
R.E.D. Laboratories

Specialty Testing for

Multifactorial Afflictions

FOCUS ON IMMUNE DISORDERS

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R.E.D. Laboratories

WHO WE ARE

- R.E.D. Laboratories is private Belgian company developing tests for patients with complex clinical picture, chronic immune diseases and intestinal dysfunctions
- We actively pursue new tests development in order to provide clinicians with updated tools



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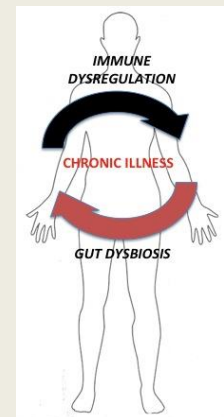


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HOW WE WORK

- At R.E.D. Laboratories, we are continuously developing new tests according to the specific needs from health care providers.
- All generated benefits are used for research and development of new assays.
- We are involved in several international groups aiming to advance knowledge in biological markers of **multifactorial afflictions** that are not optimally supported by general health care systems.



R.E.D. Laboratories

OUR PHILOSOPHY

- We focus on setting up the tests that are not (or rarely) available elsewhere.
- Personalization of testing panel leads to more efficient and rapid management of patients with complex clinical picture.
- Assay development programs at R.E.D. Laboratories focus on disorders that contribute to the onset and pathogenesis of diseases such as **chronic fatigue syndrome, autism, chronic infections or autoimmune diseases.**

R.E.D. Laboratories

WHAT WE OFFER

- More and more evidence points towards a combination of factors (genetic, infectious, environmental, etc.) being important in the development of chronic immune dysfunctions, the cardinal finding in autistic, chronically infected and CFS patients.
- In many countries these affections are still considered as psychiatric despite clear biomedical evidence.

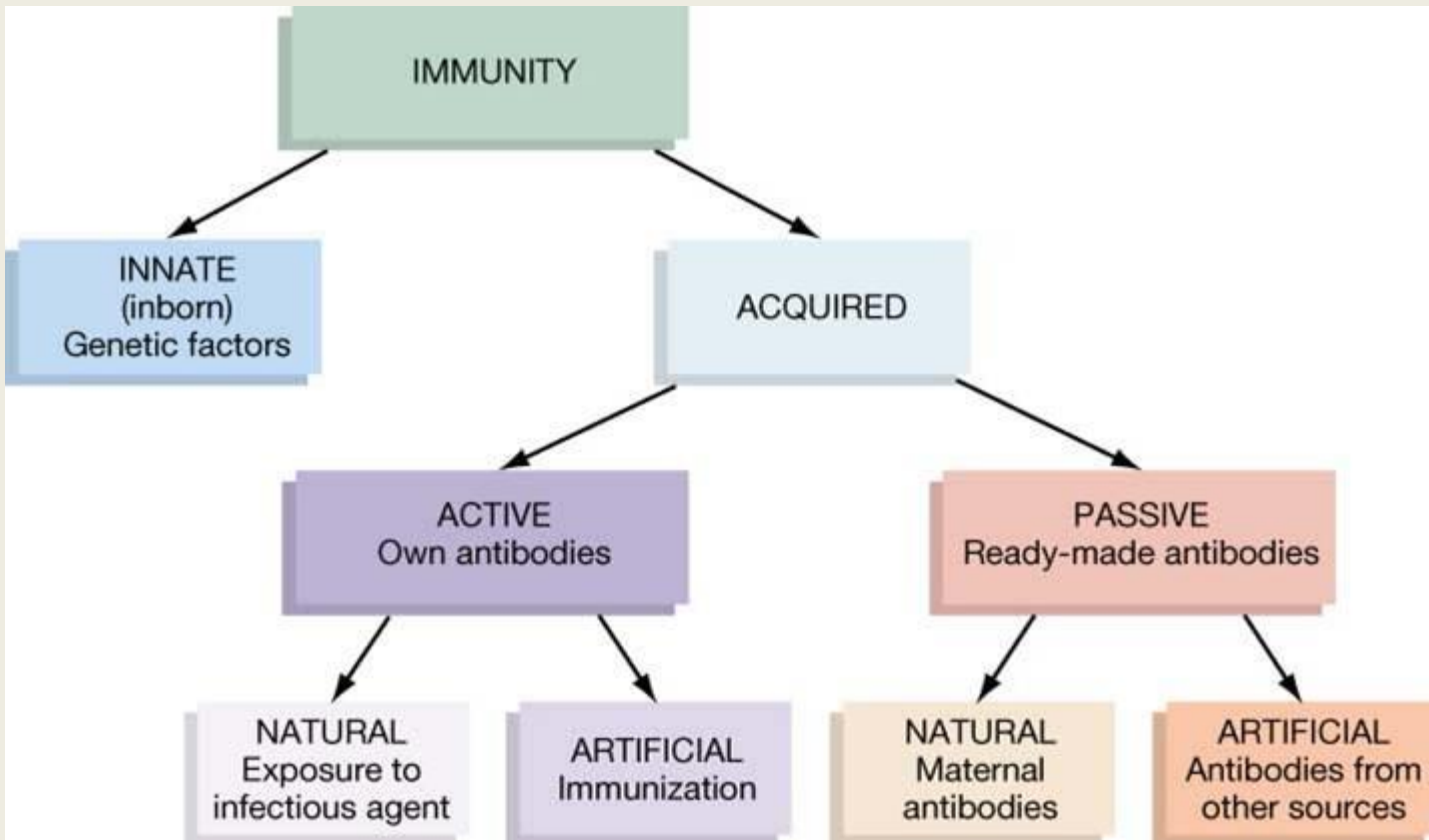
For better management of these multifactorial affections suffering from lack of medical recognition, we offer **SPECIALTY TESTS** focused on **3 major topics**:

- **1. immune disorders,**
- **2. intestinal dysfunctions and**
- **3. infections.**

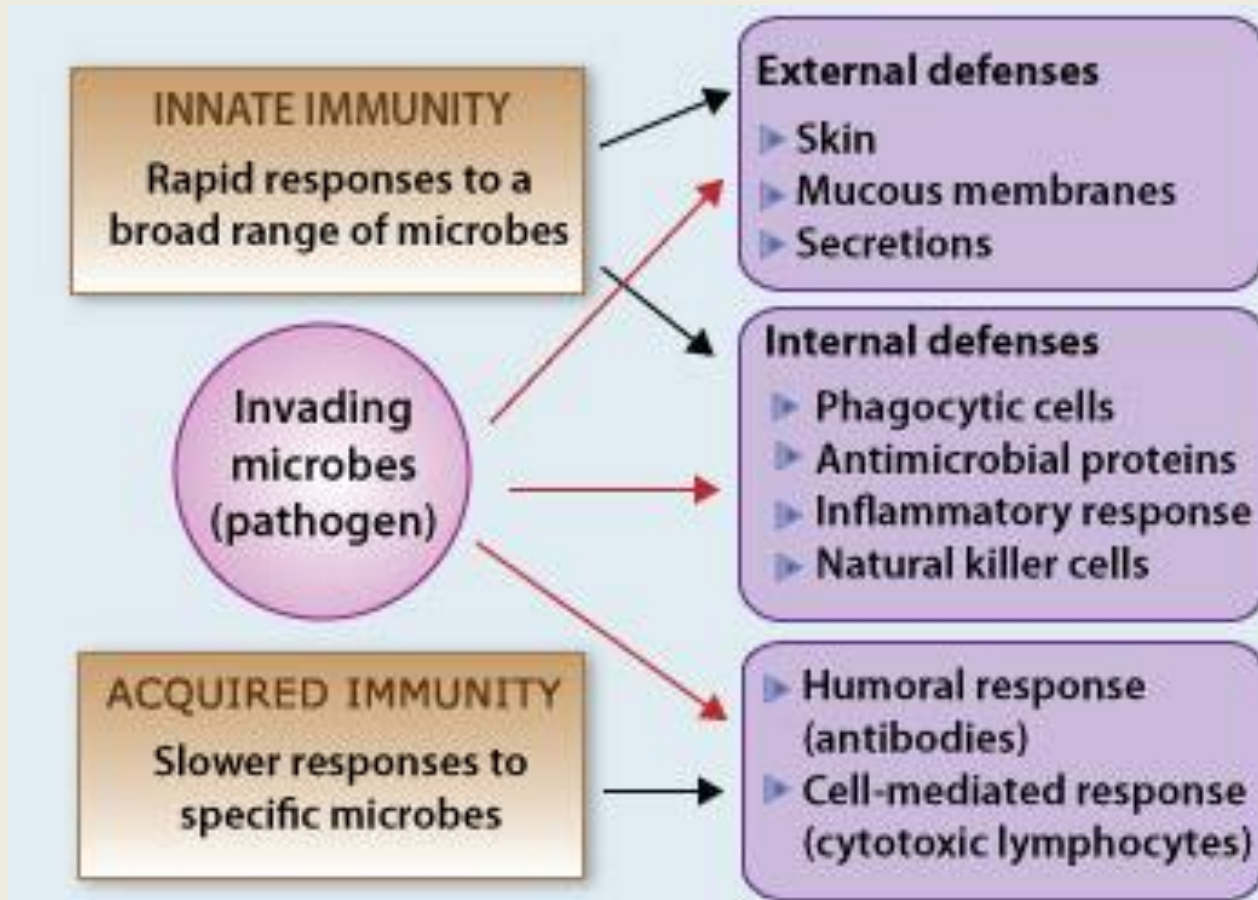
- *Check our website, our test request forms and our catalog for complete lists of tests and for newly available tests*



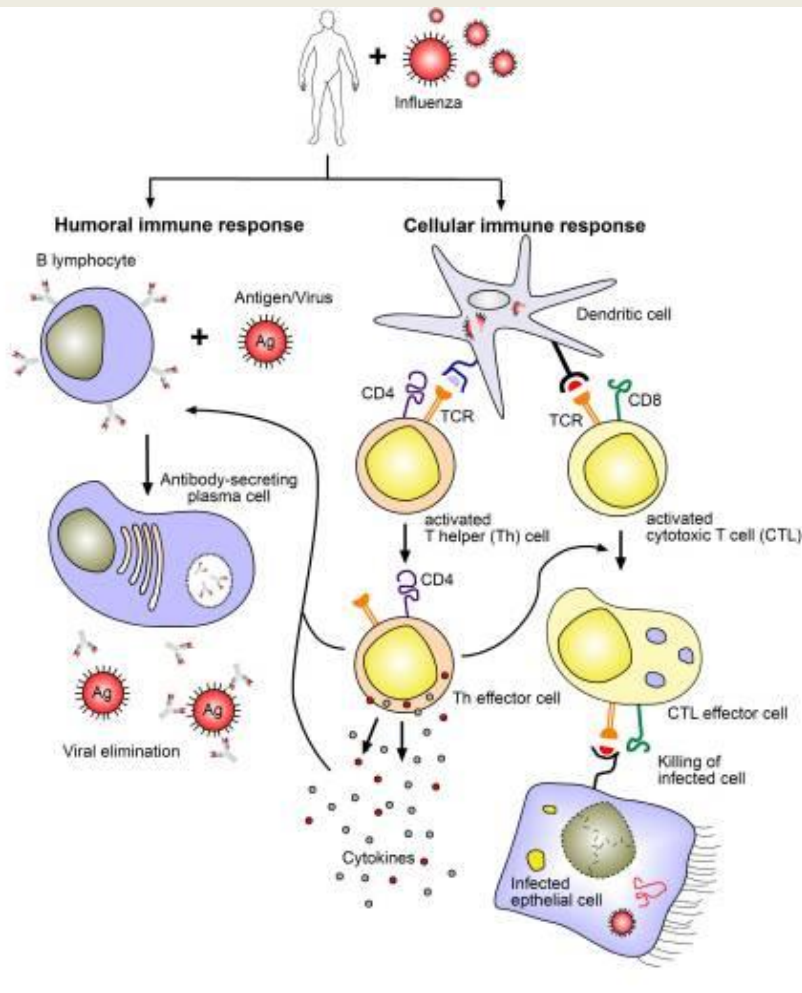
Immunity



Innate and acquired immunity



Immune response



- Cellular immune response
Th1

This is how we eliminate intracellular pathogens and non-self cells.

- Humoral immune response
Th2

This is our ability to produce antibodies to neutralize pathogens.

Th1 and Th2 should always be in balance!

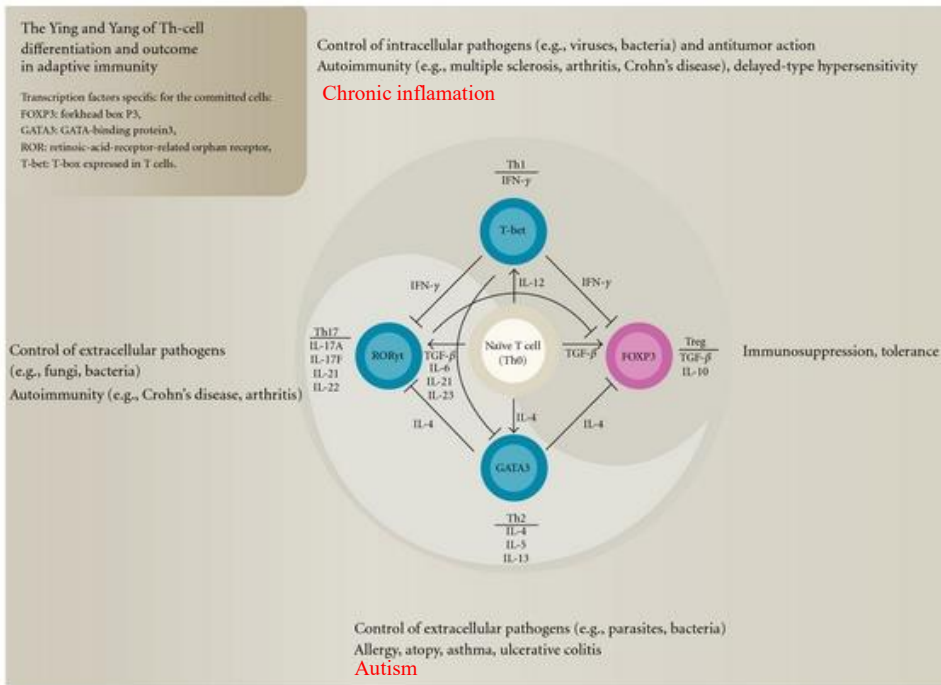
Specific immunity

- **Th1 and Th2 produce and release cytokines that trigger a domino effect leading to an immune reaction:**
- **Cytokines released by Th1** are: IL-2, IL-12, $\text{INF}\gamma$, $\text{INF}\alpha$ and $\text{INF}\beta$
- **Cytokines released by Th2** are: IL-4, IL-5, IL-10
- **Th1 cytokines suppress Th2 cytokines and vice versa**
- If the pathogen is defeated, the immune system returns to a balance between Th1 and Th2
- Unfortunately, some conditions involve chronic activation or suppression of one of the two categories.

Urine-based Th1/Th2 balance test

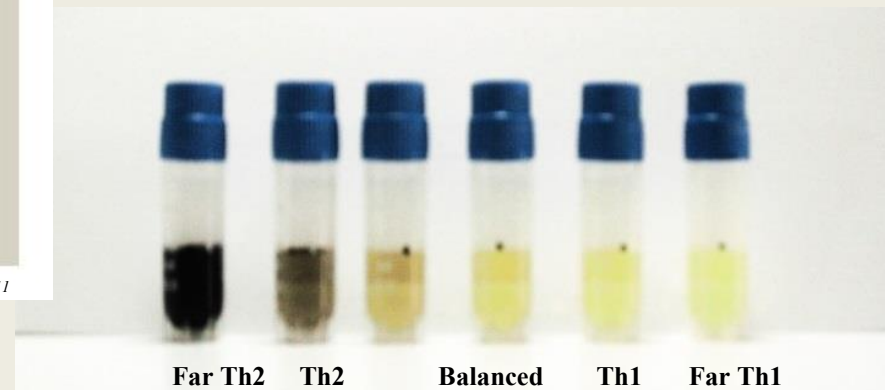
Th1/Th2 balance

urine-based Th1/Th2 balance test

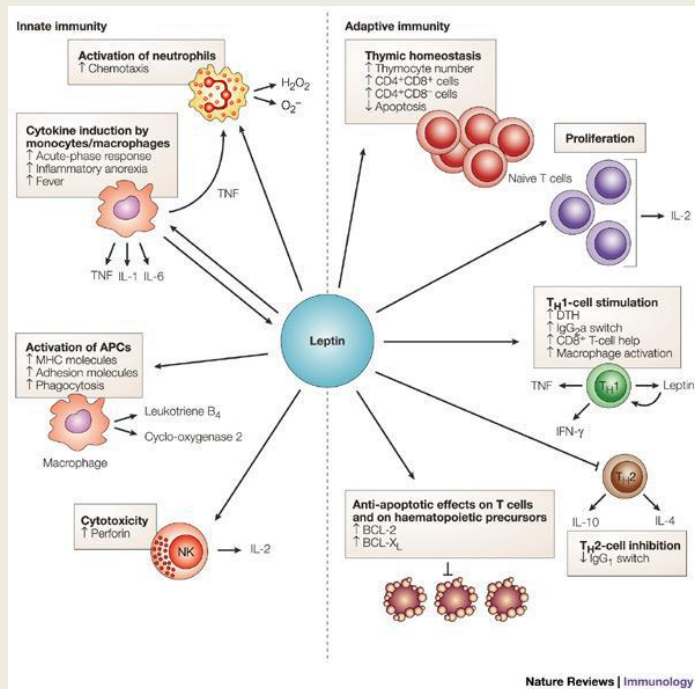


Hetland et al. *Adv Pharmacol Sci*. 2011

- may detect disturbances of this delicate equilibrium in time in order to restore balance whenever required and before irreversible conditions are developing
- allows patients to follow-up on Th1/Th2 balance during therapy (antioxidants, probiotics, nutraceuticals).



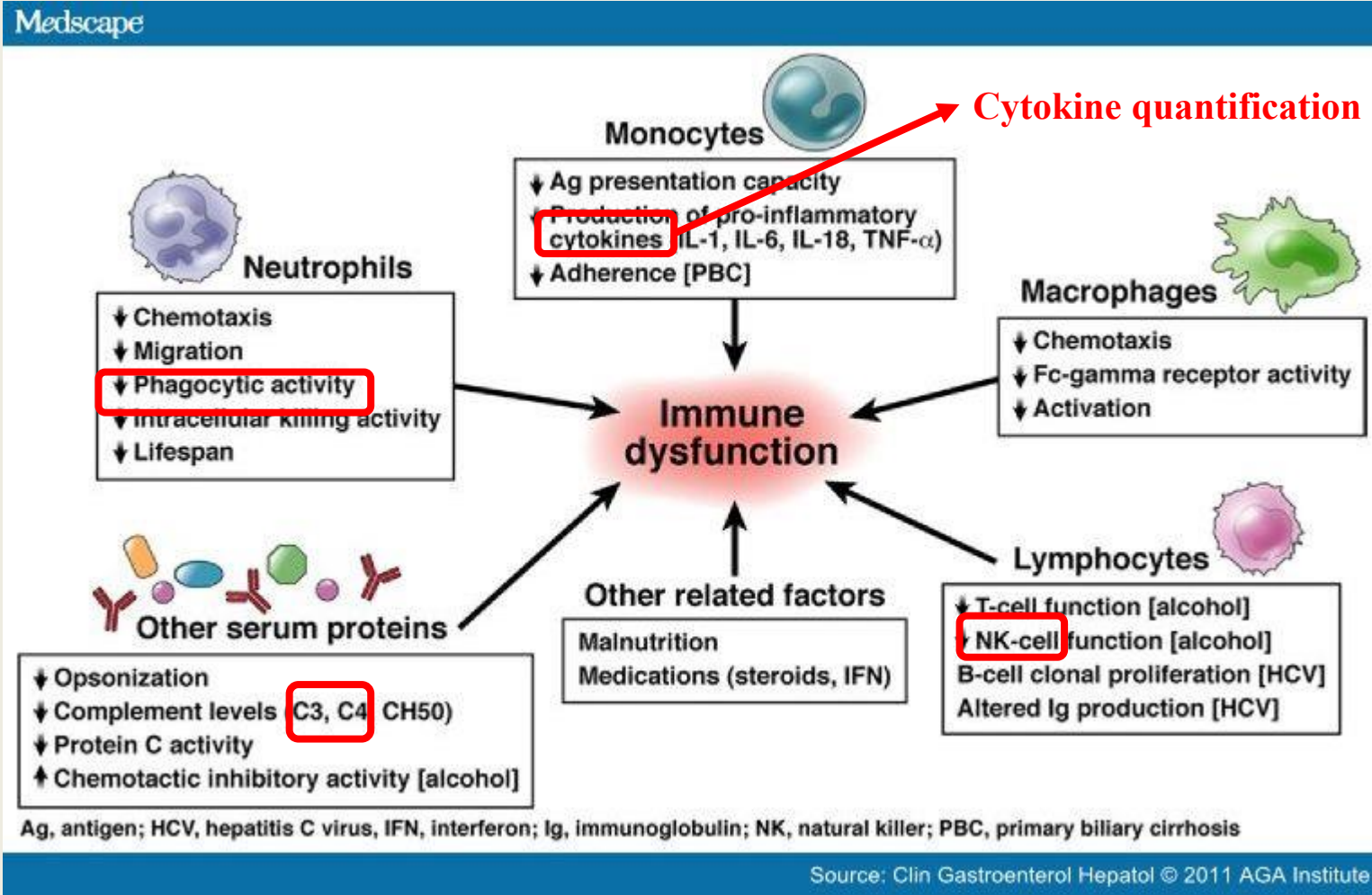
Inflammation



The response of our natural immunity to stress is called inflammation

- Neutrophils and macrophages congregate at the site of injury/infection.
- They phagocyte invaders and release toxic substances such as oxygen radicals.
- Macrophages release **pro-inflammatory cytokines** IL-6 and TNFalpha to organize further inflammatory response.
- Mast cells and eosinophils will be involved in parasitic defense and allergy.
- Natural killer cells eliminate non-self cells by releasing toxic substances.

Immune dysfunction



Testing for immune and metabolic dysfunctions

- **The extent of the global immune and / or metabolic dysfunctions are evaluated by testing:**
 - cytokine expression
 - elastase and perforin mRNA expression
 - oxidative stress, heavy metals, molds
 - macrophage phagocytic activity
 - sCD14 expression
 - C3a & C4a expression
 - CD57 cell subset absolute count
 - prostaglandine E2 (PGE2) synthesis
 - VEGF synthesis
 - ammonia accumulation
 - kynurenic and quinolinic acid accumulation, and many more

Testing for Immune dysfunctions

- **The extent of the global immune dysfunction is evaluated by testing:**

(1) *cytokine expression*

- A principal avenue of investigation has been the measurement in blood of immune signals conducted by cytokines.
- In animal studies, administration of pro-inflammatory cytokines (IL-1, TNF- α , and IL-6) directly into the brain can induce "sickness behaviors" that strongly resemble the symptoms of CFS. In particular, decreased motor activity, altered food and water intake, sleep and cognition have been linked to increases in the levels of IL-1b, IL-6 and TNF- α [Dantzer et al. Nat Rev Neurosci 2008]
- In humans, systemically administered pro-inflammatory cytokines, such as IL-6 and TNF- α typically induce a systemic inflammatory response

Cytokine imbalances in autism spectrum disorders

-Ashwood et al. (Brain Behav Immun. 2011) reported on **significant increases in plasma levels of a number of cytokines**, including IL-1 β , IL-6, IL-8 and IL-12p40 in the ASD group compared with controls.

-Suzuki et al. (PLoS One 2011) reported that the plasma concentrations of IL-1 β , IL-1RA, IL-5, IL-8, IL-12(p70), IL-13, IL-17 and GRO- α were significantly higher in subjects with ASD compared with the corresponding values of matched controls.

-Okada et al. (Prog Neuropsychopharmacol Biol Psychiatry 2007) and Ashwood et al. (J Neuroimmunol. 2008) reported on **decreased serum levels of transforming growth factor beta1 (TGFb1) in patients with autism**, with lower TGFb1 levels associated with lower adaptive behaviors and worse behavioral symptoms, suggesting that

-Al-Ayadhi LY1, Mostafa GA - J Neuroinflammation. 2012 9:158. reported that Children with autism had **significantly higher serum IL-17A** levels than healthy controls (P <0.001), with increased serum levels of IL-17A found in 48.9% of the autism group.

- Patients with severe autism had significantly higher serum IL-17A levels than those with mild to moderate autism (P=0.01), and raised serum IL-17A levels were significantly more common in children with severe autism (67.9%) than in those with mild to moderate autism (17.6%), P=0.001.
- Serum IL-17A levels were raised in the group with autism, and **the levels correlated significantly with the severity of autism**
- Further research is warranted to determine whether the increase of serum IL-17A levels plasma has a pathogenic role in autism, and whether anti- IL-17A therapy could be useful.

Testing for Immune dysfunctions

- The extent of the global immune dysfunction is evaluated by testing:

(2) elastase mRNA expression : a marker of inflammation

- Elastase is an **inflammatory protease** expressed in immune cells (monocytes, neutrophils) that contributes to immune defense by inactivating foreign bacteria but at the same time it causes damage to connective tissue, breaks down cytokines, immunoglobulins and immune cells receptors. An excess, chronic production of elastase is therefore detrimental.

(3) perforin mRNA expression : a mean to evaluate NK cell activation

- Since NK cells play a central role in the defense against viruses, decreased NK activity can lead to the development of opportunistic viral infections. NK cells exert their cytotoxic effect by releasing perforin, a protein that will **destroy the cytoplasmic membrane of target cells** and finally kill them.

Testing for Immune dysfunctions

- The extent of the global immune dysfunction is evaluated by testing:

(4) *CD57 cell subset absolute count*

- CD57+/CD3- cells are a **subset of NK cells**. Their exact function, and what differentiates them from CD56+ NK cells, is not well understood. The absolute number of CD57+/CD3- cells is low in patients suffering from chronic Lyme disease (a disease that follows an infection by a bacteria called Borrelia). **Patients with very low CD57 have significantly more co-infections and persistent immunologic defects** than patients with higher counts. In patients that respond to antibiotic therapy, the number of cells come back to normal, hence this is a useful marker to follow the effect of a therapy.

(5) *sCD14 expression*

- CD14 is expressed in monocytes/macrophages and plays a critical role in the recognition of bacterial cell wall components (LPS). The extracellular part of CD14 can be cleaved and released in the plasma, where **it will inactivate circulating LPS**. Serum soluble CD14 levels are significantly elevated in patients with inflammatory bowel disease, Crohn's disease, but also in patients suffering from Brucellosis or Lyme disease.

Testing for Immune dysfunctions

- **The extent of the global immune dysfunction is evaluated by testing:**

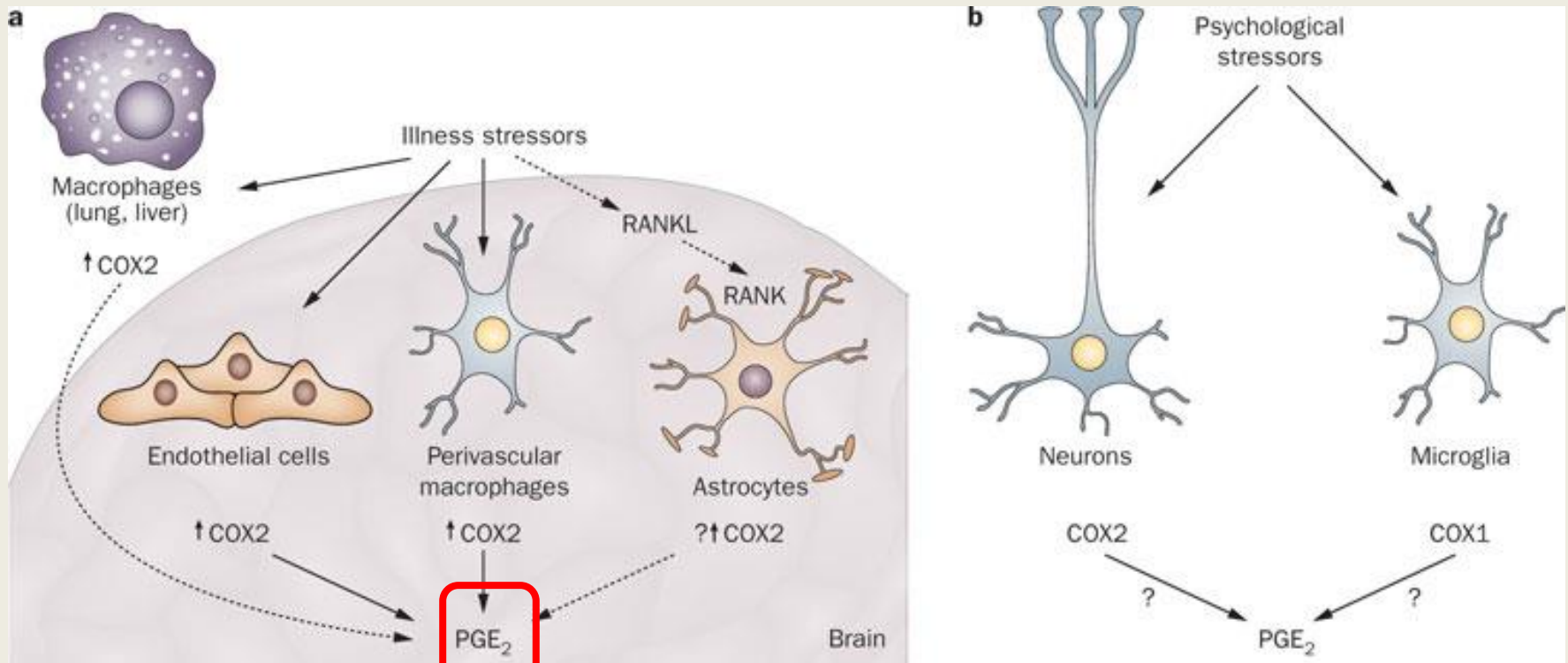
(6) *C4A expression*

- C4a is an anaphylatoxin generated by cleavage of complement component 4 (C4), upon activation of the complement system. C4a increase causes local inflammatory response and symptoms of hypersensitivity. C4a levels are elevated following exercise in CFS patients. A US study has reported that elevated complement C4a was an early marker for Lyme disease in tick bite patients.

(7) *prostaglandine E2 (PGE2) synthesis*

(8) *VEGF synthesis*

PGE₂ production during illness and psychological stress



quantification

Prostaglandine E2

prostaglandine E2 (PGE2) synthesis

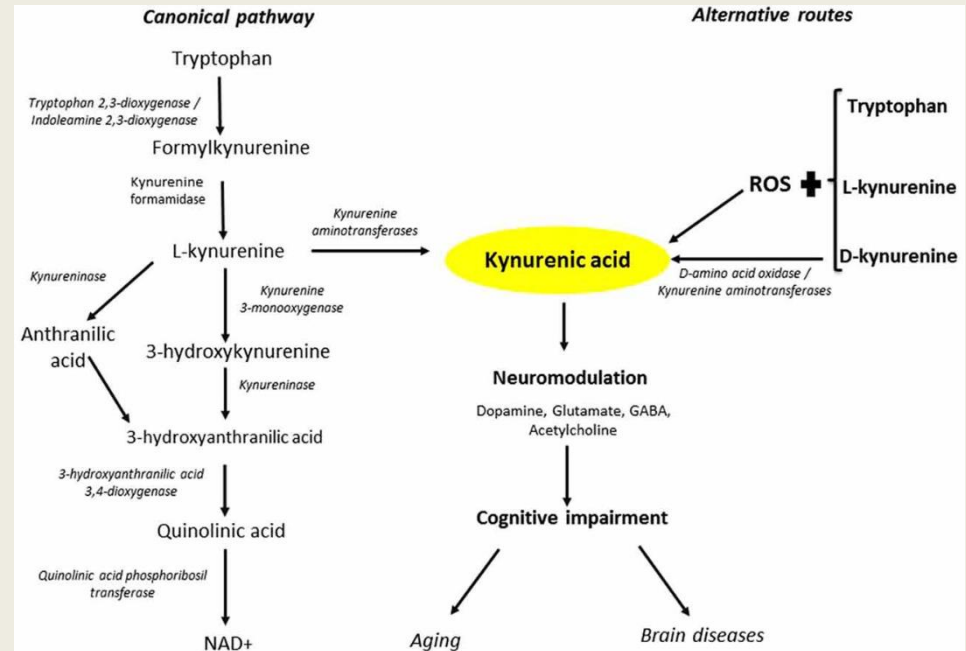
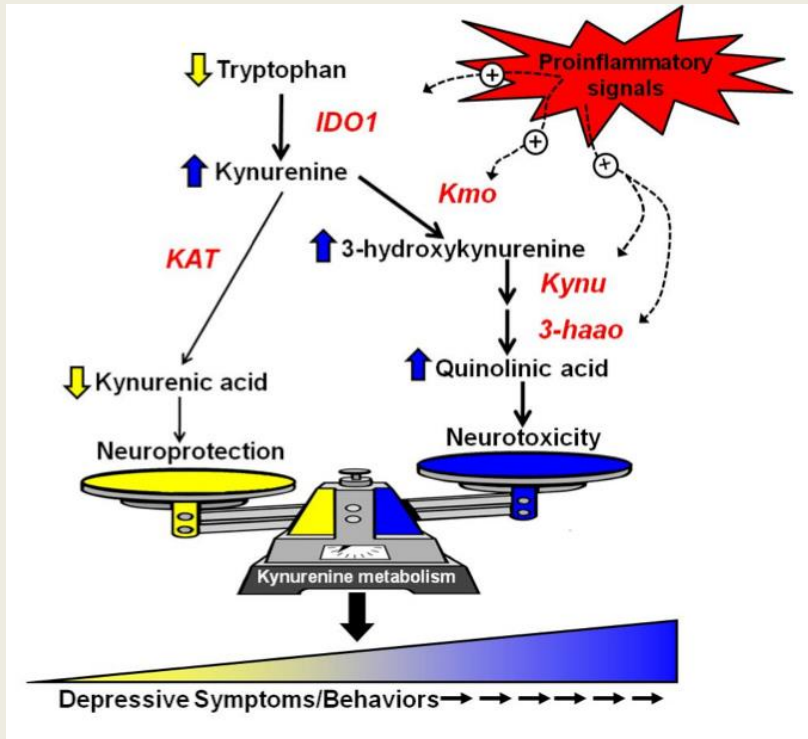
- PGE2 is a compound derived from membrane phospholipids
- PGE2 is also a **key mediator of immunopathology in chronic infections and cancer**
- PGE2 enhances its own production but suppresses acute inflammatory mediators, resulting in its predominance at late/chronic stages of immunity.
- **PGE2 selectively suppresses effector functions of macrophages and neutrophils and the Th1-, CTL-, and NK cell-mediated type 1 immunity, but it promotes Th2, Th17, and regulatory T cell responses.**
- PGE2 modulates chemokine production, inhibiting the attraction of proinflammatory cells while enhancing local accumulation of regulatory T cells and myeloid-derived suppressor cells.

PGE2 has been found as significantly higher in autistic patients

Quantification of serum levels of Prostaglandine E2

- Prostaglandin E2 (**PGE2**) has been found as significantly higher in autistic patients, recording an increase of 91.15% (El-Ansary & Al-Ayadhi, Lipids Health Dis. 2012).
- PGE2 is a compound derived from membrane phospholipids and is a **key mediator of immunopathology in chronic infections and cancer**
- PGE2 selectively suppresses effector functions of macrophages and neutrophils and the Th1-, CTL-, and NK cell-mediated type 1 immunity, but **it promotes Th2, Th17,** and regulatory T cell responses.
- PGE2 modulates chemokine production, inhibiting the attraction of proinflammatory cells while enhancing local accumulation of regulatory T cells cells and myeloid-derived suppressor cells.

Kynurenic and Quinolonic acids



- High levels of kynurenic acid have been identified in patients suffering from **tick-borne encephalitis, schizophrenia and HIV-related illnesses**. In all these situations increased levels were associated with confusion and psychotic symptoms.
- QUINO acts as a neurotoxin, gliotoxin, proinflammatory mediator, prooxidant molecule and can alter the integrity and cohesion of the blood–brain barrier. Quinolinic acid levels are increased in the brains of children infected with a range of **bacterial infections of the central nervous system (CNS)**, in patients with **poliovirus, Lyme disease with CNS involvement, traumatic CNS injury, hyperammonaemia, hypoglycaemia patients, systemic lupus erythematosus, malaria, etc.**

VEGF

- VEGF has a great role in pathological conditions that are **associated to autoimmune diseases** such as in systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis.
- Serum levels of VEGF **correlate with disease activity in a large number of autoimmune diseases** and fall with the use of standard therapy
- Possible future therapeutic strategies in autoimmune diseases with the anti-VEGF or anti-VEGFR (receptor). So far, this therapy has been used in cancer and macular ocular degeneration in diabetes.
- Abnormally **high levels of VEGF** in a mold-free environment would suggest **Bartonella infection**.
- VEGF can **go down** in the presence of indoor molds.

HIF-1a

- HIF1-alpha (HIF1A) is a subunit of HIF1, which is a transcription factor found in mammalian cells cultured under reduced oxygen tension.
- HIF1 functions as a transcriptional regulator of the adaptive response to hypoxia. Tissue hypoxia (=lack of oxygen) is a common feature during inflammation associated with infection.
- **Under hypoxic conditions**, HIF-1 activates the transcription of over 40 genes, including erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia.
- HIF1-alpha regulates hypoxia-mediated apoptosis, cell proliferation and tumor angiogenesis.
- **Activation of HIF-1 is a general phenomenon in infections with human pathogenic bacteria, viruses, fungi and protozoa.**
- HIF-1-regulated pathways might be an attractive target to modulate the course of life-threatening infections.
- Infections with the angiogenic bacterium *Bartonella henselae* (causing the vasculoproliferative disorder bacillary angiomatosis) result in the activation of HIF-1.
- HIF-1alpha has become increasingly investigated as a target against infections, a strategy that may be efficient in multidrug-resistant infections.
- **It might be considered as a kind of marker for infection-induced inflammation.**

MMP-9

-
- MMP-9 : Matrix Metalloproteinase 9
 - MMP-9 delivers inflammatory elements from the blood into sensitive tissues and can combine with PAI-1 to increase clot formation and arterial blockage.
 - MMP-9, along with elastase, appears to be a regulatory factor in neutrophil migration across the basement membrane.
 - MMP-9 plays several important functions within neutrophil action, such as degrading extracellular matrix, activation of IL-1 β , and cleavage of several chemokines.
 - MMP-9 may play an important role in angiogenesis and neovascularization.
 - MMP-9 has been found to be associated with numerous pathological processes, including cancer, placental malaria, immunologic and cardiovascular diseases.
 - Elevated MMP-9 levels can be found in the cases of rheumatoid arthritis and focal brain ischemia.
 - One of MMP-9's most widely associated pathologies is the relationship to cancer, due to its role in extracellular matrix remodelling and angiogenesis.
 - MMP-9 is a nice marker for the presence of excess cytokines production from any inflammatory disease.
 - In Lyme disease, MMP-9 levels may skyrocket as the result of treatment with antibiotics, and the resulting bacterial die-off in what is commonly referred to as a Herxheimer reaction.

Toxic Metabolites

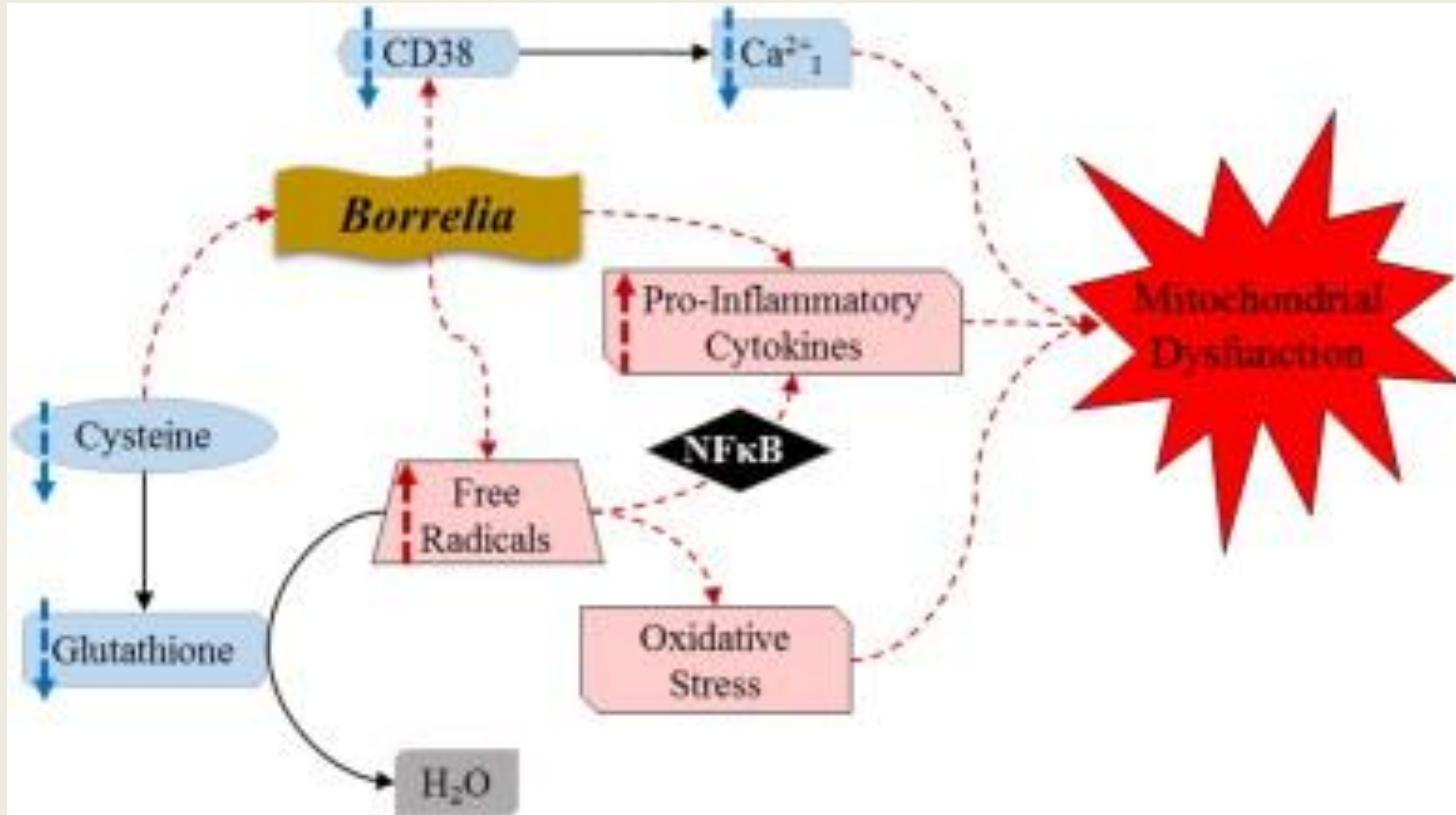
- ***D-lactate in serum***
 - a product of bacterial metabolism, it is neither produced nor metabolized by mammalian cells.
 - Typically, **elevated D-lactate levels are due to bacterial infection or short bowel syndrome** in humans.
 - Due to slow metabolism and excretion, high D-lactate can cause acidosis and encephalopathy.
- ***Ammonia in serum***
 - Ammonia is derived from bacterial enzymatic action on ingested amino acids. It is absorbed from the gastrointestinal tract and delivered through the portal vein to the liver, which converts most of it into urea.
 - Abnormally high levels of ammonia can result from colic or “**enteric hyperammonemia**” (combination of **increased bacterial production and increased gut permeability**) that occurs despite normal hepatic function. Hyperammonemia is a metabolic condition characterized by elevated levels of ammonia in the blood.
 - **Increased entry of ammonia to the brain is a primary cause of neurologic disorders, metabolic disorders and some toxic encephalopathies.**

CD38 serum levels

- **CD38 serum levels**

- CD38, which has an important role in dendritic cells (DC) chemotaxis and migration to lymph nodes, was strongly up-regulated by LPS of Gram - bacterias **but practically not at all by *Borrelia garinii*** (mostly inducing neuroborreliosis).
- *Borrelia garinii* may affect crucial DC functions by blocking the up-regulation of important molecules in DC migration to lymph nodes, thus affecting further immune responses in Lyme borreliosis infection (Hartiala et al. 2007, 2010).
- *B. burgdorferi sensu stricto* and *B. afzelii* are also unable to induce CD38 upregulation.
- Thus **low levels of CD38 might indicate *Borrelia* infection, while high levels of CD38 might indicate other Gram- infections and/or leaky gut**

CD38

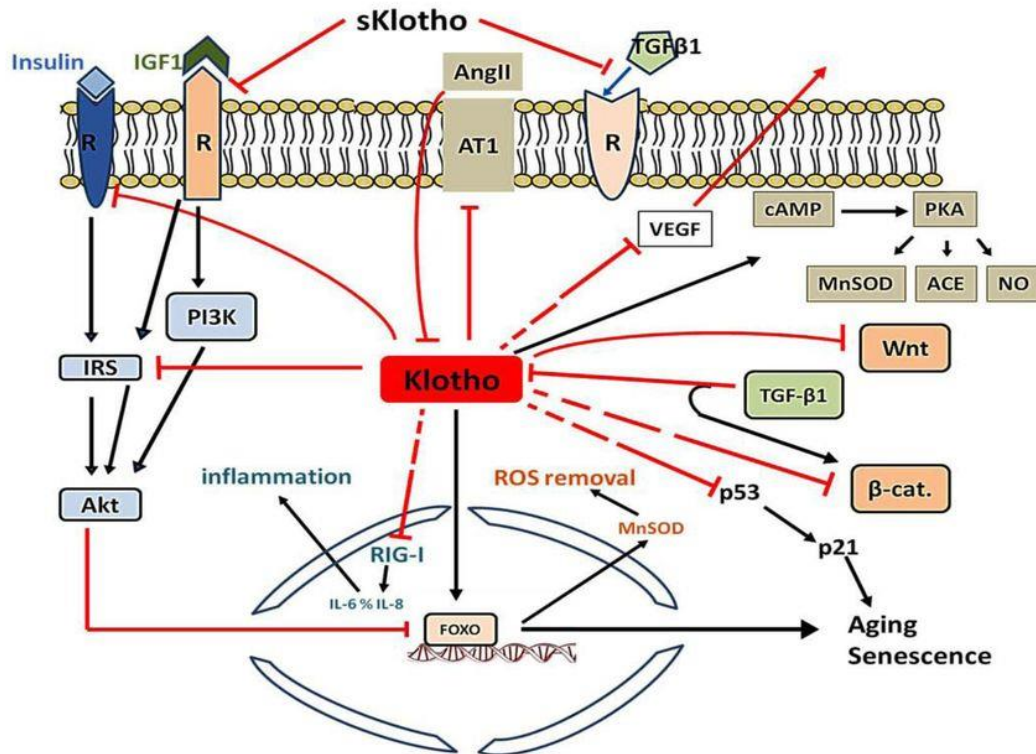


From Peacock et al. Redox Biology 2015

Antiaging - KLOTHO

- **Klotho serum levels**
 - Klotho - a marker of longevity and the body's ability to regenerate.
 - Klotho gene is the first documented aging suppressor gene in mammals that can delay aging when overexpressed and accelerate aging when disrupted.
 - The three forms of Klotho protein have distinct functions:
 - **Membrane Klotho** forms a complex with fibroblast growth factor (FGF) receptors, functions as an obligatory co-receptor for FGF23, which is involved in aging and the development of chronic diseases via regulation of Pi and vitamin D metabolism.
 - **Secreted Klotho** functions as a humoral factor with pleiotropic activities including **regulation of oxidative stress, growth factor signaling, and ion homeostasis**. Secreted Klotho is also involved in organ protection.
 - The **intracellular form of Klotho** suppresses inflammation-mediated cellular senescence and mineral metabolism.

Antiaging - KLOTHO



Schematic representation of the Klotho participating in intracellular signaling pathways. Klotho protein is involved in several intracellular signaling pathways that are essential for the regulation of many cellular processes, including aging and senescence.

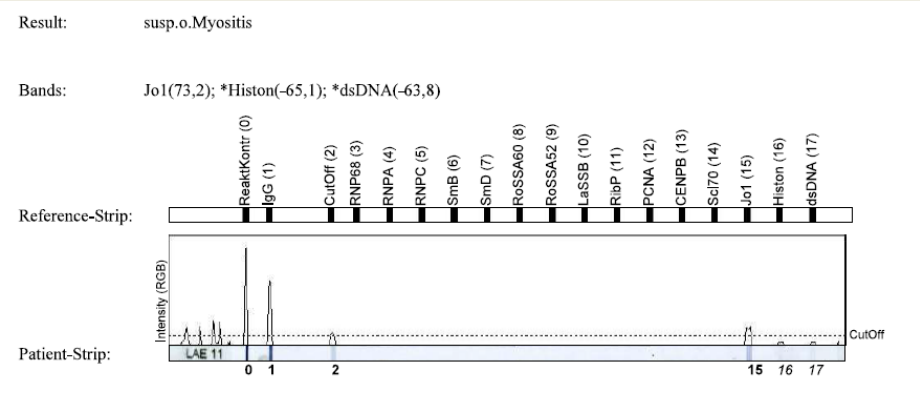
*From: M. Sopjani et al. Klotho and Intracellular Signaling
Current Molecular Medicine, 2015, Vol. 15, No. 1 33*

Klotho can act as a circulating factor or hormone, which binds to a not yet identified high-affinity receptor and **inhibits the intracellular insulin/insulin-like growth factor-1 (IGF-1) signaling cascade**; klotho can function as a novel β -glucuronidase, which deglycosylates steroid β -glucuronides and the calcium channel transient receptor potential vallinoid-5 (TRPV5); as a **cofactor essential for the stimulation of fibroblast growth factor (FGF) receptor by FGF23.**

Autoantibodies

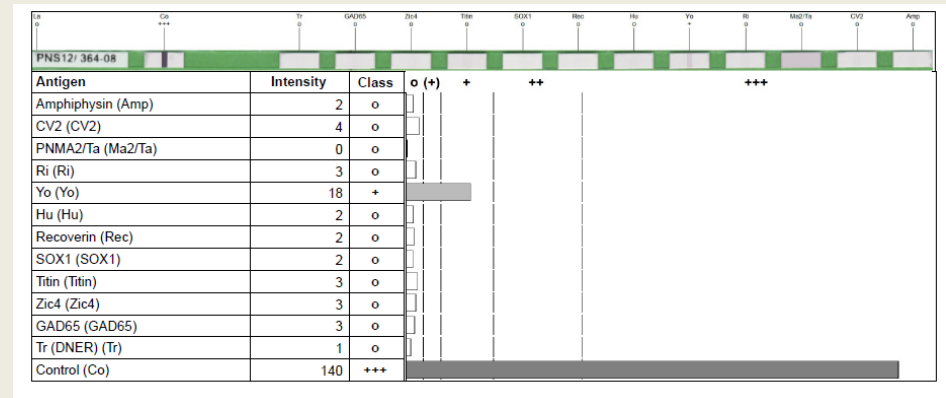
ANA/ENA

- ANA/ENA IgG immunoblot with 15 antigens is an immunoblot for autoantibodies in Connective Tissue Diseases CTD - Systemic lupus erythematosus (SLE), Sjögren's syndrom (SjS), Mixed connective tissue disease (MCTD), Progressive systemic scleroderma (PSS) & Myositis).
- ANA/ENA IgG serves for the differentiation between rheumatic autoimmune diseases and other rheumatic diseases with similar symptoms.



Autoantibodies for neuronal antigens

- AA-Neuro IgG immunoblot for autoantibodies for neuronal antigens
- The detection of these antibodies in sera of patients with neurological symptoms indicates the presence of a paraneoplastic neurological syndrome (PNS).



Genotyping

- **GFOL : Methylation cycle polymorphisms**

- 677C-T and 1298A-C are two SNPs in the gene coding for MTHFR (methylene tetrahydrofolate reductase).
- MTHFR is involved in biological pathways related to DNA synthesis, DNA methylation (important for gene regulation), neurotransmitter synthesis, myelin synthesis, glutathione synthesis (important for detoxification and antioxidant activities). Folic acid itself is a strong antioxidant.
- Pu et al. (Autism Res 2013) indicated that the **MTHFR C677T polymorphism contributes to increased ASD risk**, and periconceptual folic acid may reduce ASD risk in those with MTHFR 677C>T polymorphism.

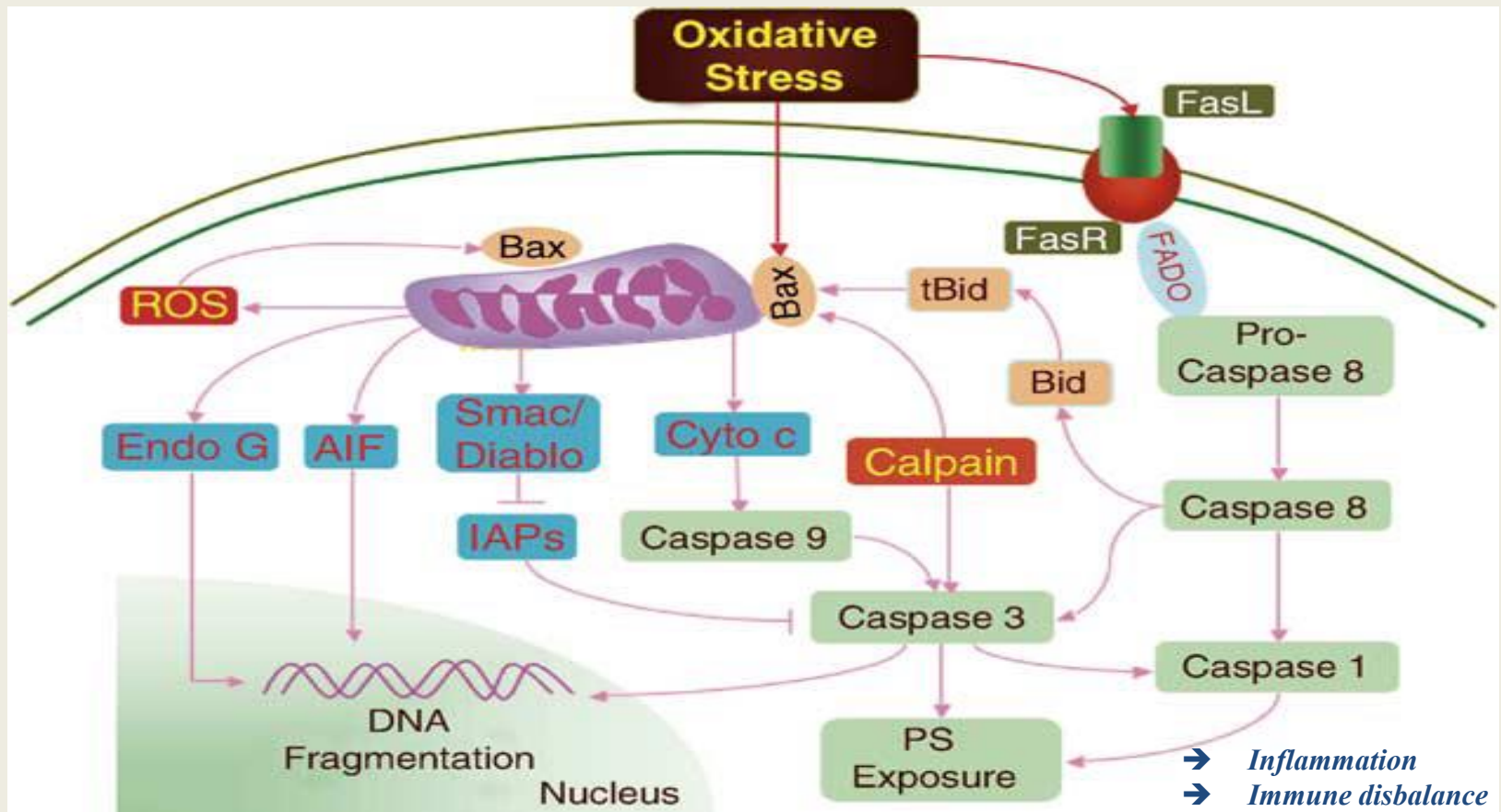
- **GVDR : Polymorphisms of Vitamin D receptor**

- Bsm1 and Fok1 are two SNPs in the gene coding for VDR (vitamin D receptor).
- VDR is involved in skeletal metabolism but also in modulation of immune response, regulation of cell proliferation and differentiation. VDR dysfunction has been linked with osteoarthritis, cancer, diabetes, cardiovascular disease

- **Lactase deficiency assay**

- a polymorphism in the gene coding for lactase, an enzyme responsible for the digestion of lactose (C/T-13910 polymorphism). In affected people, **production of the enzyme declines during or shortly after childhood, resulting in lactose malabsorption**. Undigested lactose sugars affect the development of gut microflora, leading to dysbiosis.

Oxidative Stress Tests



Environmental exposure and oxidative stress

- Oxidative stress is implicated in a large number of diseases, including neurodegenerative diseases and **autoimmune diseases**
- Indicators of oxidative stress have been detected in muscles and blood
- Oxidative damage to cellular membranes can alter the permeability of the blood-brain barrier, which could lead to some of the **cognitive symptoms** observed in patients.
- Increased oxidative stress could have several origins: **chronic inflammation** (activated neutrophils release pro-oxidative molecules), **excess nitric oxide production** (NO reacts with free radicals to produce peroxynitrite, a potent oxidant), or **exposure to environmental toxins** (exposure to certain chemicals leads to the depletion of essential antioxidants such as glutathione and selenium; heavy metals can also directly inhibit antioxidant enzymes like superoxide dismutase or glutathione reductase).
- Oxidative stress markers are useful to evaluate the need for antioxidant therapy.

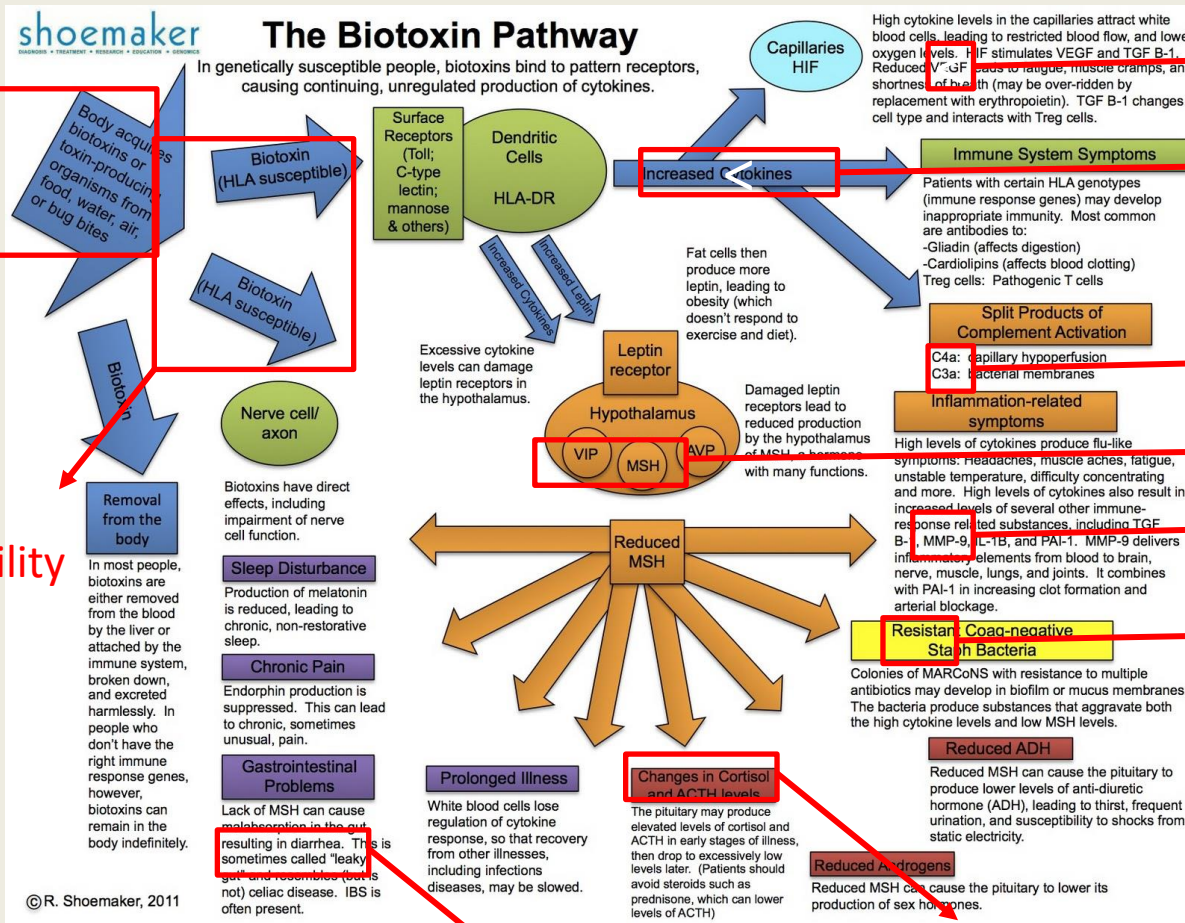
Environmental exposure and oxidative stress testing options

- Colorimetric assay that measures the **total antioxidant capacity** of a sample (e.g., serum), based on the reduction of Cu^{++} to Cu^{+} (**ANOX** assay).
- Lipid peroxidation is evaluated by **OXFA** assay, based on the capacity of MDA to react with thiobarbituric acid (TBA), forming an MDA-TBA adduct that can be quantified spectrophotometrically.
- Additional extensive testing offer through Great Plains Laboratories, US.

Focus on CIRS

- CIRS = Chronic Inflammatory Response Syndrome
- Dr. Ritchie Shoemaker: Pioneer in CIRS, Mold & Biotoxins
 - ➔ Body acquires biotoxins or toxin-producing organisms from food, water, air or bug bites
 - ➔ Biotoxins cause continuing, unregulated production of cytokines
 - ➔ Multiple damages to the body with inflammation-related symptoms, immune system symptoms, resistant bacteria, chronic pain, sleep disturbance, gastrointestinal problems,....
 - ➔ Investigation of several markers from Shoemaker biotoxin pathway

CIRS-related Testing



VEGF

Cytokines

C3A & C4A

VIP & a-MSH

MMP-9

MARCoNS

Altered hormone production

Gastrointestinal problems

Questions and Contacts

- Material available on the website (www.redlabs.com)
- Check regularly our website (www.redlabs.com) for the updates



- Questions and contact:
 - General queries, logistics : E-mail to info@redlabs.be
 - Scientific questions : E-mail to tmijatovic@redlabs.be