New biomedical approach for laboratory testing of chronic fatigue syndrome (CFS) patients

Madrid, October 14 2013

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R.E.D. Laboratories

- R.E.D. Laboratories is a biotechnology company developing tests for chronic immune diseases
- We actively pursue new tests development in order to provide clinicians with updated tools to assess immune dysfunctions

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New biomedical approach for laboratory testing of chronic fatigue syndrome patients : *Objectives*

- Over the past several years, scientists at R.E.D. Laboratories have been deciphering the biochemical and molecular mysteries surrounding CFS
- The main goal of these efforts is **to create a number of objective clinical laboratory assays** that can assist the physician in not only making the diagnosis of CFS, but can also help in staging the severity of disease and monitor the effect of a therapy
- Based on the scientific literature and extensive collaboration with physicians specialized in CFS diagnostic and treatment, **we developed a new testing panel that aims to contribute to a better management of CFS patients**

New biomedical approach for laboratory testing of chronic fatigue syndrome patients : *Our approach*

- More and more evidence points towards a combination of factors (genetic, infectious, environmental, etc.) being important in the development of chronic immune dysfunction, the cardinal finding in all CFS patients
- We identified **3 major groups of factors pointing to the subtype of the CFS-related disorders**
  - ***global immune dysfunctions***
  - ***persistent and/or chronic infections***
  - ***intestinal dysfunctions***

CFS-related disorders subgroups: *1. global immune dysfunction*

- **The extent of the global immune dysfunction is evaluated by testing:**
  - cytokine expression
  - elastase mRNA expression
  - perforin mRNA expression
  - macrophage phagocytic activity
  - alpha-N-acetylgalactosaminidase activity (Nagalase testing)
  - sCD14 expression
  - C4a expression
  - CD57 cell subset absolute count
  - prostaglandine E2 (PGE2) synthesis.

CFS-related disorders subgroups: *1. global immune dysfunction*

- **The extent of the global immune dysfunction is evaluated by testing:**
  - cytokine expression
    - *CFS has a propensity to over-produce pro-inflammatory cytokines (e.g., TNF-α coupled with a decreased production of anti-inflammatory cytokines.*
    - *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 where one of the major symptoms is intense fatigue*

CFS-related disorders subgroups: *1. global immune dysfunction*

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

CFS-related disorders subgroups: *1. global immune dysfunction*

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

CFS-related disorders subgroups: *1. global immune dysfunction*

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

CFS-related disorders subgroups: *1. global immune dysfunction*

- **The extent of the global immune dysfunction is evaluated by testing:**
  - macrophage phagocytic activity
    - *Macrophages and neutrophils constitutes one of the main effectors of nonspecific immunity, phagocytosing conventional and opportunistic microorganisms and presenting their antigens to T lymphocytes for inducing specific immune functions*
  - alpha-N-acetylgalactosaminidase activity (Nagalase testing)
    - *Nagalase is a component of viral hemagglutinin and is released by the action of trypsin on hemagglutinin. In the absence of recent viral infection or malignancies, elevated Nagalase activity likely represents a marker of viral production of hemagglutinin protein being acted upon by inflammatory cell mediated trypsin activity; as such* ***it may represent viral persistence, active transcription, and inflammation***
    - *Beyond this, Nagalase is an enzyme that deglycosylates the Gc protein also known as vitamin D binding protein (VDBP), rendering it incapable of conversion to active GcMAF (Gc protein-derived Macrophage Activating Factor) and thereby* ***preventing its regulation of macrophage activation.***

CFS-related disorders subgroups: *1. global immune dysfunction*

- **The extent of the global immune dysfunction is evaluated by testing:**
  - prostaglandine E2 (PGE2) synthesis
    - *Prostaglandin E2 (PGE2) is a compound derived from membrane phospholipids and an important mediator of synaptic plasticity, pain response, sleep/ awake cycle and is believed to be associated with inflammation in the brain.*
    - *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. PGE(2) modulates chemokine production, inhibiting the attraction of proinflammatory cells while enhancing local accumulation of regulatory T cells cells and myeloid-derived suppressor cells.*
- **The PGE2 overexpression and increased Nagalase activity emerged as very specific CFS markers**

CFS-related disorders subgroups:

2. persistent and/or chronic infections

- A number of pathogens have been associated with CFS
- **A majority of CFS patients describe an infectious onset of their disease**
- Pathogens that are often found in CFS patients include herpesviruses like HHV-6, HHV-7, and EBV; Borrelia, Bartonella, Mycoplasma and Chlamydia species
- Some of these pathogens can be detected in the blood, but infections may also be localized in specific tissues. In a recent study, persistent Parvovirus B19 infections were detected in the gastric and intestinal mucosa of CFS patients
- Persistent infections, and especially chronic zoonoses (like chronic Lyme disease) in CFS patients can contribute to the maintenance of the disease and to the worsening of the symptoms
- **R.E.D. Labs scientists developed a range of serology and PCR-based assays to support chronic infection testing**.

CFS-related disorders subgroups:

2. persistent and/or chronic infections

- **Chronic Lyme disease can mimic every disease process including Chronic Fatigue Syndrome (Myalgic Encephalomyelitis), Fibromyalgia,** Autoimmune conditions including sero-negative rheumatoid arthritis and MS, Psychiatric conditions including depression and anxiety, and cause significant memory and concentration problems mimicking early dementia. **It is called the "Great Imitator“**
- If an individual has **any chronic health condition**, ranging from arthritis to chronic fatigue syndrome to fibromyalgia, it is **important to rule out or diagnose Lyme disease.** It is apparent that **many cases of fibromyalgia and chronic fatigue syndrome are actually Lyme disease in disguise**
- **Chronic Lyme sufferers also frequently house “co-infections”** such as Mycoplasma, Chlamydias, Ehrlichia, Bartonella and Babesia. These are different types of “bugs” that enjoy the company of B. burgdorferi. Each infection must be addressed in the treatment protocol.

CFS-related disorders subgroups:

2. persistent and/or chronic infections

- **R.E.D. Labs scientists developed a range of serology and PCR-based assays to support chronic infection testing**:
  - **Immunoblot for Borrelias**
  - **Immunoblot for Chlamydias (pneaumoniae, trachomatis, psittacii)**
  - **Immunoblot for Yersinia**
  - **PCRs for Mycoplasma spp, Mycoplasma fermentans, Mycoplasma pneumoniae, Bartonella, Brucella, Coxiella, Babesia, Anaplasma, Ehrlichia, Chlamydias**
  - **PCRs for viral infections:** herpesviruses like HHV-6, HHV-7, HHV-8, Parvovirus, EBV

CFS-related disorders subgroups:
3. intestinal dysfunctions

Evidence for GI dysfunction in CFS

- ***Gastro-intestinal symptoms in patients***
  - More than 90% of CFS patients will present IBS symptoms during their life
  - co-occurrence of CFS and IBS is associated with increased levels of inflammatory cytokines *[Aaron et al., Arch Internal Med 2000; Scully et al., Am J Gastroenterol 2010]*
- ***Endoscopic evidence***
  - Endoscopic examination of duodenum or stomach almost systematically reveal the existence of inflammed areas of the mucosa
- ***Inflammatory markers in stools***

CFS-related disorders subgroups:
3. intestinal dysfunctions

Regulation of immune function in the gut

- **70% of our immune cells reside in the gut**
  - Gut associated lymphoid tissue (GALT)is spread along the intestinal mucosa (Peyer's patch in the small intestine, lymphoid follicles in the colon) and hosts 70% of the body's immune cells
  - These immune cells permanently interact with mucosa-associated micro-organisms (bacteria, viruses…)
  - A delicate balance is maintained between tolerance to gut antigens (down-regulation of inflammation,…), and defense against pathogens (production of defensins,…)
- **Imbalance of gut immunity affects the whole body**
  - ***Gut barrier integrity is essential :*** Increased permeability of the mucosa causes systemic endotoxemia (chronic low grade inflammation) and abnormal immune reactions to gut antigens
  - ***Interactions host/gut flora :*** the gut microbial flora plays a major role in maintenance of host health, but can be affected by abnormal host immune function

CFS-related disorders subgroups: *3. intestinal dysfunctions*

Establishment and composition of the gut flora

- ***Human GI tract is colonized by 10^{14} microorganisms***
  - 10 times more than human cells, 1.5kg of biomass.
- ***Colonization starts immediately at birth***
  - **Establishment of specific populations** will depend on many factors, (normal birth or cesarean section, breast- vs formula feeding, hygiene conditions during the first months of life, early use of antibiotics, genetics…). At 1-2 years of age the ecosystem stabilizes.
  - **Extreme diversity** (500 to 1,000 different bacterial species)
  - **Specificity**: each individual displays a unique pattern of microbial diversity
  - **Apparent stability** over life, good resilience, but can be affected by drugs, infections, diet changes…

CFS-related disorders subgroups:
3. intestinal dysfunctions

Gut flora and health

Germ-free mice can survive but present developmental and morphological alterations, as well as abnormalities in their immunological and nutritional functions. Although some bacteria are potentially pathogenic, host-bacterial interaction is mainly symbiotic

The microbiome contributes to the processing and metabolization of food

- digestion and absorption of nutrients

- sugar metabolism

-synthesis of short chain fatty acids (eg, synth. of butyrate by Faecalibacterium, Roseburia… is important for colon health… but gives a lot of energy -> obesity)

- synthesis of amino acids and vitamins (vit B12, vit B9, vit K)

-detoxification of pollutants and toxic molecules present in the food

- regulation of immune function

CFS-related disorders subgroups: *3. intestinal dysfunctions*

Dysbiosis in CFS

A frequent disorder of intestinal function is dysbiosis, i.e. the overgrowth of pathogenic bacteria in the intestine.

- ***Several published studies suggest that CFS is associated with dysbiosis***

  - Culture-based assays revealed **increased levels of *Streptococcus* and *Enterococcus spp*.** in faecal samples of ME/CFS patients *[Sheedy et al., In Vivo 2009]*

  - **Probiotic supplementation** (*L. casei* in one study, *L. paracasei* + *L. acidophilus* + *B. lactis* in another study) **resulted in improved** emotional symptoms and neurocognitive functions *[Rao et al., Gut Pathog 2009; Sullivan et al., Nutr J 2009]*

CFS-related disorders subgroups:

3. intestinal dysfunctions

We use the following assays to investigate intestinal dysfunctions:

- ***IgA/IgM against intestinal bacteria***

  -an antibody screening assay for antibodies (IgA and IgM) directed against antigens from intestinal pathogens. IgA are secreted from intestinal cells, IgM are produced by immune cells in the blood. In healthy individuals pathogenic bacteria are only found in low quantities in the gut, and antibody titers in the blood are very low. In case of bacterial overgrowth however, large quantities of IgA are produced and some IgA will be found in the bloodstream. In case of leaky gut, bacterial proteins may make their way to the bloodstream, and specific IgM will be produced. Therefore, high titers of IgM for intestinal bacteria is an indicator of increased intestinal permeability.

- ***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.

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CFS-related disorders subgroups:

3. intestinal dysfunctions

We use the following assays to investigate intestinal dysfunctions:

- ***sIgA ELISA tests in stool samples***

  -sIgA key function is to bind to invading micro organisms and toxins and entrap them in the mucus layer or within the epithelial cells, so inhibiting microbial motility, agglutinating the organisms and neutralising their exotoxins and then assist in their harmless elimination from the body in the fecal flow.

  -sIgA also 'tags' food as acceptable, so low sIgA leads to increased sensitivity to foods.

The concentration of sIgA gives us information about the intestinal immune defense:

A lack of sIgA indicates a **diminished activity** of the intestinal immune system

An increased level of sIgA shows **intestinal inflammation**.

- ***Inflammation markers in stool samples***

  -Hemoglobin : *discharged with the feces in gastrointestinal bleeding diseases*

  -Transferrin*: a blood-derived component ; a good marker for gastrointestinal bleeding*

  -Calprotectin: *a neutrophil cytosolic protein with antimicrobial properties, which is present at increased concentration in stool during bowel inflammation*

  *-**Lactoferrin:** a glycoprotein component of neutrophil secondary granules, a primary component of the acute inflammatory response released from fecal leukocytes; may serve as a marker of inflammation in the intestine*

CFS-related disorders subgroups:
3. intestinal dysfunctions

R.E.D. Labs scientists have developed and validated a new procedure to analyze bacterial populations in a stool sample : MSA assay

- ***New molecular technique involving sequencing of specific regions of bacterial DNA (metagenomics)***
  - Can be performed on dead organisms (exposure to oxygen, freezing are not a problem)
  - Identification of each bacteria by comparing sequence with public databases: extremely precise, not subjective
  - High-throughput technology allows identification of tens or even hundreds of thousends organisms in a single sample
- ***Methods***
  - Bacterial DNA was extracted from stool samples, PCR amplification was performed on 16S rRNA gene regions, and PCR amplicons were sequenced using Roche FLX 454 sequencer. Bacteria were classified by phylum, family and genus. Data were analyzed using Mann-Whitney test and linear discriminant analysis.

CFS-related disorders subgroups: *3. intestinal dysfunctions*

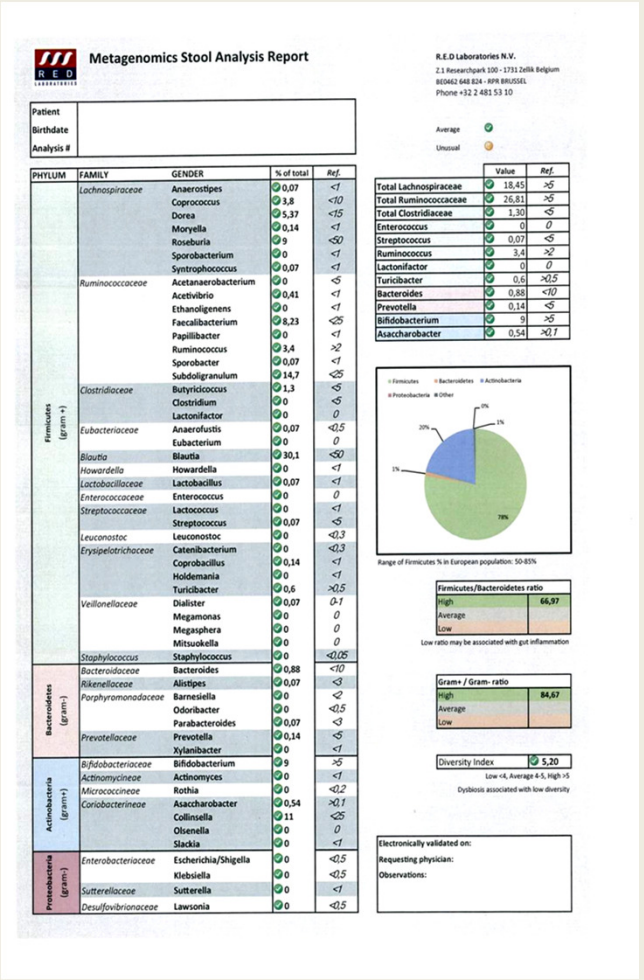
Intestinal microbiota analysis : from culture to high-throughput sequencing

- ***Until recently research into microbiota composition relied almost exclusively on culture***
  - *40 to 80% of gut bacteria cannot be cultured*
  - *Identification of colonies can be difficult*
  - *Bacteria must be alive: studies of anaerobes very difficult, major loss during collection and processing of samples*
  - *Culture approach may address only a small fraction of all bacterial species (10%?)*
  - *E.coli once thought to be a dominant species, is a minor member…*
- ***R.E.D. Labs scientists have developed and validated a new procedure to analyze bacterial populations in a stool sample***

CFS-related disorders subgroups:

3. intestinal dysfunctions

- **MSA assay : results example for a control subject**
- Bacteria are classified by phylum (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria) and family.
- For each bacterial genus, percentage of total is reported.
- Diversity index is also calculated.



Metagenomics Stool Analysis Report

R.E.D Laboratories N.V.
Z.1 Researchpark 100 - 1731 Zellik Belgium
BE0462 648 824 - RPR BRUSSEL
Phone +32 2 481 53 10

Patient
Birthdate
Analysis #

Average
Unusual

PHYLUM	FAMILY	GENDER	% of total	Ref.
Firmicutes (gram +)	Lachnospiraceae	Anaerostipes	0,07	<1
		Coprococcus	3,8	<10
		Dorea	5,37	<15
		Moryella	0,14	<1
		Roseburia	9	<50
		Sporobacterium	0	<1
		Syntrophococcus	0,07	<1
	Ruminococcaceae	Acetanaerobacterium	0	<5
		Acetivibrio	0,41	<1
		Ethanoligenens	0	<1
		Faecalibacterium	8,23	<25
		Papillibacter	0	<1
		Ruminococcus	3,4	>2
		Sporobacter	0,07	<1
		Subdoligranulum	14,7	<25
	Clostridiaceae	Butyricoccus	1,3	<5
		Clostridium	0	<5
		Lactonifactor	0	0
	Eubacteriaceae	Anaerofustis	0,07	<0,5
		Eubacterium	0	0
	Blautia	Blautia	30,1	<50
	Howardella	Howardella	0	<1
	Lactobacillaceae	Lactobacillus	0,07	<1
	Enterococcaceae	Enterococcus	0	0
	Streptococcaceae	Lactococcus	0	<1
		Streptococcus	0,07	<5
	Leuconostoc	Leuconostoc	0	<0,3
	Erysipelotrichaceae	Catenibacterium	0	<0,3
		Coprobacillus	0,14	<1
		Holdemania	0	<1
		Turicibacter	0,6	>0,5
	Veillonellaceae	Dialister	0,07	0-1
		Megamonas	0	0
		Megasphera	0	0
		Mitsuokella	0	0
	Staphylococcus	Staphylococcus	0	<0,05
Bacteroidetes (gram-)	Bacteroidaceae	Bacteroides	0,88	<10
	Rikenellaceae	Alistipes	0,07	<3
	Porphyromonadaceae	Barnesiella	0	<2
		Odoribacter	0	<0,5
		Parabacteroides	0,07	<3
	Prevotellaceae	Prevotella	0,14	<5
		Xylanibacter	0	<1
Actinobacteria (gram+)	Bifidobacteriaceae	Bifidobacterium	9	>5
	Actinomycineae	Actinomyces	0	<1
	Micrococcineae	Rothia	0	<0,2
	Coriobacterineae	Asaccharobacter	0,54	>0,1
		Collinsella	11	<25
		Olsenella	0	0
		Slackia	0	<1
Proteobacteria (gram-)	Enterobacteriaceae	Escherichia/Shigella	0	<0,5
		Klebsiella	0	<0,5
	Sutterellaceae	Sutterella	0	<1
	Desulfovibrionaceae	Lawsonia	0	<0,5

	Value	Ref.
Total Lachnospiraceae	18,45	>5
Total Ruminococcaceae	26,81	>5
Total Clostridiaceae	1,30	<5
Enterococcus	0	0
Streptococcus	0,07	<5
Ruminococcus	3,4	>2
Lactonifactor	0	0
Turicibacter	0,6	>0,5
Bacteroides	0,88	<10
Prevotella	0,14	<5
Bifidobacterium	9	>5
Asaccharobacter	0,54	>0,1

Range of Firmicutes % in European population: 50-85%

Firmicutes/Bacteroidetes ratio	
High	66,97
Average	
Low	

Low ratio may be associated with gut inflammation

Gram+ / Gram- ratio	
High	84,67
Average	
Low	

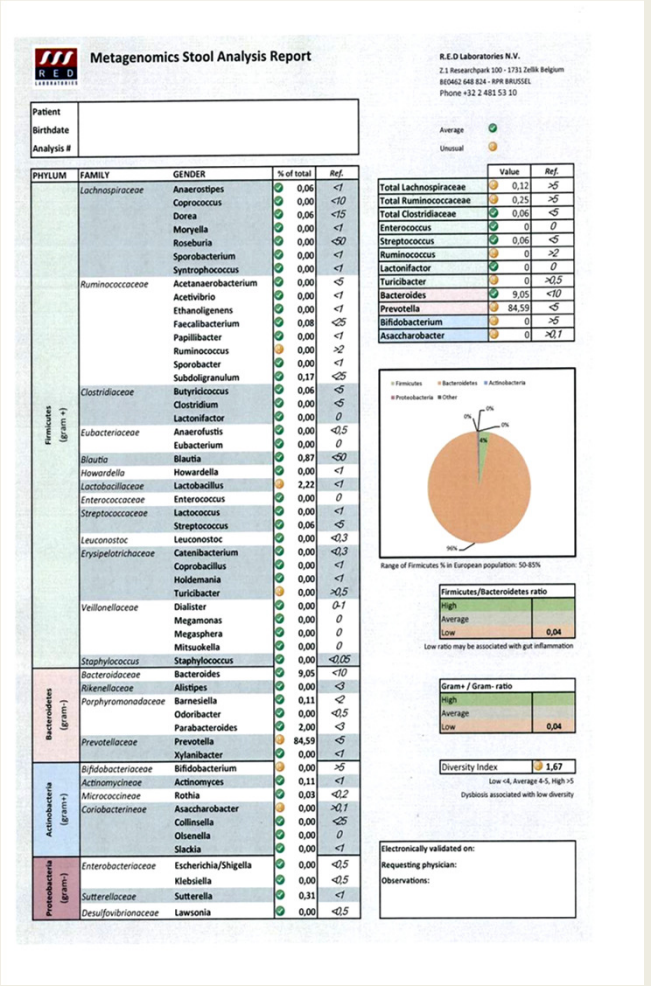
Diversity Index	5,20

Low <4, Average 4-5, High >5
Dysbiosis associated with low diversity

Electronically validated on:
Requesting physician:
Observations:

CFS-related disorders subgroups:
3. intestinal dysfunctions

- **MSA assay : results example of a patient (I)**
- Highly abnormal sample: mostly Prevotella.



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Patient
Birthdate
Analysis #

Average
Unusual

PHYLUM	FAMILY	GENDER	% of total	Ref.
Firmicutes (gram +)	Lachnospiraceae	Anaerostipes	0,06	<1
		Coprococcus	0,00	<10
		Dorea	0,06	<15
		Moryella	0,00	<1
		Roseburia	0,00	<50
		Sporobacterium	0,00	<1
		Syntrophococcus	0,00	<1
	Ruminococcaceae	Acetanaerobacterium	0,00	<5
		Acetivibrio	0,00	<1
		Ethanoligenens	0,00	<1
		Faecalibacterium	0,08	<25
		Papillibacter	0,00	<1
		Ruminococcus	0,00	>2
		Sporobacter	0,00	<1
		Subdoligranulum	0,17	<25
	Clostridiaceae	Butyricicoccus	0,06	<5
		Clostridium	0,00	<5
		Lactonifactor	0,00	0
	Eubacteriaceae	Anaerofustis	0,00	<0,5
		Eubacterium	0,00	0
	Blautia	Blautia	0,87	<50
	Howardella	Howardella	0,00	<1
	Lactobacillaceae	Lactobacillus	2,22	<1
	Enterococcaceae	Enterococcus	0,00	0
	Streptococcaceae	Lactococcus	0,00	<1
		Streptococcus	0,06	<5
	Leuconostoc	Leuconostoc	0,00	<0,3
	Erysipelotrichaceae	Catenibacterium	0,00	<0,3
		Coprobacillus	0,00	<1
		Holdemania	0,00	<1
		Turicibacter	0,00	>0,5
	Veillonellaceae	Dialister	0,00	0-1
		Megamonas	0,00	0
		Megasphera	0,00	0
		Mitsuokella	0,00	0
	Staphylococcus	Staphylococcus	0,00	<0,05
Bacteroidetes (gram-)	Bacteroidaceae	Bacteroides	9,05	<10
	Rikenellaceae	Alistipes	0,00	<3
	Porphyromonadaceae	Barnesiella	0,11	<2
		Odoribacter	0,00	<0,5
		Parabacteroides	2,00	<3
	Prevotellaceae	Prevotella	84,59	<5
		Xylanibacter	0,00	<1
Actinobacteria (gram+)	Bifidobacteriaceae	Bifidobacterium	0,00	>5
	Actinomycineae	Actinomyces	0,11	<1
	Micrococcineae	Rothia	0,03	<0,2
	Coriobacterineae	Asaccharobacter	0,00	>0,1
		Collinsella	0,00	<25
		Olsenella	0,00	0
		Slackia	0,00	<1
Proteobacteria (gram-)	Enterobacteriaceae	Escherichia/Shigella	0,00	<0,5
		Klebsiella	0,00	<0,5
	Sutterellaceae	Sutterella	0,31	<1
	Desulfovibrionaceae	Lawsonia	0,00	<0,5

	Value	Ref.
Total Lachnospiraceae	0,12	>5
Total Ruminococcaceae	0,25	>5
Total Clostridiaceae	0,06	<5
Enterococcus	0	0
Streptococcus	0,06	<5
Ruminococcus	0	>2
Lactonifactor	0	0
Turicibacter	0	>0,5
Bacteroides	9,05	<10
Prevotella	84,59	<5
Bifidobacterium	0	>5
Asaccharobacter	0	>0,1

Firmicutes Bacteroidetes Actinobacteria
Proteobacteria Other

Range of Firmicutes % in European population: 50-85%

Firmicutes/Bacteroidetes ratio	
High	
Average	
Low	0,04

Low ratio may be associated with gut inflammation

Gram+ / Gram- ratio	
High	
Average	
Low	0,04

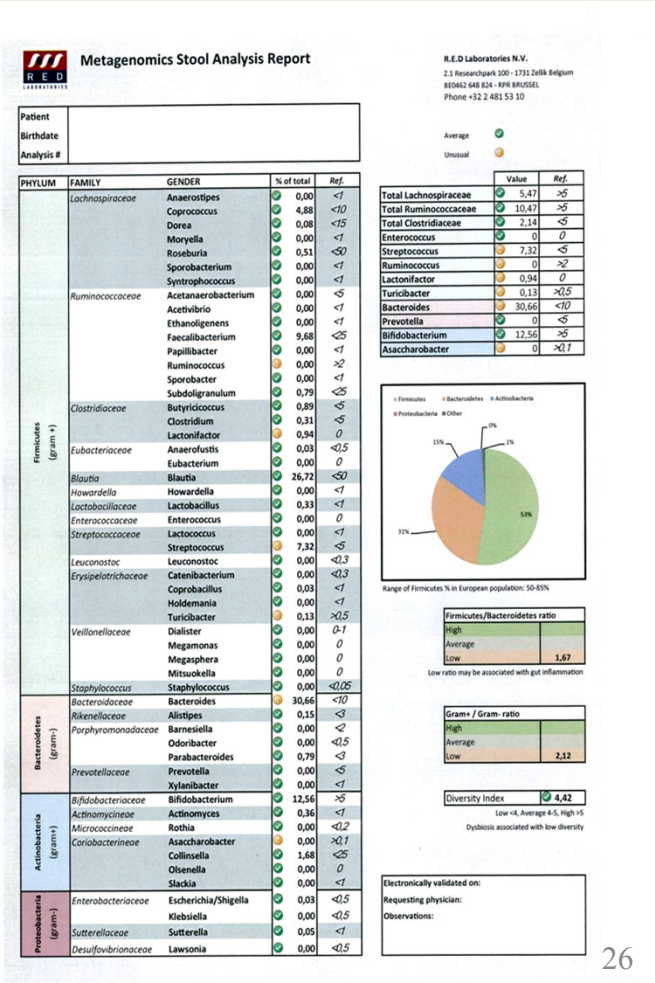
Diversity Index	1,67

Low <4, Average 4-5, High >5
Dysbiosis associated with low diversity

Electronically validated on:
Requesting physician:
Observations:

CFS-related disorders subgroups:
3. intestinal dysfunctions

- **MSA assay : results example of a patient (II)**
- Presence of Streptococcus and Lactonifactor.
- High Bacteroides.



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		Coprococcus	4,88	<10
		Dorea	0,08	<15
		Moryella	0,00	<1
		Roseburia	0,51	<50
		Sporobacterium	0,00	<1
		Syntrophococcus	0,00	<1
	Ruminococcaceae	Acetanaerobacterium	0,00	<5
		Acetivibrio	0,00	<1
		Ethanoligenens	0,00	<1
		Faecalibacterium	9,68	<25
		Papillibacter	0,00	<1
		Ruminococcus	0,00	>2
		Sporobacter	0,00	<1
		Subdoligranulum	0,79	<25
	Clostridiaceae	Butyricicoccus	0,89	<5
		Clostridium	0,31	<5
		Lactonifactor	0,94	0
	Eubacteriaceae	Anaerofustis	0,03	<0,5
		Eubacterium	0,00	0
	Blautia	Blautia	26,72	<50
	Howardella	Howardella	0,00	<1
	Lactobacillaceae	Lactobacillus	0,33	<1
	Enterococcaceae	Enterococcus	0,00	0
	Streptococcaceae	Lactococcus	0,00	<1
		Streptococcus	7,32	<5
	Leuconostoc	Leuconostoc	0,00	<0,3
	Erysipelotrichaceae	Catenibacterium	0,00	<0,3
		Coprobacillus	0,03	<1
		Holdemania	0,00	<1
		Turicibacter	0,13	>0,5
	Veillonellaceae	Dialister	0,00	0-1
		Megamonas	0,00	0
		Megasphera	0,00	0
		Mitsuokella	0,00	0
	Staphylococcus	Staphylococcus	0,00	<0,05
Bacteroidetes (gram-)	Bacteroidaceae	Bacteroides	30,66	<10
	Rikenellaceae	Alistipes	0,15	<3
	Porphyromonadaceae	Barnesiella	0,00	<2
		Odoribacter	0,00	<0,5
		Parabacteroides	0,79	<3
	Prevotellaceae	Prevotella	0,00	<5
		Xylanibacter	0,00	<1
Actinobacteria (gram+)	Bifidobacteriaceae	Bifidobacterium	12,56	>5
	Actinomycineae	Actinomyces	0,36	<1
	Micrococcineae	Rothia	0,00	<0,2
	Coriobacterineae	Asaccharobacter	0,00	>0,1
		Collinsella	1,68	<25
		Olsenella	0,00	0
		Slackia	0,00	<1
Proteobacteria (gram-)	Enterobacteriaceae	Escherichia/Shigella	0,03	<0,5
		Klebsiella	0,00	<0,5
	Sutterellaceae	Sutterella	0,05	<1
	Desulfovibrionaceae	Lawsonia	0,00	<0,5

	Value	Ref.
Total Lachnospiraceae	5,47	>5
Total Ruminococcaceae	10,47	>5
Total Clostridiaceae	2,14	<5
Enterococcus	0	0
Streptococcus	7,32	<5
Ruminococcus	0	>2
Lactonifactor	0,94	0
Turicibacter	0,13	>0,5
Bacteroides	30,66	<10
Prevotella	0	<5
Bifidobacterium	12,56	>5
Asaccharobacter	0	>0,1

Range of Firmicutes % in European population: 50-85%

Firmicutes/Bacteroidetes ratio	
High	
Average	
Low	1,67

Low ratio may be associated with gut inflammation

Gram+ / Gram- ratio	
High	
Average	
Low	2,12

Diversity Index	4,42

Low <4, Average 4-5, High >5
Dysbiosis associated with low diversity

Electronically validated on:
Requesting physician:
Observations:

CFS-related disorders subgroups: *3. intestinal dysfunctions*

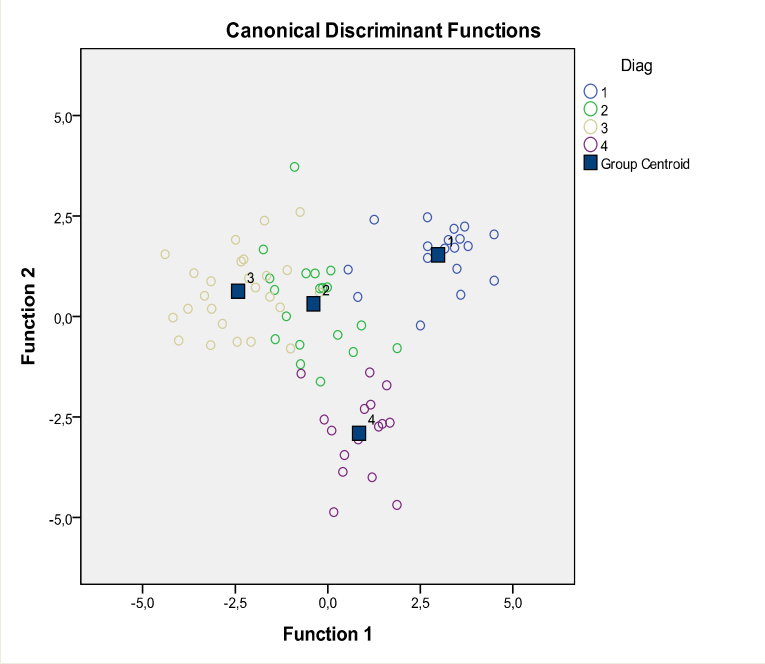
- **MSA ASSAY: First study**
- ***Patient and controls***
  - 19 Belgian healthy controls (mean age 41±12,6), 18 Belgian patients (mean age 38,5±13),
  - 17 Norwegian healthy controls (mean age 45±19), 25 Norwegian patients (mean age 41±12,5) were included in the study.

CFS-related disorders subgroups: *3. intestinal dysfunctions*

- **MSA ASSAY: First study**
- ***Separation of samples in four distinct groups***
  - Linear discriminant analysis can separate the four different groups of samples

Patients separate from controls, but people from different geographical origins (Belgians vs. Norwegians) also show differences in their gut microbiota.

The separation is statistically significant (p=0,022, Wilk's Lambda test).



CFS-related disorders subgroups:
3. intestinal dysfunctions

MSA Study : Differences between Norwegian and Belgian controls

- ***Irrespective of disease status, the gut flora composition differs between Norwegian and Belgian samples***
- Norwegian controls present a significantly lower percentage of Bacteroidetes bacteria and a 3-fold increase of the Firmicutes / Bacteroidetes ratio

Norwegian controls vs. Belgian controls		
Genus	**Increase/Decrease**	**Sign.**
Roseburia	x1,7	0,048
Holdemania	X3	0,008
Bacteroides	x0,36	0,015
Alistipes	x0,2	0,009
Barnesiella	x0,2	0,01
Parabacteroides	x0,26	0,008
Prevotella	x0,025	0,002

CFS-related disorders subgroups:
3. intestinal dysfunctions

MSA Study : Microbiota alterations in patients

- Norwegian patients presented a decrease of several Firmicutes genera, an increase of Alistipes (a Bacteroidetes genus), and a strong increase of Lactonifactor.
- There were less variations in Belgian patients, but interestingly Lactonifactor was also more frequently detected, and in higher levels, in patients than in controls.

Norwegian patients vs. Norwegian controls		
Genus	**Increase/Decrease**	**Sign.**
Roseburia	x0,54	0,029
Syntrophococcus	x0,4	0,015
Lactonifactor	x20	0,003
Holdemania	x0,02	0,0001
Dialister	x0,6	0,04
Alistipes	x3,8	0,013

Belgian patients vs. Belgian controls		
Genus	**Increase/Decrease**	**Sign.**
Lactonifactor	x45	0,006
Asaccharobacter	x0,25	0,041

Mann-Whithney test was used to compare patient samples with healthy control samples

CFS-related disorders subgroups:
3. intestinal dysfunctions

MSA Study : Conclusions

- **ME/CFS patients present significant alterations of gut flora composition**
- In Norwegians patients, the observed alterations are consistent with intestinal dysfunction and inflammation. **Roseburia, which is decreased in patients**, is a major butyrate-producing genus, as such contributes to colon health and gut mucosa integrity. **Alistipes, which is increased in patients**, has been associated IBS symptoms.
- A striking observation is the **strong increase of Lactonifactor**, which is seen in both Norwegian and Belgian patients. Lactonifactor is able to metabolize phytoestrogens; abnormal production of lignans or sterol metabolites could interfere with estrogen receptor pathways, with effects on immunity and inflammation.
- More research is ongoing to elucidate the relations between specific bacterial populations and clinical symptoms. The relation between viral infections and microflora composition will be investigated.

CFS-related disorders subgroups:
3. intestinal dysfunctions

MSA Assay: Interest and potential applications

- New technology: first lab to offer this test as commercial IVD service
- Collection procedure easy, samples can be stabilized: easy access to patients
- Large number of applications: not only CFS but all intestinal diseases, obesity, allergies, cancer…
- Can follow the effect of therapeutic interventions: pre- or probiotics, antibiotics, plant extracts, diet changes…
- Can be used for clinical trials

CFS-related disorders subgroups: *3. intestinal dysfunctions*

Viral infections : Several viruses have been associated with CFS

- Human Herpesvirus 6 and 7 [Chapenko et al., J Clin Virol 2006]
- Enteroviruses [Chia et al., J Clin Pathol 2010]
- Parvovirus B19 [Kerr et al., J Gen Virol 2010]
- Bornavirus [Nakaya et al., FEBS Lett 1996]
- Epstein-Barr virus [Lerner et al., In Vivo 2004]
- *Not specific for CFS; none of these viruses found in all CFS patients*
- Absence of detection could in some cases be explained by viral localization.
- **Persistent viral infections can affect intestinal immunity**
  - HHV-6 is immunosuppressive, causes depletion of CD4 cells, down-regulation of CD3 in infected T cells, alteration of cytokine expression (TNFa, IL-1b, IL-10, IL-12). Parvovirus infection associated with altered IFNg response.
- **Consequences on intestinal health**
  - Immunosuppression may favor development of other viruses or pathogens; alteration of gut immunity can also affect the gut flora.

CFS-related disorders subgroups: *3. intestinal dysfunctions*

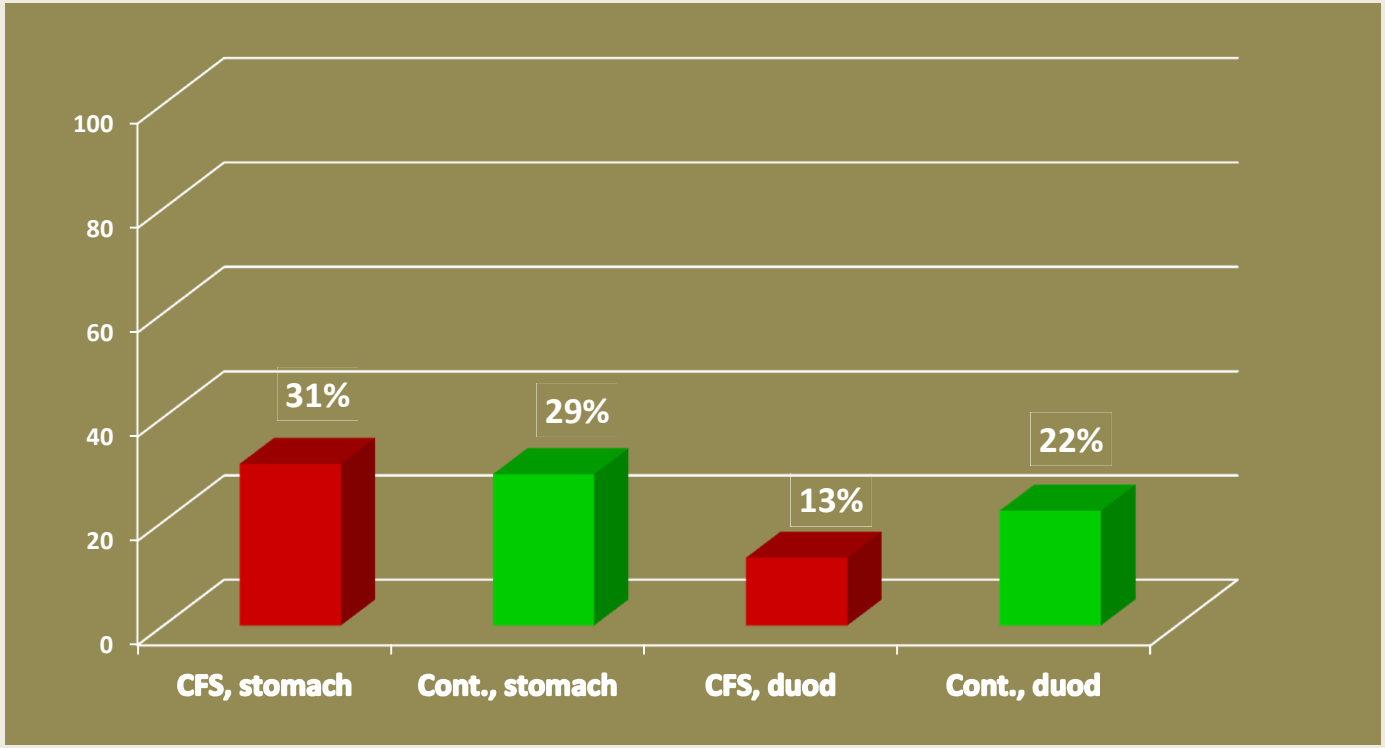
Viral infections : Search for viruses in the gut mucosa : rationale and experimental approach

- The gastro-intestinal mucosa is a known reservoir for several viruses
  - HHV-6, HHV-7, CMV are found in intestinal biopsies of HIV patients and transplant recipients;
  - EBV is found in the gastric mucosa, associated with gastritis and gastric cancer; chronic
  - enteroviral infections have been found in the stomach of CFS patients.
- A study has been conducted at RED Laboratories to investigate the presence of specific viral infections in the GI tract of CFS patients
- ***Experimental approach***
- Determination of HHV-6, EBV and parvovirus B19 viral loads in gastric and intestinal biopsies of CFS patients and non-CFS controls, by real-time quantitative PCR. 48 patients, 35 controls.

CFS-related disorders subgroups: *3. intestinal dysfunctions*

Viral infections : HHV-6 in stomach and duodenum biopsies

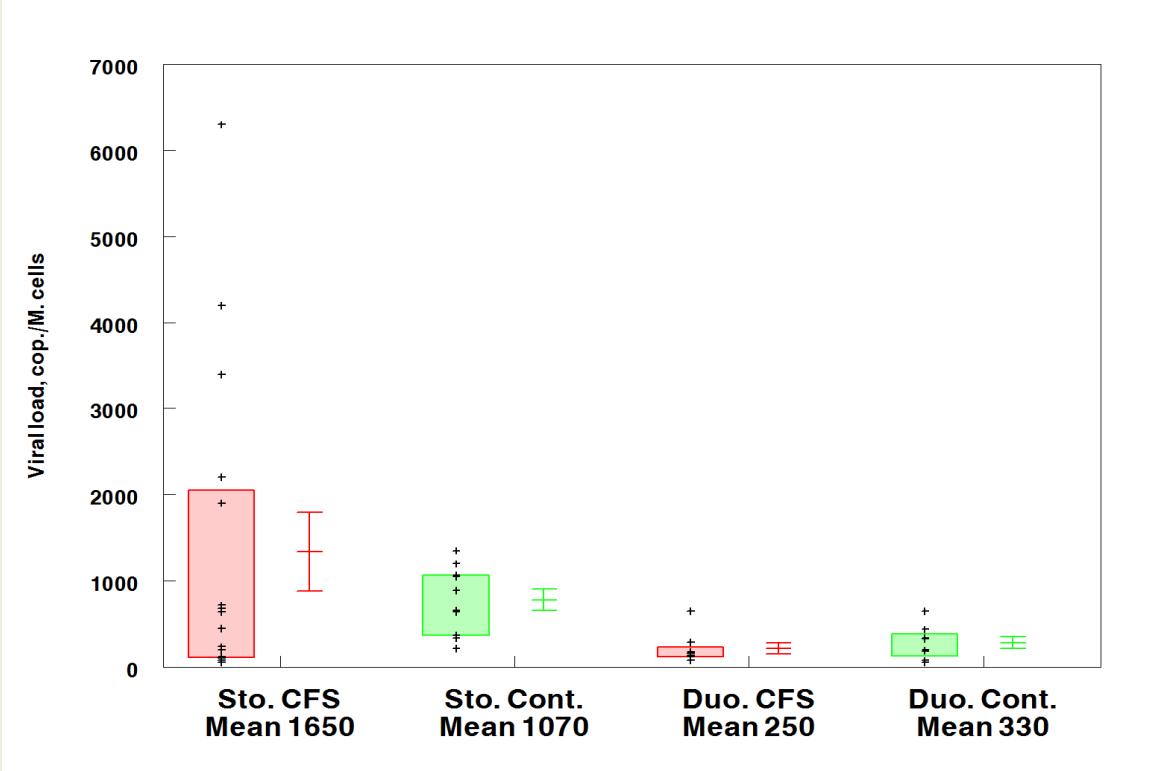
- 13 to 31% of all biopsies are positive. Similar proportions in controls and patients.



CFS-related disorders subgroups:
3. intestinal dysfunctions

Viral infections : HHV-6 viral loads in positive biopsies

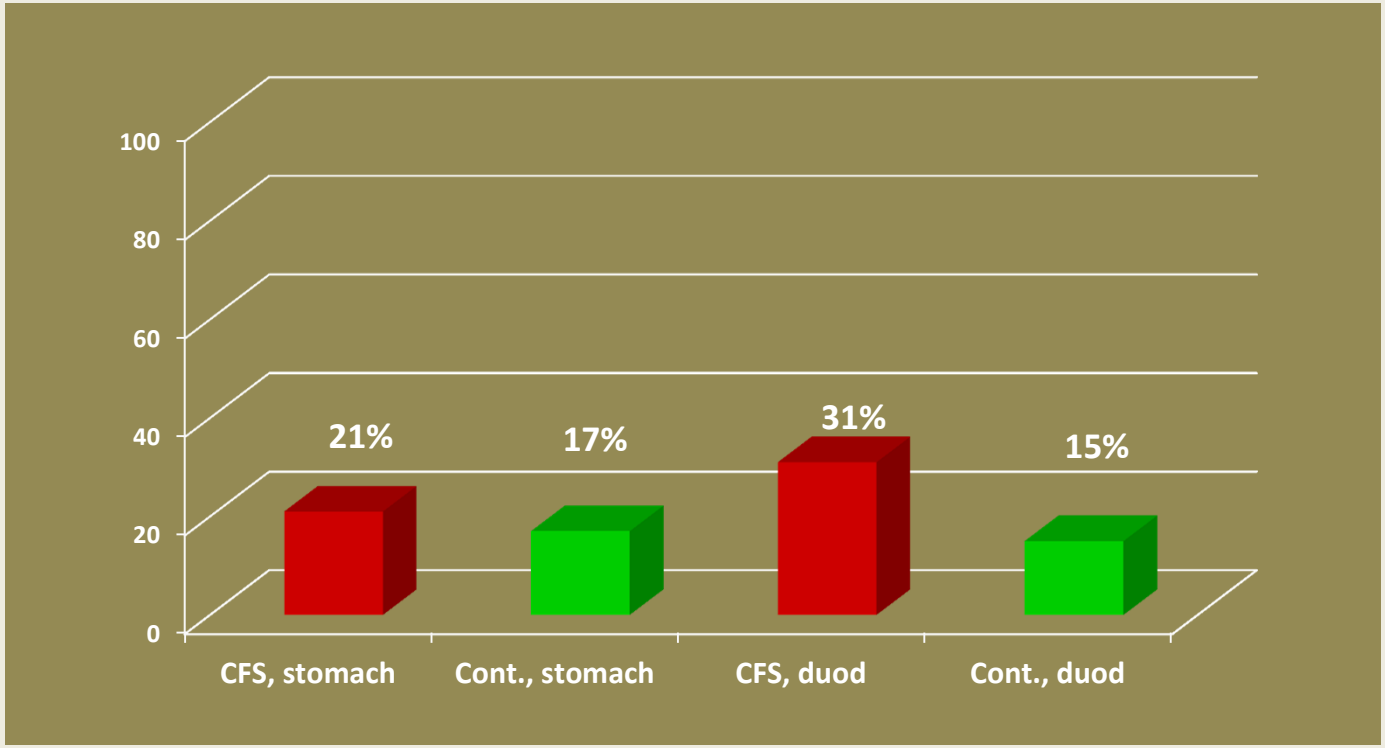
- Several highly positives in the gastric mucosa of CFS patients.
- Higher loads in stomach than in duodenum.



CFS-related disorders subgroups:
3. intestinal dysfunctions

Viral infections : EBV in stomach and duodenum biopsies

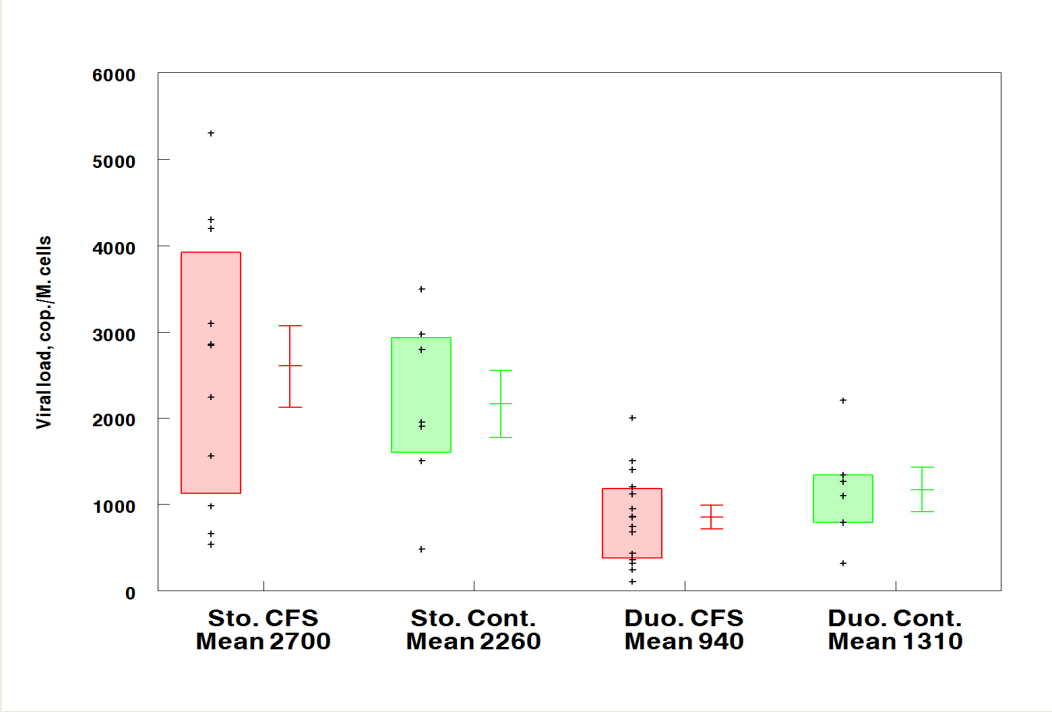
- EBV detected in 15 to 31% of all biopsies. No significant differences CFS/controls.



CFS-related disorders subgroups:
3. intestinal dysfunctions

Viral infections : EBV viral loads in positive biopsies

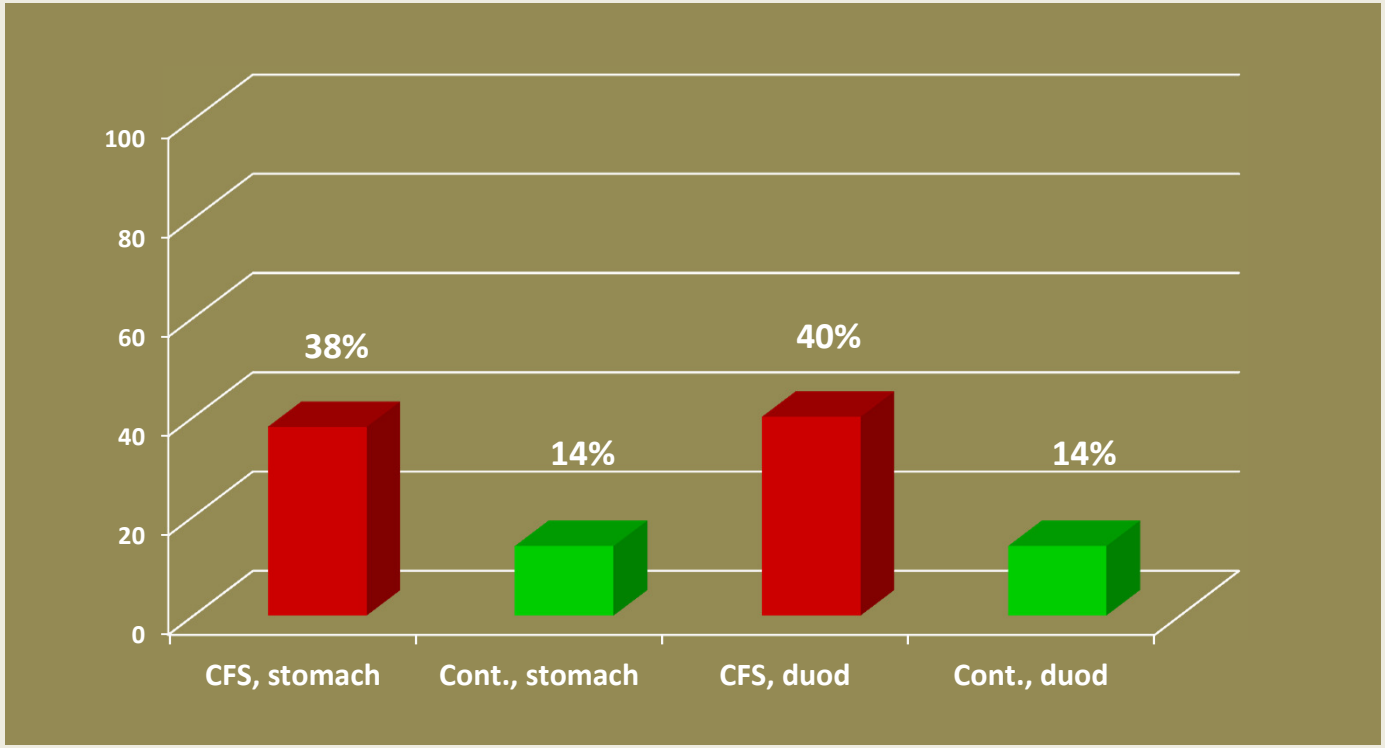
- No difference between CFS patients and controls.



CFS-related disorders subgroups:
3. intestinal dysfunctions

Viral infections : Parvovirus B19 in stomach and duodenum biopsies

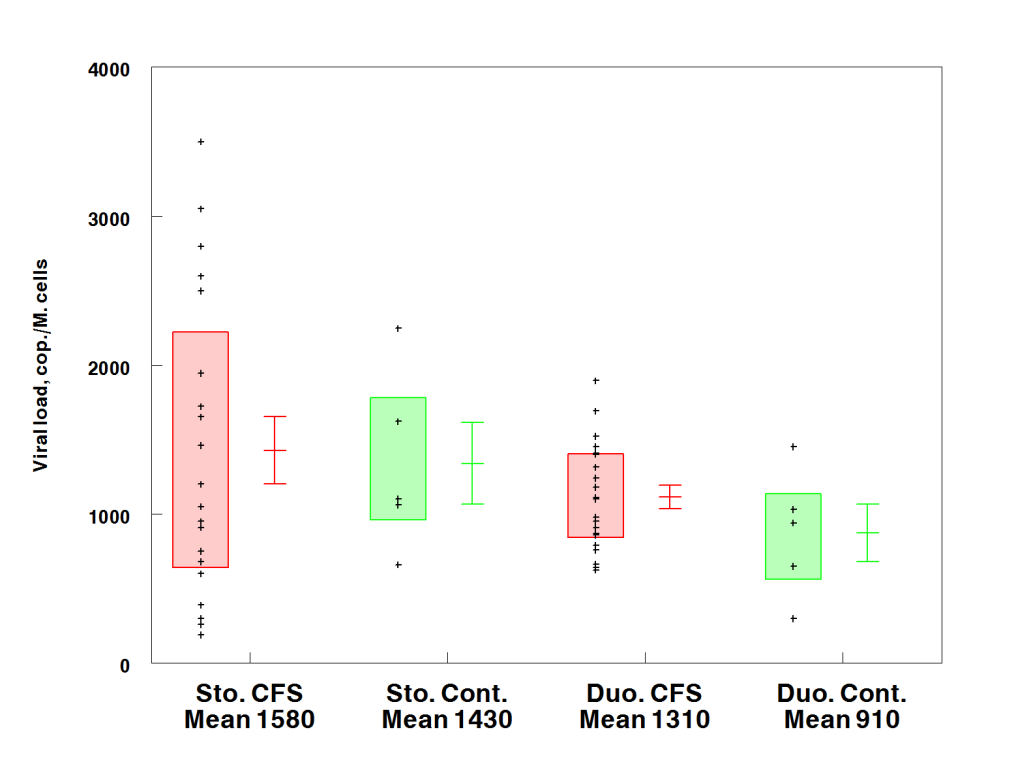
- **Higher frequency of Parvovirus B19** in both gastric and duodenal mucosa of patients compared to controls.



CFS-related disorders subgroups:
3. intestinal dysfunctions

Viral infections : Parvovirus B19 viral loads in positive biopsies

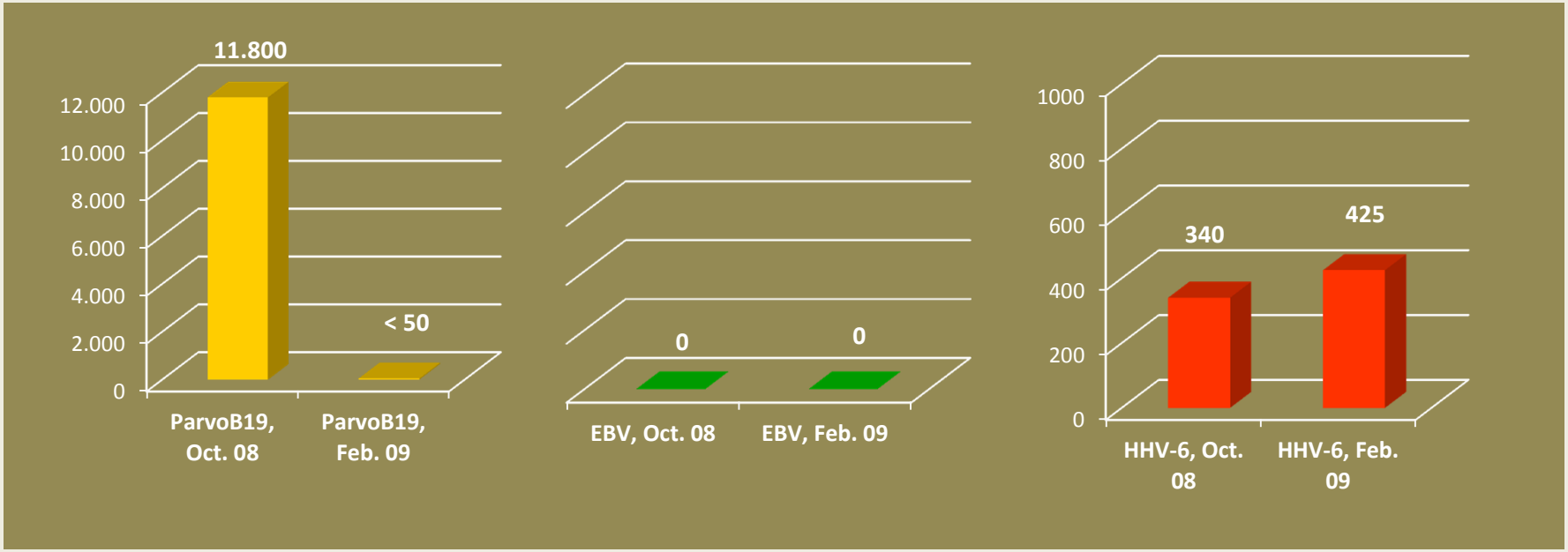
- Very similar loads in stomach and duodenum



CFS-related disorders subgroups:
3. intestinal dysfunctions

Viral infections : Case study

- CFS/ME male patient, born 1989
- Tested October 31st, 2008 for Parvovirus B19, EBV and HHV-6 in gastric mucosa.
- Started SUBCUVIA S.C. (6.4 g of gammaglobulins weekly). Retested February 12th, 2009.
- Evolution of viral loads in stomach biopsies :



New biomedical approach for laboratory testing of chronic fatigue syndrome patients : *Conclusions*

- We identified **3 major groups of factors pointing to the subtype of the CFS-related disorders**
  - ***global immune dysfunctions***
  - ***persistent and/or chronic infections***
  - ***intestinal dysfunctions***
- **New testing panel developed aiming to contribute to a better management of CFS patients**
- Although expensive, the use of this panel-based approach enabled more specific diagnostic and better management of CFS patients