, using metabolite concentrations to predict disease activity, progression or severity. Similarly, the related technique PLS discriminant analysis (PLS-DA) uses multivariate metabolomic peak data to describe the assignment of samples to binary groups provided. By focusing on the variation that describes the differences between control and test samples, it is possible to tease out the clinically relevant variation in the face of significant unrelated noise. The output of these approaches is markedly similar: a statistical model from which is it possible to accurately predict disease state or patient group from similarly processed samples from new individuals. However, these models are complicated, including peaks on the basis of correlation. Forward selection regression analysis may therefore be employed to iteratively discard peaks resulting in a small number of metabolites while maintaining predictive capability – a requirement for the development of cheap specific bioassays. Selection of controls, accounting for confounding effects such as diet, smoking, and medication history, is key to the development of accurate models.

A simple example of the output of a metabolomic study is shown in figure 2, where we assessed NMR-derived metabolic fingerprints of serum from a small cohort of healthy elderly individuals (age > 75 years) in comparison to young donors (< 25 years). The groups are clearly separated in a PLS-DA model and analysis of the loadings plot (which indicates which regions of the NMR spectra contribute to the separation) indicates several distinguishing metabolites including methylamine and ketone bodies which have been associated with ageing in rats probably as a result in changes in pyruvate and energy metabolism [11]. Ketone bodies are also generated by inflammatory leukocytes and have been observed in the synovium in RA [2]. Therefore here they may be present as a result of low grade inflammation which is characteristic of inflammaging and so may provide novels markers of this process [12].
By applying these metabolomics techniques to inflammatory disease the goal is to determine the role of metabolic processes in the development of normal and aberrant inflammatory states. Metabolite markers, assayed from the various tissues and fluids, are accessible and simple to obtain from patients while having the potential to provide deeper indications of underlying disease processes.

**Metabolic markers**

In the following sections we take a systematic approach to outline key markers identified by metabolomic studies to date which are summarised in Table 1.

In spite of the early state of the field, there already exist a number of key areas of investigation where metabolic patterns are emerging, both in general inflammatory disease and in specific pathologies. Here we focus on the most important of these which include energy sources, hypoxia, regulation of inflammation, tissue degradation, waste and drug metabolites.

**Lactate: the Warburg effect and Hypoxia**

In the presence of adequate oxygen, differentiated cells metabolize glucose through mitochondrial oxidation of glycolytic pyruvate in the tricarboxylic acid (TCA) cycle, with resulting NADH fuelling oxidative phosphorylation to produce 36 ATP per glucose molecule. Under hypoxic conditions the oxidative pathways are inhibited and glycolytic pyruvate is instead excreted as lactate, with a decreased 2 ATP output per glucose molecules in the cell. Physiological levels of oxygen range between 5-12% depending on tissue location. Structures in the body held naturally at the lower end of this range, by virtue of low perfusion or vascularization, include the lymph nodes, eye and synovium. In normal inflammatory conditions hypoxia results as a side effect of blood vessel occlusion due to inflammation or as a result of increased cell density following infiltration. Hypoxia is a key driver of cell migration into the tissues and perfusion may act as a signalling mechanism to invading cells [13]. Reductions in oxygen lead to stabilisation of transcription factor hypoxia-inducible factor (HIF) providing an elegant cellular oxygen detection system. Stabilisation of HIF drives major changes in cellular gene expression including the promotion of glycolytic enzymes. Peripheral blood CD4+ T cells exposed to hypoxia show dramatic induction of genes involved in metabolism and homeostasis [14]. Innate immune cells, including neutrophils and macrophages are thought better adapted to function at low oxygen tensions with preferential use of glycolysis to provide ATP even under normal physiological oxygen [15]. Macrophages tend to accumulate in the hypoxic sites of chronic inflammation and hypoxia is associated with the activation of infiltrating and tissue-resident macrophages resulting in up-regulation of pro-inflammatory IL-1, IL-6, IFN-γ and TNF-α [16-20]. Most infections or injuries are cleared or repaired quickly with perfusion and metabolic homeostasis soon restored. However, in poorly perfused tissues such as the rheumatoid synovium and sclerotic tissues oxygen perfusion can fall as low as 0.5-2.5% and this regulation may be perturbed leading to continued recruitment [21,22]. Lactate is a waste product of normal metabolism reversibly converted from glycolytic pyruvate via the enzyme lactate dehydrogenase. Excess cellular lactate is excreted and re-circulated via conversion in the live to glucose (the Cori cycle). In sufficiently oxygenated cells it may also be converted
back to pyruvate via reaction with intracellular NAD+ to fuel the citric acid cycle. However, in poorly perfused tissues the production of lactate outstrips this homeostatic control and lactate accumulates. In hypoxic conditions the HIF-mediated up-regulation of lactate dehydrogenase A further promotes this accumulation [23].

Lactate therefore is a key marker of inflammatory sites under hypoxia. However, this relationship is not restricted to inflammation. In 1924 Otto Warburg first observed that cancer cells metabolize glucose by glycolysis even under aerobic conditions [24]. It has been suggested that that all differentiating and replicating cells must use this same mechanism in order to produce the requisite biomass for cellular replication [25]. By this mechanism, glucose and glutamine can adequately supply the majority of carbon, nitrogen and energy for cell growth and division. Indeed, under growth conditions, metabolizing glucose to CO₂ via oxidative phosphorylation runs counter to the needs of the cell. Therefore, non-proliferative, highly ATP-dependent organs such as the brain favour free-energy production via oxidative phosphorylation of glucose [26], while in contrast, under resting conditions, skeletal muscle metabolism is predominantly fatty acid dependent, switching to glycolysis and oxidative phosphorylation under activity [27]. In proliferative tissues such as the lymph nodes, there is instead a tendency towards metabolism of glucose to lactate, pyruvate and CO₂, and glutamine to ammonia, glutamate and aspartate. Importantly this may also apply to cells, such as polymorphonuclear leukocytes, that invade inflammatory sites [28,29]. The switch to aerobic glycolysis therefore compounds the production of lactate, locally and distally – including where hypoxia is absent.

The role for lactate as a universal marker of inflammation is borne out clinically. Increases in the lactate:glucose in blood plasma and synovial fluid in rheumatoid arthritis have been shown to correlate with active inflammation and oxidative damage to the joint for example [8]. Meanwhile, in a wide-ranging study of cerebrospinal fluid (CSF) from patients with a number of neurological conditions, high lactate was a distinguishing feature of idiopathic intracranial hypertension (IIH) hinting to a potential inflammatory basis for the disease with pressure occlusion of the intracranial vasculature. Increased CSF lactate and creatinine are also seen in multiple sclerosis patients versus controls, potentially reflecting leukocyte infiltration into the lesions [30]. Such alterations in pyruvate and lactate can in turn drive downstream effects on the citric acid cycle. Pyruvate may be converted to intermediates of the citric acid cycle, malate and oxaloacetate [31] by pyruvate carboxylase and malic enzyme respectively. Raised urine malate is a strong predictor of disease activity in the Hartley guinea pig model of OA [32]. Raised oxaloacetate and reduced citrate are also associated with disease in idiopathic intracranial hypertension together with increases in ketone bodies 3-hydroxybutyrate, suggestive of an anaerobic carbohydrate-deficient environment [9].

Alternative energy sources

The base relationship between resting energy expenditure and body mass is described by the Kleiber formula (REE = 293M⁰.75). Organ-specific metabolic rates and preferred substrates are also well established (Figure) with the heart and kidney consuming the major proportion of energy in the resting adult, followed by the brain and liver [33,34]. However, these basal
rates are significantly altered by malnutrition, illness, smoking, and inflammation and changes in substrate availability can have disproportionate effects [34]. Recent work on a cohort of RA patients found that their global basal metabolic rate (BMR) was 1500kcal/day, which was 8% higher than the value from a control group. Importantly a group of RA patients who smoked had a 20% higher BMR of 1718kcal/day (Figure 4) [35]. This indicates how large the effects of inflammation and factors promoting it can be and explains why changes in blood and tissue metabolites are easily observed. Inflammation is a highly energy-dependent process and acutely reliant on adequate energy availability to function correctly [36]. Fever is associated with an 11% increase in energy consumption per 1°C, while phagocyte production during an infectious episode consumes approximately $7.9 \times 10^5$ joules [37,38]. In sepsis the metabolic rate can reach up to 30-60% above baseline levels [39]. The converse is also true, with the energy surplus associated with obesity and other metabolic syndromes driving altered immune responses and chronic inflammation [40].

Energy availability is therefore a key regulator of immune activation and resolution. Resting energy requirements for non-brain and non-proliferative tissues, particularly the skeletal muscle and heart, are predominantly met by the oxidation of free fatty acids [27]. Due to the oxidation requirement their use is typically limited to well-perfused tissues, however damage to the joint in inflammatory and osteoarthritis appears to be associated with an increased perfusion of macromolecular fatty acids into the synovium. In osteoarthritis increased lipoprotein associated fatty acids, together with metabolism products glycerol, ketones and pyruvate, are all indicative of lipolysis occurring as an energy source. Similarly, a study of RA synovial fluid samples has shown reduced chylomicron and very-low-density-lipoprotein associated triglycerides compared to controls, suggesting increased utilisation of fats as an energy source in the joint in spite of the hypoxic environment. Blood plasma levels of cholesterol, acetylated glycoprotein and lipids were also raised in RA [41].

Fatty acids are additionally broken down in the liver and kidney, producing ketone bodies for recirculation to the tissues. Ketone bodies acetoacetate and 3-hydroxybutyrate provide a key energy source for the heart and brain, through reconversion to acetyl-CoA to feed the citric acid cycle in the tissues, while acetone is readily excreted as waste. In normal fasting conditions plasma levels of ketone bodies are low, as tissues with lower metabolic needs sustain themselves on local reserves of fatty acids, but these sources are not sufficient for active, highly energy-dependent tissues and ketone body production rises significantly. After 3.5 days of glucose starvation the brain can obtain as much as 25% of total energy from ketone bodies [26]. However, alterations in ketone body production and metabolism have also been observed in inflammatory diseases, in the absence of glucose restriction. Elevated 3-hydroxybutyrate has also been seen in MS with samples containing as much as 32μM concentration of this metabolite [42,43]. As previously described chronic inflammation may drive metabolic changes resulting from inflammation, particularly deriving from cachexia-associated and the presence of the same metabolite in urine has been linked to respiratory chain deficiency leading to impaired NADH oxidation [44]. Local changes may also be informative: raised synovial fluid concentrations of ketone bodies, particularly 3-hydroxybutyrate, in patients with rheumatoid arthritis are suggested to reflecting the degradation of local acetyl-CoA under hypoxic conditions [12]. This was also suggested by
the low glucose NMR signal in synovial fluid which was 20% lower than paired sera, which is a direct measure of the decrease in this metabolite.

**Tissue degradation and waste**

Normal tissue homeostasis requires the turnover of both cellular and molecular biomass. In the inflammatory state however, these processes are often altered offering the possibility of characteristic excess or paucity of specific biomarkers. Specific tissue-derived metabolites may provide the clearest markers of gross tissue destruction. Hyaluronic acid for example, is a major component of articular cartilage proteoglycan aggregate and essential for the functional integrity of extracellular matrix. Hyaluronidase activity is absent in both normal and inflamed synovial fluid, and therefore breakdown does not occur. However, in rheumatoid arthritis the action of reactive oxygen species results in detectable levels of hyaluronic acid in the synovium providing a direct local marker of joint destruction [45].

Essential amino acids, those that cannot be synthesized *de novo* in the human body, also offer a useful general marker for protein degradation. On a background of steady dietary intake any alterations in tissue concentrations must derive from catabolism of existing proteins – sourced from gross tissue destruction, apoptosis or cellular autophagy. This principle has been clearly demonstrated in a mass spectrometry study of serum from patients with OA. Valine concentration in the OA patients was 306 μM while in the control group it was 266 μM, giving rise to a significant alteration in the ratio of serum valine to histidine and leucine to histidine in patients versus controls [46]. In a study of comparing faecal extracts from patients with ulcerative colitis (UD) and Crohn’s disease (CD), higher levels of alanine, isoleucine, leucine, lysine and valine were all seen in patients with CD [47]. Gas chromatography-mass spectrometry analysis of urinary metabolites in a mouse model of Crohn’s disease similarly identified alterations in tryptophan metabolism [48]. This appears to be a general feature of inflammatory processes, as demonstrated using adenoviral vector to induce chronic expression of inflammatory IL-1B or TNFα, the IL-1B treated group similarly saw increases in leucine, isoleucine, valine, n-butyrate and glucose [49]. Interestingly, dietary supplementation with a subset of essential amino acids, the branch chain amino acids, has shown to drive an increase in Th1-like responses via IL-1, IL-2, TNF and interferon suggesting the possibility of a link between local release from degradation and chronic inflammation [50].

The catabolism of amino acids, either as an energy source or resulting from protein degradation, results in the local and systemic accumulation of ammonia. This toxic metabolite is rapidly converted to urea, predominantly in the liver and kidney but also elsewhere, for subsequent excretion via urine [51]. In tissue destruction other nitrogenous base carriers, such as trimethylamine, perform broadly similar functions. Excretion of nitrogenous waste is key to tissue homeostasis, however, under excess production or in sites of poor circulation these metabolites can accumulate. For example in a study of ocular inflammatory diseases compared vitreous humour samples from patients with various retinal disorders distinctions could be drawn with urea, oxaloacetate and glucose all significantly raised in lens-induced uveitis (LIU) compared to chronic non-infectious uveitis (CU) [52]. Similarly, a study of urinary metabolites in a IL-10 deficient mouse model of inflammatory
bowel disease (IBD) showed an increase in trimethylamine (TMA) compared to controls, with the increase in urine TMA (1540 μM in the IL10 deficient compared to 308 μM in the wild-type at 20 weeks) running parallel to the progression of IBD [53], a finding corroborated in human studies using both urine and faecal extracts [47].

**Xenobiotics and the Microbiome**

So far we have dealt only with metabolites of human biological origin. However, two other broad metabolite classes deserve further mention in the context of inflammatory disease: xenobiotics and the microbiome.

Xenobiotics are chemicals found in an organism that are not derived from normal biological processes or dietary intake – a classification that includes most drug treatments. Metabolism of xenobiotics is a complex process, predominantly occurring in the liver and typically involving multiple steps of oxidation, reduction, hydrolysis, cyclization, and decyclization with the end goal of detoxification and excretion. Metabolism is associated with characteristic profile of intermediates and excretion products that are unique to the drug or class. In a clinical context these metabolites simply restate what is already known – the treatment regimen of the patient. More interesting are the effects of drug metabolism on disease progression and resolution. For example, in a study of diclofenac significantly altered levels of oxylipids were seen in those patients that responded to the drug, with arachadonic acid metabolite 5,6-DHET identified as a novel marker of inflammation [54]. Similarly, analysis of patient responses to simvastatin successfully discriminated responders and non-responders by metabolic profiles of cholesterol esters and phospholipid metabolites [55]. Interestingly, this study was also able to discriminate between metabolites associated with drug effects on LDL-C or C-reactive protein independently – characterizing mechanism of action within an individual. Potentially, the baseline metabolic characteristics of an individual may have important implications for drug lifetime, and effectiveness, and provide a route to tailoring dosing or drug choice to an individual in accordance to not just genetic variability but also lifestyle and diet.

The microbiome is a collective term for the totality of commensal bacteria within our bodies. Bacteria outnumber our own cells by a ratio of 10 to 1 [56], are responsible for many essential metabolic processes for our health, and respond to both inflammatory and immune processes. In an analysis of faecal extracts from patients with Crohn’s disease (CD) and ulcerative colitis (UC) reduced levels of butyrate, acetate, methylamine and TMA were detected compared to controls [47]. Urinary metabolites derived from alterations in gut metabolism also allow distinction to be drawn between the two diseases with hippurate and 4-cresol low in CD versus UC and controls, and formate higher in CD [57]. While it is perhaps unsurprising that inflammatory gut conditions would drive alterations in gut bacterial metabolism, changes have also been seen in other conditions with no known gut involvement. In a case-control study of type-1 diabetes a switch in gut bacteria from mucin-degrading, butyrate producing bacteria, to non-butyrate and lactate-utilizing bacteria has been associated with development of the disease [58]. The role of the microbiome in cardiovascular conditions was likewise demonstrated by the finding that metabolism of dietary lecithin by gut flora leads to absorption and accumulation of derivatives of choline.
which in turn promotes disease [59]. This close relationship between the metabolism of gut bacteria and elsewhere in the body provides a key challenge in our understanding of disease processes through analysis of body fluids.

**Conclusion**

Metabolomics is a rooted firmly in the ‘systems approach’ to deriving information from large and complex datasets. However, as outlined here seemingly disparate markers can be broadly arranged into functional groupings that offer insight into local and global disease processes. The key difficulty is in separating these local and global effects when assaying through a single bio-fluid. However, in the context of diagnostic or prognostic markers, this is not necessarily required. Metabolic processes do not occur in isolation, and through the noise the interaction of the multiple systems involved in an inflammatory response emerges a characteristic fingerprint of disease. In developing diagnostic assays we are aided by the fact that these fingerprints frequently consist of the same relatively small subset of metabolites, yet maintain their discriminative capacity, sensitivity and specificity. Metabolomics offers a novel non-invasive approach to rapid diagnosis, prognosis and prediction of inflammatory disease outcomes.

**References**

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Figure 1. Levels of plasma antioxidant glutathione decrease with age whereas markers of oxidative damage lipid hydroperoxides increase
This provides an example of the magnitude of changes in metabolite levels that can be observed in humans which, in the case, may be consequence of inflammaging. Adapted from [62].
Figure 2. Metabolomic analysis of serum samples from old (●) and young (○) healthy volunteers. 1D spectra were acquired in a 500MHz spectrometer and processed with NMRLab/Metabolab and analysed with partial least squares discriminant analysis (PLS-DA). The two groups (<25 and >75 years) were well separated on PLSDA (A) with loadings (B) showing amine derivatives and ketone bodies are raised in ageing, which may be markers of underlying inflammaging.
Figure 3. Metabolic requirements of the major tissues
While the overall resting energy expenditure falls in old age, relative contributions of organs to the total remains stable. Adapted from [34].
Figure 4. Smoking has a significant effect on the basal metabolic rate of rheumatoid arthritis patients (RA)
Metabolic rate is higher in RA compared to healthy controls and this is enhanced even further by smoking, indicating the complexity of interactions between metabolism and disease. Adapted from [35].
Table 1

Key metabolites and their association with inflammatory diseases in current literature arranged according to the major themes discussed. RA – Rheumatoid arthritis; IA – Inflammatory arthritis; OA – Osteoarthritis; IIH – idiopathic intercranial hypertension; MS – Multiple sclerosis; LIU – Lens induced uveitis; CD – Crohn’s disease. CSF – Cerebospinal fluid, VF- Vitreous fluid, SF - Synovial fluid.

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† Animal model