Study of tear composition in multiple sclerosis patients

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Abstract

Multiple sclerosis (MS) is the first cause of progressive neurological disability among young adults living in occidental countries. Its diagnosis is mostly based on clinical evaluation, neuroimaging and in some cases cerebrospinal fluid (CSF) analysis. The presence of different subtypes associated to a large broad of nonspecific symptoms makes the diagnosis difficult. In addition, no definitive biomarker diagnostic test exists to date. We proposed here that the exploration of tear fluid (TF) using omics strategies may be useful for discovering new biomarkers in MS.

TFs will be collected in Schirmer bands from control and MS patients. Independent studies including proteomics and metabolomics, will be carried out and the profiles obtained between TFs from MS patients and control patients will be compared. Metabolites or proteins which will be found to be differentially expressed between MS and control patients, or between the different subtypes of MS, will be integrated into cellular and functional pathways to bring the light their involvement in potential common pathways. The differential expression of the selected candidates will be verified and validated by complementary targeted strategies including western blot, ELISA and qRT-PCR on a larger cohort of independent patients. Finally, their clinical performances will be evaluated individually and/or in combination.

Background

Multiple sclerosis (MS) is an unpredictable and often disabling disease that affects preferably young people (average: 20-40 years old). MS is presented as a chronic immune-mediated disease of the central nervous system (CNS) leading to neuro-inflammation, demyelination and subsequent irreversible neuronal damages. Even if the exact pathophysiological mechanisms are not yet elucidated, the disease is probably initiated by external factors including environmental factors and/or virus in patient presenting MS genetically sensitivity. Current MS diagnosis is based on clinical history and examination supported by neuroimaging and in some situations cerebrospinal fluid analysis. Due to its complex presentation -every patient experience his own symptoms-, early MS diagnosis remains difficult. Furthermore, MS clinical presentation differs from patient to patient with different clinical subtypes (relapsing remitting MS; secondary progressive MS; primary progressive MS and clinical isolated syndrome (CIS)) associated with a large spectrum of non-specific symptoms, individual clinical course and variable responses to treatment. In addition, no prognosis or treatment-efficiency tool exists. In this context, a biomarker differentially expressed in MS patients could be highly useful for helping physicians in diagnosis but also maybe for better understanding MS pathophysiological events. Interestingly, initial symptoms are often in relation with vision: blurred or double vision, color distortion, optic neuritis.

In this context we speculate that specific modifications in tear content can reflect the MS status of the patient and could replace lumbar puncture. Indeed, a lot of studies investigating different biological samples (blood and CSF among the most largely screened) as source of MS biomarkers were made until nowadays. The presence of oligoclonal bands in CSF is probably the most wide-spread diagnostic biomarker, as considered as sensitive but not specific of MS. Furthermore, a variety of potential biomarkers in these fluids including neurofilaments, glial fibrillary acidic protein (GFAP), myelin basic protein, tau, neural cell adhesion molecule (NCAM), CXCL-13, have been extensively reported in the past. However, so far, none of them displayed sufficiently robust results to be definitively established as clinically useful biomarkers. Interestingly, with the availability of omics technologies and the bioinformatics possibility to combine omics data, completely new and unexpected candidates emerge in plasma, CSF and urine and could probably resolve open questions associated to multiple sclerosis.

To date, tear fluids (TFs) are marginally investigated, and MS is not an exception. Typically, those studies evaluated only the presence of OCB in tears of MS patients confirming that the composition of tears can be
modified in presence of MS. Indeed, tears may mirror potential disturbances occurring in CSF, including glial, neuronal, myelin as well as inflammatory modifications associated to the disease.

**Aim and impact**

The field of biomarkers (whatever their biological nature, including proteins, genes, metabolites, lipids, etc) in tears remains largely under-investigated despite the great clinical potential of this fluid. Analysing tears using proteomics and metabolomics offers the highest chances of obtaining a comprehensive and unbiased view of biomarkers. Here, the project aims to identify new omics-biomarkers in tears that could: (i) allow an early detection of pathological events (and eventually tools for their prevention); (ii) discriminate the subtype of MS diseases; (iii) detect proteins associated pro-inflammatory conditions but also proteins associated with neurodegeneration, demyelination but also neurorepair that could provide crucial information for outcome prediction and (iv) allow to physicians to evaluate the therapeutic response.

**Research plan and method**

*Selection of patients.* The study will be located at the HUG (Neurology division and CRC - Centre de Recherche Clinique), Geneva, and will last 2 years between 2016-2018. The selection of patients is based on the following criteria: Subjects aged ≥18 and ≤70 years, of any gender and ethnicity will be included in the study. Subjects presenting any eye allergy or conjunctivitis or acute infectious diseases the day of tears collection will be excluded from the study. Tears will not be withdrawn from subjects having make-up or contact lenses at the time of sampling. No subject with cancer and/or chronic autoimmune disease will be enrolled. Patients having a recent history (< 3 months) with IV Immunoglobulin, steroids or antidepressant drugs will be excluded. Chronic immunosuppression with MS disease modifying therapies is not an exclusion criterion. This group will contain 50 MS patients which have already enrolled in the study 12-210 SMSC cohort in order to have standardized clinical data. As described elsewhere, different subtypes of MS exist and all are expected to be included (CIS, RR-MS and SP-MS will represent approximately 85% of patients, and PP-MS the remaining 15%).

This study has also two control groups: 50 healthy volunteers (control group, but no particular analysis or medical visits are foreseen to assess the healthy condition of volunteers) and 100 patients admitted to neurology division but without history of multiple sclerosis. These patients typically suffer from other neurological disorders (OND) including CNS inflammatory diseases other than MS, seizure, stroke, neupathies, amyotrophic lateral sclerosis (ALS), migraines, and neuromuscular junctions disorders (myasthenia gravis). They will be recruited during their stay in the “hôpital de jour” or during hospitalisation in the division of neurology (HUG). The reason to include 100 patients in this specific group is that we plan to differentiate OND patients with and without inflammatory diseases.

*Sampling.* The patients who meet the above criteria are included in the ongoing study. Professionals in charge of tear collection work wear gloves in order to avoid any contamination. The tear collection will be performed according to the Standard Operating procedures (SOP) described by the Sjörgen International Collaborative Clinical Alliance (SICCA) (SICCA manual of Operation, San Fransisco, 2012). Briefly, a Schirmer-Plus® (Dina-Hitex for Gecis, Lamotte-Beuvron, France) paper strip method is placed carefully over the lower lid margin. No external stimulation or anesthesia is done to collect the basal fluid. Care is taken to avoid damage to the conjunctive surface and local eye irritation. For each patient, tear samples is taken from both eyes. As soon as the Schirmer strips are in place, the time should be noted and the strip allow to remain in place for a maximum of 5 minutes, or until the strips are completely satured with tears, if sooner. If the strips are not saturated after 5 minutes, the amount of wetting should be reported. If complete saturation of the strips occurs before 5 minutes, the amount of time should be recorded. The patients should keep their eyelids closed during the Schirmer test. For biomarkers that are influenced by circadian rhythms, time of withdrawal is important. Since it is often difficult to accomplish standardization of withdrawal time in everyday clinical practice, documentation of withdrawal time is necessary to select the appropriate samples to minimize the effect of this variable. At the end, the strips should be carefully removed, placed into a vial (polypropylene tubes for storage, with their low protein binding potential, for collecting biological fluids (e.g. Sarstedt, Ref. 72.730.006; 0,5ml), labelled with the participant ID number and frozen immediately at -20°C until their later transfer to the Serotheque (HUG). There, they will be definitively stored at -80°C until analyses.

*Omics analyses.* For each type of ‘omics’ analysis, we plan to test 12 patients presenting MS and 12 age/gender matched control patients or 12 OND patients, corresponding to 4 independent experiments.
Metabolomics: As only marginal published studies reported metabolites analyses in tears, no validated protocol and workflow exist and probably some technical improvements or adaptation should be planned. This part of the project will be supported by a strong and close collaboration with the Proteomics Core Facility (Faculty of medicine, Geneva) and with interaction already existing between Dr. Natacha Turck and Prof. Aurélien Thomas (CURML, CHUV/HUG, Lausanne/Geneva, Switzerland).

Proteomics: Isobaric labelling (TMT®) quantitative proteomics experiments have been described elsewhere. The quantification values will be extracted from the reporter peak intensities and a more detailed process is described elsewhere. The calculations, as well the determination of a p-value <0.05 are made automatically by Easyprot or Isobar softwares. The protein ratios that differ significantly between the two conditions (ratio >1.5) will be selected for further experiments.

Integration of omics data. The candidate biomarkers will be incorporated into KEGG, DAVID, or IPA and pathways mining will be undertaken. An example of such an analysis can be found in the paper by Huang et al. Ideally these steps will guide us in the selection of 8-10 biomarkers (metabolites and/or proteins) with the highest clinical potential for further validation.

Verification and validation steps. These most promising 8-10 candidates will be verified with orthogonal approaches (western blot, immunoassay if commercially available or alternatively with selected reaction monitoring, SRM) using independent samples. For this verification step, we will then use a larger study including at least 25 patients per disease and an age/gender matched healthy subgroup). For the validation step, only the candidates (probably 4-5) who perform satisfactorily in the verification step will be included. Here, the objective will be to test independent patients in order to assess whether the performances of biomarkers can be generalised to a large scale population.

Statistics. Sample size and statistical power for the verification and validation study was calculated using the pROC tool in TIBCO Spotfire S+ version 8.2 software (TIBCO Software Inc, Somerville, MA, USA) for proteins and metabolites. The clinical performance of biomarkers will be measured using IBM SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA).

Appropriate statistical tests will be performed to assess the ability of biomarkers to discriminate diseased and control patients. The assumption of normality will be verified using Normal plot and skewness and kurtosis tests. Moreover, the variability of biomarkers in each group of patients is expected to be different with the controls patients probably presenting less variation than diseased patients. For this verification step, we will then use a larger study including at least 25 patients per disease and an age/gender matched healthy subgroup. For the validation step, only the candidates (probably 4-5) who perform satisfactorily in the verification step will be included. Here, the objective will be to test independent patients in order to assess whether the performances of biomarkers can be generalised to a large scale population.

Risks and feasibility.

Clinics: Recruitment of patients is planned to be accomplished within the first year of the study. Most multiple sclerosis patients are followed in the clinic through the “swiss MS cohort” and will be easily accessible to the study. Other patients and controls will be included according to the recruitment protocol described in the attachment (see files submitted to the ethical committee).

The recruitment will be highly facilitated with the support of a 10% “attachée de recherche scientifique” that will be hired for the study (Mme Mariagrazia Di Marco; CRC). This will help also for all administrative, ethical and data collection, especially in connection with the Swiss MS cohort.

A homogenous collection, handling and storage of ocular samples associated to a non-ambiguous clinical profile of the patients should minimize the biological variability and therefore provide robust omics comparison between the different groups of patients.

Proteomics: The generation of preliminary data in global tear proteome associated to the power of the new proteomics methods should reduce the risk of having a limited number of identified proteins as well as the lack
of differential proteins in the different comparison investigated (Note: two new performant mass spectrometers have been recently acquired in the Proteomics Core Facility that should guaranty high-throughput and sensitive analyses).

**Metabolomics**: Multiple strategies will be combined for ensuring a large cover of the metabolome. It is however well-known that to date even with a combination of complementary profiling methods the really comprehensive profiling of the human metabolome is not achievable. It is however important to maximize the ways of profiling the body fluids since each detection method will present partial overlap with the others but a large proportion of features will be unique to a given type of detection. The statistical analysis of the metabolomics profiles from one sample type and technology might deliver a very limited discriminating power between sample populations. Nevertheless, as we have an appropriate number of samples and those analyzed by different analytical platforms, the risk of complete failure to deliver candidate metabolomics signature is limited. The identification rate of discriminating metabolite signals might be low and could limit the number of pathways to curate, therefore also limiting the number of candidate metabolites to submit to development of quantitative assays. The number of targeted compounds to quantify is sufficiently low (10 to 30) so that one can expect to extract enough candidates. In addition, the literature can help in consolidating the choice.

**Verification and validation steps**: The major risk resides on the lack of commercially available antibodies/kits or antibodies/kits working poorly. If necessary, these antibodies will be produced in the dedicated Core Facility in UniGe (phage display technology, Prof. Cosson, duration: 2-3 weeks). Furthermore, the failing of antibody should be counter parted using alternative targeted proteomics strategy such as multiple reaction monitoring (MRN) or single reaction monitoring (SRM) and label-free technology.

**References**


