COMBIMS

A NOVEL DRUG DISCOVERY METHOD BASED ON SYSTEMS BIOLOGY: COMBINATION THERAPY AND BIOMARKERS FOR MULTIPLE SCLEROSIS

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SEVENTH FRAMEWORK PROGRAMME

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Executive summary

Complex diseases are particularly complicated to treat as they involve multiple processes and patients are very heterogeneous. Such features make it very likely that combination therapies will be required to target the distinct mechanisms and pathways involved. However, this currently represents an important therapeutic challenge, as the effects of combining drugs have been little studied, both in terms of their benefits and the potential risks. For this reason, we set out to define a method where clinical data and molecular information were taken from patients, representing a faithful reflection of the condition, and they were integrated into computational models that reflect the processes affected in the disease (adopting what is considered to be a systems biology perspective).

Multiple Sclerosis (MS) is a prototypic and debilitating complex disease in which an autoimmune attack is launched against the nervous system. Current therapies for MS are far from effective and target only part of the immune response. Hence, we used this as a prototypic disease to determine whether the method we have designed could identify combination therapies with good safety profiles, offering the opportunity to better control this condition by adopting a more holistic approach.

The method involves first understanding the biological networks that are at play in the disease and how current MS therapies affect these networks. The immune response is mediated by interactions between cells, many of which involve the transmission of signals. Thus, we measured biological features of cells involved in dealing with these signals (using proteomics) and that are involved in the disease, comparing these to the same features in cells from healthy individuals. In this way, we could define the differences between the healthy and disease state, and attempt to identify combinations of drugs or compounds that reverse the changes in the diseased cells so that they appear more like the healthy cells. Prior to validating and adopting these combination therapies, suitable algorithms are employed to determine the safety issues and possible side-effects associated with the combinations identified and in addition, the potential commercial viability.

By applying this methodology to MS, we have been able to define different combination therapies that we predict will provide additional benefits in the treatment of MS. We have tested these combinations in isolated cells and in animals, and the results obtained to date indicate that we may well have identified therapies that offer additional benefits in MS. Thus, it would appear that the method we have designed may represent a useful approach to identify new treatments for complex diseases.

This approach and the tools developed will be applicable to other immune diseases and eventually, to other types of complex diseases (e.g., cancer), helping to improve their future therapeutic options. In addition, assessing the potential commercial interest in these drug combinations is fundamental to their possible development and future uptake. It is noteworthy that some of the changes we detected may be specific to specific sub-groups of patients, allowing us to better define the distinct stages of the disease and to possibly identify signs that indicate a patient is passing from one stage to another. Therefore, our technology also contributes to the development of personalized medicine, a possibility that is currently beyond the reach of specialist physicians.

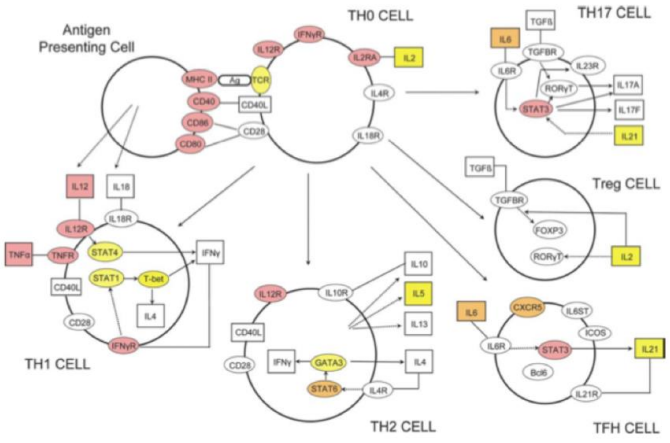
A summary description of project context and objectives

Complex diseases pose a challenge to the current drug development strategies that generally target specific aspects of these pathologies, without achieving complete control of the disease. Due to the multiple pathways implicated in such diseases and the heterogeneity between patients, approaches directed at single targets have provided limited efficacy, as many important pathogenic mechanisms remain unmodified by the therapy. Hence, a promising therapeutic approach to target complex diseases involves combining different therapies, each addressing a distinct pathway, in order to modify the pathways involved in the pathogenesis without augmenting or producing side-effects.

Despite the potential interest in such combination approaches, there are currently no standard procedures to identify potentially beneficial drug combinations that might offer suitable strategies to treat complex or any other disease. Indeed, when addressing this issue it is also important to take into consideration not only the positive effects that may be gained from but also, any enhancement or appearance of novel side-effects. Thus, it is important that methods and pipelines that aim to identify beneficial combinations of available or novel drugs include an analysis of the potential safety aspects involved.

MS is a prototypic complex disease in which the immune system launches an autoimmune attack against the brain, resulting in significant disability[1,2]. MS is the second cause of disability in young people in the EU, and it imposes a significant social and health burden to EU citizens and governments[3-5]. Current therapies for MS aim to modulate the immune response, yet while to some extent they may delay the progress of the disease, they fail to modify the mechanisms that cause the disease (the multiple pathways leading to increased inflammation and neurodegeneration). Thus, while the benefits to patients are well established, they are modest and the situation is far from satisfactory. Moreover, these therapies have common side-effects (flu-like symptoms, allergic reactions, increased liver enzymes and decreased in leukocyte counts) that limit the patient's quality of life and may even provoke serious adverse events. Thus, the challenge is how to improve treatments by combining drugs while preventing further adverse events.

Targeting complex diseases would benefit from an improved understanding of their pathogenesis, enabling us to confront the challenge of identifying potential targets. Several genetic polymorphisms have been associated with susceptibility to MS, which sheds light on some of the main pathways involved in the disease and provides a base on which to build more realistic network models of interacting pathways driving MS (see Fig. 1). Now the challenge is to integrate this information and these pathways into a coherent model of the pathogenesis of the disease, and take advantage of this knowledge to develop more efficacious and safer therapies for MS. We consider that a systems biology (SB) approach may allow us to achieve this integration, combining the current data from the area of 'omics technologies with clinical information and data on the therapeutic response[6,7]. Network and pathway analysis are two methods to integrate such information in order to identify possible combinations to be tested experimentally[8,9]. Defining combinations in this manner allows us to understand the effects of such drugs on their targets (pharmacodynamics) and puts us in a position to assess their possible side-effects (toxicology).



Term ID	Term name
GO:0046651	lymphocyte proliferation
GO:0032943	mononuclear cell proliferation
GO:0070661	leukocyte proliferation
GO:0002376	immune system process
GO:0050863	regulation of T cell activation
GO:0042110	T cell activation
GO:0050865	regulation of cell activation
GO:0046649	lymphocyte activation
GO:0050867	positive regulation of cell activation
GO:0050870	positive regulation of T cell activation
GO:0042098	T cell proliferation
GO:0001775	cell activation
GO:0051249	regulation of lymphocyte activation
GO:0045321	leukocyte activation
GO:0006955	immune response
GO:0002694	regulation of leukocyte activation
GO:0051251	positive regulation of lymphocyte activation
GO:0002696	positive regulation of leukocyte activation
IPA:THC_DIFF	IPA:T Helper Cell Differentiation
GO:0002520	immune system development
GO:0023033	signaling pathway

Fig. 1. Genes and pathways identified in the MS GWAS study[10]. The role of the genes identified is depicted in the pathways related to T cell activation and the differentiation into Th subpopulations (Th0, TH1, TH17, Th2, Treg, Tfh). The table indicates the pathways identified in Gene Ontology associated with the genes identified in the GWAS.

Our strategy is based on: 1) drug discovery from a systems perspective, considering the response to therapy of the whole cell or body, and not just of a given receptor; 2) not applying reductionist approaches to identify the best ligand for the target but rather, analyzing the effect of drugs on the entire biology of the cell; 3) the use of human primary cells from patients for in vitro assays, avoiding inter-species differences and allowing assays to be performed in a physiological context, thereby improving the accuracy and efficacy of the results; 4) testing therapies in patients in the early stages of the discovery process, even if performed ex-vivo, in order to diminish the number of false positives; 5) focusing on the signalling networks, which allows us to work at a level that best correlates with cell stimulation and with the cellular response to drugs; 6) the benefits of using extended knowledge of the human proteome and the availability of new methods to accurately quantify representative phosphoproteins in the signalling pathways; 7) a method that uses advanced computational tools, such as dynamic networks, genetic algorithms and logic networks, to deal with the complexity of the cell and provide useful physiological insights.

To date, there are no new approved drugs that have been identified through systems drug discovery, an approach our method looks to benefit from. In terms of toxicology, our network analysis will deliver predictive toxicology that can be validated experimentally. The problem of off-target effects pervades drug development, even when using highly specific methods to target single molecules. This issue can be addressed by using specific computational tools to build biological networks that can predict drug effects and identify side-effects from all the available information regarding drugs, molecules and biomedical research from datasets, and the power of network analysis[11] (Fig. 2).

Of the many molecular technologies that can be used to interrogate the state of the body (e.g., genetics, gene expression, cytomics, etc…), the analysis of the phosphoproteome provides important information regarding the activation of signalling pathways. Such information often reflects the communication between different cell types or the interaction of cells with their environment, and their correspondence with the response to therapy[12]. Several studies of MS have identified abnormalities in the activation of phosphorylation cascades involving the TCR, NFκB and IFN (Stat1-2, IRF). Moreover, some therapies for MS act through specific cell surface receptors (e.g., INF-beta, fingolimod) and they alter the

phosphorylation of their downstream signalling pathways[13-15]. Here, we will utilize computational modelling techniques to represent the phosphorylation networks and pathways involved in MS, in order to understand the disease and the mechanism of action (MoA) of therapies[11,16-19].

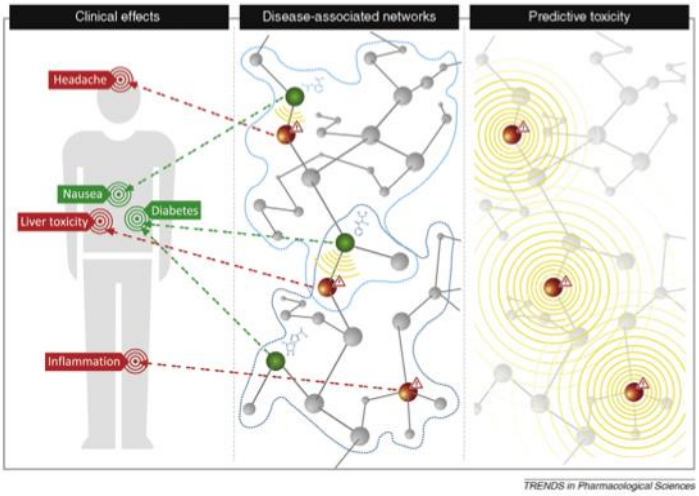


Fig. 2. Network approach to drug discovery. Information available in clinical database (side-effects, indications), drug databases (chemical structure, indications, side-effects), molecular databases (protein-protein interaction, gene expression) are integrated using network formalisms and explored in order to predict new indications and toxicity using advance computational methods.

As indicated previously, new therapeutic approaches to MS involving neuroprotection may offer better control of this disease. The brain's susceptibility to damage, its critical role in survival and quality of life, and its poor reparative capacity set the stage for neuroprotection as a key area in CNS research. Currently, no neuroprotective therapies are available for MS or other brain diseases. However, Bionure pharma (BN) has recently identified a set of 9 small chemical entities that exert neuroprotective effects in vitro and in animal models of MS, including when combined with immunomodulatory MS therapies (WO 2011/024078A1; WO 2011024079A1; P2010/61378823). BN is now advancing its research programme from the preclinical to the clinical phase in combination with immunomodulatory therapy, in the hope it may achieve significantly better control of the disease. Among other attempts to develop neuroprotective therapies, the use of natural antioxidants such as the green tea extract Epigallocatechin-3-gallate (EGCG) are being considered, and at Charité the potential of EGCG therapy is currently under study in a pilot phase II trial.

Thus, we set out to provide a proof-of-concept that a SB drug discovery approach represents a novel and improved drug discovery process, with a particular emphasis on deriving a methodology to develop efficient and safe combination therapies. In addition to obtaining a better understanding of the events controlling the pathogenesis of MS, in this project we aimed to focus on the cohort of available treatments and repositioning of drugs for MS by utilizing clinical and molecular data, and predicting their positive and negative effects. Accordingly, the main goal of the CombiMS project was to identify and validate a new method for drug discovery based on SB and apply this to the identification of novel combination therapies for MS, a goal that was to be achieved through the following objectives:

1. To improve and develop new computational pathway models capturing the immunomodulatory and neuroprotective processes causing MS;
2. To redefine the clinical phenotypes of MS based on molecular and dynamic signalling parameters;
3. To predict putative combinatorial therapeutic targets and to experimentally validate at least one of these sets of targets;
4. To develop an integrated experimental–computational method that enables efficient and safe identification of combination therapies and biomarkers that may be of wider use within biomedical research, and in the pharmaceutical and biotech industry.

To accomplish these objectives, this consortium brought together biotech companies with R&D expertise that are currently developing new therapies for MS (BN) and that have expertise in the application of SB models to complex diseases (AX & PAO). In addition, groups with experience in phosphoproteomics analysis and in the modelling of signalling pathways participated (PAO & EBI). We set out to study the human phosphoproteome (using xMAP technology) as this provides one of the best relationships between molecular measurements and cell function. Furthermore, we counted on a group of clinical centres (KI, UZH, IDIBAPS & Charité), with demonstrated expertise in clinical care and MS research, and with the capacity to test the models' predictions *in vitro* and in animal models. Due to the several limitations of animal models and many 'omics technologies in translational research, we focused on human samples, specifically peripheral blood mononuclear cells (PBMC) from subpopulations of MS patients. The aim of these studies was to gain a more comprehensive understanding of the biological mechanism of action of current MS therapies and to develop new tools to design combination therapy targeting MS in particular and complex diseases in general.

In summary, the CombiMS consortium set out to design an integrated computational modelling pipeline to be applied to the discovery of combination therapies for complex diseases such as MS. This pipeline is based on well-established tools developed by CombiMS participants, integrated such that the outcome of one model is the input to the following one. As such, network analysis of databases and the scientific literature will provide a map of proteins, clinical indications and side effects. The analysis of this map drives the selection of the pathways to be modelled with logic models and the results obtained through these logic models are refined by ODE models. Finally, predictions from signalling models are again tested in network models to predict side effects and efficacy, as well as at the experimental level. **Therefore, this will be one of the first times networks, logic and ODE models will be integrated in an 'application' to resolve clinical/biological questions**. This is possible because such a modelling approach has been validated and optimized in previous studies by members of CombiMS consortium, **representing a true advance over the state-of-the-art**.

A description of the main S&T results/foregrounds

In order to develop a new method to identify drug combinations using network and dynamics models of signalling pathways involved in MS, the key strategy embarked upon involved:

1) Examination of the pathogenic changes and the response to selected therapies in primary cells isolated from patients in order to define the molecular signature of clinical phenotypes;

2) Definition of the phosphoproteome network at the molecular level that reflects the cellular and clinical phenotypes;

3) Production of network models that reflect the clinical situation, i.e.: the relevant phosphoproteomic changes in the immune system and genotypes relevant to the diseases, and that can be used to evaluate the effects of therapeutic drugs and their capacity to revert these changes towards the healthy state.

4) Applying further network analysis to evaluate the potential safety issues and side-effects that the selected combination therapies might provoke.

5) The use of in vitro human cell assays from patients or animal models to evaluate the effects of the combination therapies identified, improving the translational yield to clinical application.

This drug discovery **method** established by applying this strategy aimed to improve the efficacy in selecting suitable drug combinations for human use in complex diseases. In addition, it should enable biomarkers to be identified for disease progression and prognosis, as well as to predict side-effects. The overall strategy following the general scheme indicated above was divided into four principal Work packages that were aimed to be completed over 2 years.

Formulation of computational network models of pathways relevant to Multiple Sclerosis (MS)

Phosphoproteomic data of MS associated pathways obtained from peripheral PBMCs

In order to formulate the computational network models of pathways relevant to MS four specific tasks were defined. The first of these involved defining the specific signalling pathways that are of interest in MS and that can be measured through xMAP assays (phosphoproteomics) of human peripheral PBMCs, CD4+ cells and macrophages. This involved identifying the proteins to be studied in response to specific stimuli (i.e.: defining how immune system cells from healthy controls and MS patients respond to stimulation). In addition, it was necessary to develop the assays to measure the phosphoproteome for which healthy PBMCs were used. To select antibodies (Abs) that could be used in the assays and that gave a sufficiently good signal-to-noise ratio (SNR), approximately 200 cytokine and 70 phosphoprotein Abs were tested, and a total of 28 stimuli were initially tested. To define the list of xMAP signatures, the following parameters were taken into account:

- Relevance to the general pathways of interest in the context of MS;
- Direct references to the gene/protein in the context of MS;
- Expected changes in response to drugs of interest (IFNb, fingolimod, dimethyl fumarate or BN201);
- References from GWAS study (see Fig. 1);
- Relevance to the environmental factors related to MS risk (vitamin D).

A final approved list contained 22 proteins of interest, taking into account that not all of them were suitable for the final assay and that targets of BN's drug in development BN201 would be added later once the MoA of BN201 was available (See deliverable D1.1 for more details). The donor-to-donor variability was then assessed in healthy volunteers and given that considerable variability was observed

in the healthy donors, this would also be expected among MS patients. Thus, measures to normalize the data were evaluated and to ensure that the stimuli used to provoke the phosphoproteomic changes in the cells would prove useful. As a result, the stimuli and drugs, as well as the readouts were established.

When defining the procedure to perform the assays on the cells collected from healthy controls and MS patients, it was decided that a standard kit would be developed and distributed to the clinical centres in order to reduce the heterogeneity in the results obtained. This kit was designed by PAO to obtain both the samples to evaluate the phosphoproteome and the genotypes of the subjects.

Creation of new computational models based on MS related pathways

Assembly of a Prior Knowledge Network (PKN)

Based on the literature, a novel protein signalling network was assembled that consisted of interactions reported to be related to MS. A previously published network of well-established qualitative knowledge derived from primary T cells was initially considered[16]. This network was extended by including the phosphosignals identified by PAO and the targets of the therapeutic treatments currently under study. Finally, the literature was mined to determine points of crosstalk between the initial network and these additions. All the newly included interactions and proteins in the network were filtered for their tissue of origin and those originally found in human immune cells were prioritized. In this way, a protein signalling network was assembled that was highly specific for the regulation of human immune cells, and that at the same time was tailored to the experimental aims of the CombiMS study. The final Preliminary Knowledge Network (PKN) consisted of 120 nodes and 182 interactions.

Creation of a Logic ODE model of MS pathways

The PKN protein signalling network was converted into a logic model using the CellNOpt platform developed by members of the CombiMS consortium (EMBL-EBI). CellNOpt was used to remove all nodes and interactions that did not interact with nodes that could be identified experimentally (generally lying on terminal branches of the pathway). So-called non-controllable nodes were also removed (i.e.: those that were neither observable nor targeted by the experimental perturbations considered). This analysis aimed to identify the sections of the protein signalling network from which we could not learn about structure given the experimental coverage. Finally, a structure of logic gates was created using CellNOpt: (i) AND gates, in which multiple upstream nodes are necessary to regulate downstream nodes; (ii) OR gates, in which one of the multiple upstream nodes is sufficient to regulate the behaviour of downstream nodes; and iii) NOT gates to analogously represent inhibition. Therefore, the model could readily be trained against experimental data to identify the signalling network regulating the cellular response to treatment in MS and it was adapted to the stimuli and signals to measure phosphorylation established by PAO.

An existing dynamic model of the IFNb pathway generated by IDIBAPS could be extended to other drugs, and they developed an ODE model of SGK-FOXO3 signalling in order to predict the dynamics of the activation of this pathway by BN201, as well as its interaction with other MS pathways and drugs. Similarly, an ensemble approach developed at KI was used to overcome the current limitations of the type I IFN ODE model built by IDIBAPS that they used as a starting point. The idea was to maintain the model simple while making it more comprehensive, avoiding detailed pathway models, while trying to use a data-driven approach and extending it to several types of data (SNPs, phenotypes, cells, etc…). The aim was to generate families of solutions and to acknowledge that these are different, thereafter treating these different classes of solutions as sufficient explanations under a particular scheme. Using

these models, the difference between healthy controls and MS patients could be assessed in terms of the genotype and phosphoproteome data collected.

Computational network models of pathways relevant to MS that identify new treatment options

A final approach adopted at Anaxomics (AX) involved characterizing MS at the protein level through bibliographic searches, and predicted potential combinations after constructing a MS network and generating a network model. Those predicted combinations could then be subjected to a patentability analysis, as well as study of their safety profile and a MoA prediction by sampling methods. The characterization of MS identified 5 distinct motifs:

1) Autoreactivity and multifocal inflammation;

2) Blood Brain Barrier (BBB) adhesion and destruction;

3) Demyelination;

4) Axonal damage and neuronal loss; and

5) Remyelination.

These MS motifs were characterized at the protein level, identifying 237 different proteins that could be used as seeds to construct the network, generating a map with a total of 2608 proteins. The construction and topological analysis of the MS protein interaction network showed that the pathology was correctly characterized and it highlighted known relationships with other pathologies or treatments, as well as highlighting some relationships that may not be so obvious. Static, topological information is only the first step towards a complete understanding of any biological process and thus, the construction of mathematical models will help identify and characterize new therapeutic strategies to treat MS.

After applying the appropriate filters, a list of 33 potential drug combinations was obtained from the network model that met the criteria of synergy, patentability and MoA rationally based on the drug target. These include combinations of current therapies (e.g., Dimethyl fumarate and IFNb) with non-MS drugs. Combinations of Fingolimod could not be identified because their individual predictive value is very high, so it was impossible to find combinations that met the threshold of the synergy filter. After analysing the safety profile of each combination, the list of candidates was ranked and they were finally filtered to select candidate combinations the MoA of which might involves proteins included in the phosphoproteome defined by PAO (See Fig. 3). Gene expression was further introduced into this system in order to provide more accurate results.

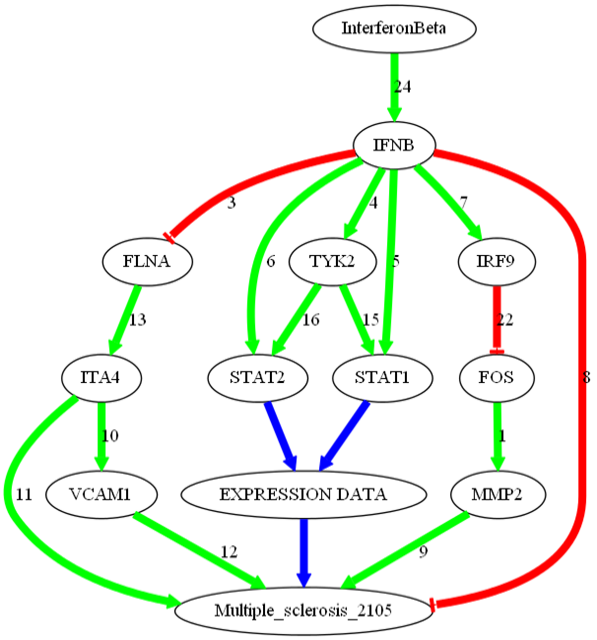


Figure 3. Example of the MoA predicted by the sampling methods used by AX. *Links:* Red lines indicate Inhibition and green arrows Activation. *Nodes (proteins or functions):* White background in a node reveals the involvement of the node in the synergistic MoA of a drug combination.

Summary of the most significant results

- Validated xMAP phosphoassays and very clean validated cytokine assays for MS studies with good consensus and low background noise;
- A preliminary list of phosphoproteome signals that may be relevant to MS;
- Creation of a logic model of MS pathways ready to be trained against experimental data in order to identify the signalling network regulating the cellular response to treatment in MS.
- A protein interaction network of MS with a total of 2608 proteins that represents the biological knowledge of MS pathophysiology.
- A list of 27 potential combinations that meet the criteria of synergy, patentability and MoA rational, established based on drug targets for which the safety profile has been also analysed.

Redefining the clinical phenotypes of MS based on parameters from data collection and modelling

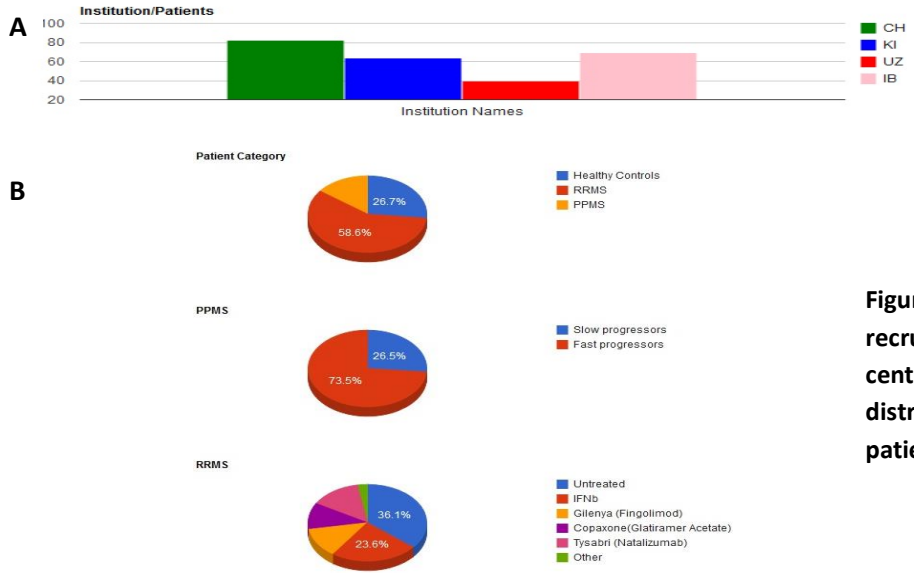


Figure 4. (A) Number of patients recruited at the four clinical centres and (B) percentage distribution of individuals by patient category.

Phosphoproteomic data from PBMCs of untreated MS patients or those treated for at least 1 year with any first or second line therapies

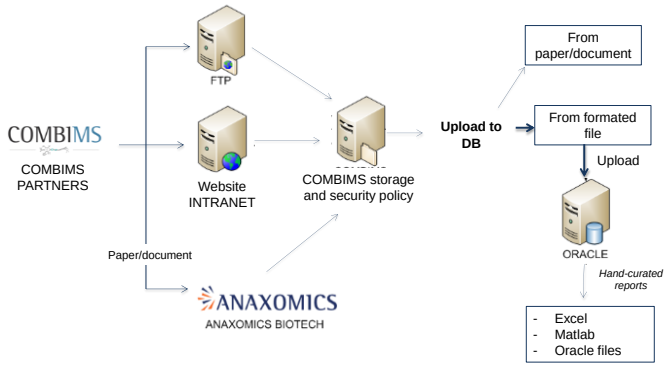
Unified standard operating procedures (SOPs) for PBMC stimulation and lysis, as well as for sample storing and shipping were established, along with a kit to perform the procedure in order to reduce external variability in the results. A total of 255 individuals, MS patients and healthy controls, were recruited at the clinical centres involved in the consortium (UZH, KI, Charité and IDIBAPS). Of these 255 individuals, 26.7 % were healthy controls, 58.6 % were patients with relapsing-remitting MS (RRMS - either untreated or treated with different drugs), and 14.7 % were patients with primary progressive MS (PPMS) including both slow and fast progressors. Most patients were being followed and treated at these clinical centres before enrolment in the study, although some patients were referred by physicians at local hospitals, private practices or by patient advocacy groups (UZH).

The patients that participated in the study were explicitly informed about the project and signed the informed consent form. Moreover, in compliance with the Ethics requirements related to research involving humans, approvals by the competent Institutional Review Board (IRB) were obtained at each participating centre that require such permission. We ensured that these approvals covered transfer of

human biological samples and data between partners. The patient's clinical data collected was subjected to Quality Controls and stored in a specific database. The PBMC lysates from healthy controls and MS patients obtained at the 4 clinical centres were sent to PAO to perform the phosphoprotein and cytokine assays, and DNA was extracted from the blood samples for genotyping and delivered to IDIBAPS.

Clinical data management and patient information database

A protocol for managing clinical information was prepared and the databases generated in dedicated data storage equipment. An online electronic CRF was designed to register patient information (sample donors) according to the project needs. During the time the patients' database was open, it was reviewed and curated. The statistics section allowed the consortium to control the samples and identify whether more patients of any given phenotype should be recruited.



The Clinical MS Phenotypes to be considered in the study were defined by all the clinical partners (IDIBAPS, UZH, KI, Charité), and the acceptable treatments to be used and the number of samples needed were also defined. A hierarchy for the analysis to be performed was agreed:

- Cases (two cohorts of patients) vs controls, age and sex matched at each centre.
- One group of untreated MS patients early in their diagnosis should be obtained (< 3 years after first symptoms).
- Use Fingolimod or Dimethyl Fumarate to define the phosphoproteome profile but not the response, since the action of these drugs is faster than that of IFNb. Recruit patients who have been on Fingolimod or Fumarate at least for 3 months.
- Regarding the definitions of MS patient subgroups, it was agreed to include all the samples available from untreated early MS patients and Fingolimod treated MS patients (without dividing them into responders and non-responders), and to then perform a post-hoc analysis afterwards to stratify.
- Focus on IFNb to study differences in response.
- Define the phosphoproteome profile of non-responders to IFNb (or insufficient responders). To define a response or insufficient response to IFNb, the standard definition will be accepted:
  Responder: no relapses and no change in EDSS in the 2 year follow-up after the onset of therapy;
  Non-responder: 1 relapse or increase of 1 point in the EDSS in the 2 year follow-up after the onset of therapy.
- If possible, analyse disease subtypes.

Regarding the number of samples it was agreed to recruit 100 controls and 200 untreated MS patients, to study two time points (5 and 20 min, which means 24 treatments in each plate) and not to carry out replicates on the subjects.

Phosphoproteomic and cytokine data, and the genotyping of healthy controls and MS patients

The phosphoproteomic and cytokine data from healthy controls and MS patients was obtained through xMAP assays. Cytokine profiles were measured first as they were considered to be less complicated and

cheaper to learn from. A pipeline for data acquisition was devised that involved 5 quality control (QC) check points to increase the robustness of phosphoproteomic and cytokine measurements:

1) **Evaluation of donor and sample characteristics as QC checkpoint of the isolation process.** Those with low cells counts or rare donor phenotypes were set aside, leaving a sample size of 208.

2) **QC checkpoint of the stimulation process**. Positive controls of alignment and stimulation for phosphoproteomeand cytokine measurements.

3) **QC checkpoint of data acquisition process.** Based on the Bead Count and "irregular" distributions.

4) ***QC checkpoint of the Biological noise*** through replicates and negative control beads.

5) **QC checkpoint of instrument noise**

The phosphoprotein data was also visually curated to exclude single well errors that might distort the data, excluding wells that represented outliers. Patients "enriched" in outlying wells were also excluded from the dataset. This process filtered out a total of 7 patient samples and 37 wells.

To take into account the inter-individual genetic variability, genotyping data was also obtained from DNA samples collected from the subjects. The patients were genotyped for a total of 120 SNPs validated as associated with MS (identified by the International Multiple Sclerosis Genetics Consortium - IMSGC), applying appropriate quality control and data exclusion measures to ensure the quality of the data. Regarding the samples, 8 of the 244 samples analysed did not pass all the quality controls and were removed from the results file. Likewise of the 120 genotyped, 8 SNPs did not pass the quality controls and were also removed from the results file.

Fitting the Boolean models developed to the experimental data to identify an MS signalling signature

Based on the logic model generated, an optimization strategy was defined to identify one signalling network for each patient that could explain the phosphoproteome dataset acquired. Further analysis of the predictive model characterized the signalling MoA of single drugs according to the patient's clinical treatment for MS. A data processing pipeline was established to normalise the data and account for the differences between patients and clinical centres. The change in phosphorylation for each protein and each patient was calculated with respect to the control conditions (Fig. 5).

The normalised data was then used to generate a robust signalling network for each patient. For optimization, the network was transformed into a logic model and all the interactions expanded to assess all the possible combinations by replacing them with logic gates. Finally, an identifiability analysis revealed the portion of the expanded network that could be identified, and unidentifiable parts were removed. These expansion and reduction steps yielded a final network of 83 nodes and 462 reactions.

To predict which signalling mechanisms within this initial network produce a signal transduction pattern most similar to the experimental observations, 100 randomly-modified networks were generated and the best solution was found for each patient. This process was repeated 10 times for each patient, which enabled robust merged solutions to be calculated (Fig. 6).

To identify a MS signalling signature associated with the disease and the response to current therapies, the patient-specific models were compared. **A set of interactions was identified that might explain the differences between the groups and the predicted signatures of interest as therapeutic targets for experimental validation**. Characteristic interactions for each group of patients were determined according to the disease subtype and drug response signalling patterns, and the patient-specific models were compared between healthy and MS individuals. Accordingly, signatures of interest were identified,

i.e. interactions that differ strongly between MS patients and healthy donors and that constitute a promising therapeutic target.

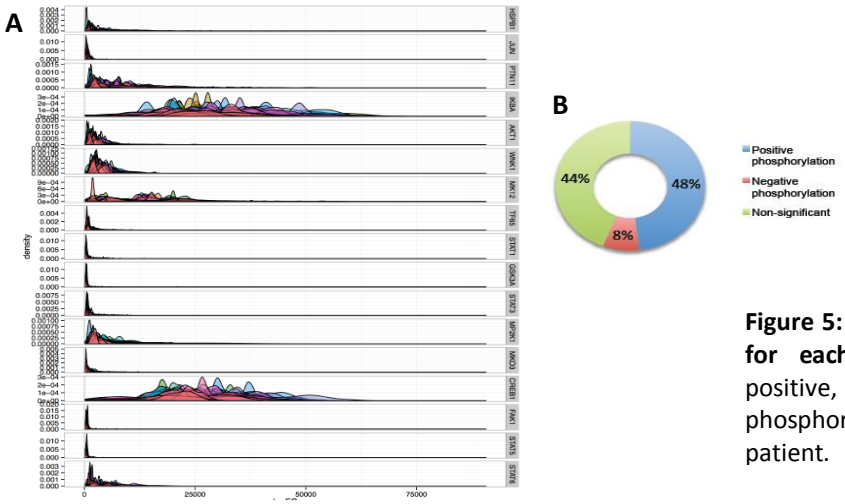


Figure 5: Phosphoproteomic dataset for each patient. Assessment of positive, negative and non-significant phosphorylation by protein for each patient.

Fitting of ODE models of MS pathways to the experimental data

A multi-scale modelling approach was developed, introducing the concept of MS disease course and progression into a dynamic model suitable for different phases of MS (disease subtypes), and that was useful to define new therapeutic strategies for the disease. The model focused on general knowledge about MS, and it combines previous ODE models with imaging and clinical datasets in order to decipher the influence of the inflammatory attacks produced by immune cells on the neurological symptoms observed in MS patients.

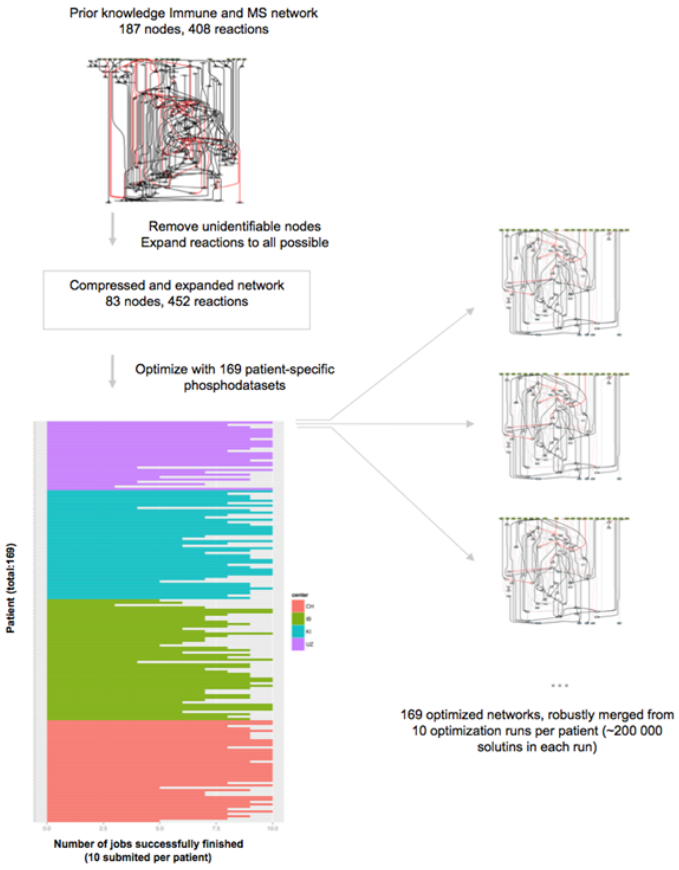


Figure 6: Workflow for the calculation of a patient-specific network.

Computational models – Mathematical Modelling of immune cell/CNS interactions

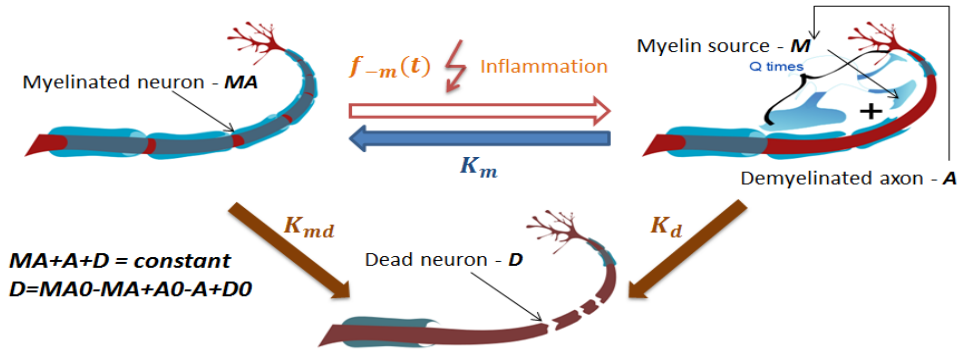


Figure 7. Ordinary differential equation (ODE) model of the MS

In another approach to integrate highly informative and functional 'omics data (phosphoproteomics) with other 'omics data (genotyping, cytokines), and to correlate this with the clinical MS phenotype, a minimal mathematical model was used that describes the disease course based on the interaction of three well-known disease components: inflammatory, anti-inflammatory and the CNS. The model was developed to consider the interplay among the cell types governing the course of MS. In this model individual genetic and molecular information could be linked to the clinical phenotypes, thereby exploring putative clinical scenarios of the individual with MS. The different hypotheses of how each cell type can exerts its effects on the system were tested mathematically.

Using these models the similarities and differences in phosphoprotein responses to given stimuli were analysed among the sub-groups of MS patients, which led to a prediction of the best combination therapies for each sub-group. Likewise, the genotyping data was analyzed to find the most significant SNPs in each sub-group, producing an overall picture of the distance between the sub-groups that was consistent with that obtained for the phosphoproteomic data.

Using Support vector machine and random forest to find molecular signatures of each phenotype, as well as to define new subtypes for stratification, molecular signatures were found separately in each dataset and at different levels. The relationships between the molecular signatures of the different phenotypes were modelled to develop personalized mathematical models.

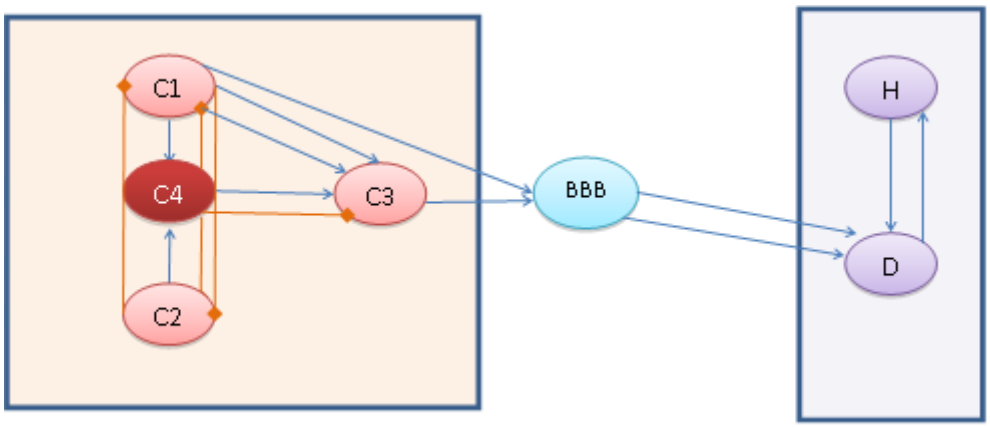


Figure 8: The schematic representation of MS-Model. D stands for demyelinated axons and H represents healthy or repaired axons.

Summary of the most significant results

- The clinical phenotypes, treatments and sample size for the study were defined, and the procedures established, producing a specific Kit to obtain samples from the blood provided by healthy controls and MS patients to be analysed in xMAP assays, and for genotyping.
- A secured electronic CRF was established to handle patient data and clinical information
- Phosphoproteomic, cytokine and genotyping data was collected from healthy controls and MS patients
- Data-driven logic and ordinary differential equation (ODE) models of MS pathways were generated and optimized to predict the signatures of interest as drug targets for experimental validation. Two complementary mathematical models of MS were developed: one to predict the best therapeutic strategy for each individual; the second to predict the effect of such treatment for individuals in function of their genotype.

Predictive modelling of combination therapy and biomarkers

Identification of potential drug combinations through a literature, database and network analyses

The computational signalling models developed specifically for the different MS phenotypes were used to identify the most promising new combinatorial treatments and to predict their safety profile. An intensive literature search was carried out to update the information available and include articles related to MS published in 2013 (Fig. 9). Accordingly, 16 new proteins were identified and added to the MS network.

Table 1. Disease characterization update: 16 new proteins have been included in the AX database.

Motive	# Proteins June 2013	# Proteins Jan.2014
Autoreactivity and Multifocal inflammation	98	111
BBB adhesion and destruction	47	50
Demyelination	41	41
Axonal damage and neuronal loss	93	93
Remyelination	39	39
Total:	**227**	**243**

The AX system was prepared to incorporate the clinical data as a biological restriction that the model must meet, generating different sub-models for each disease phenotype that will allow it to identify the best drug combination for each phenotype (Fig. 10).

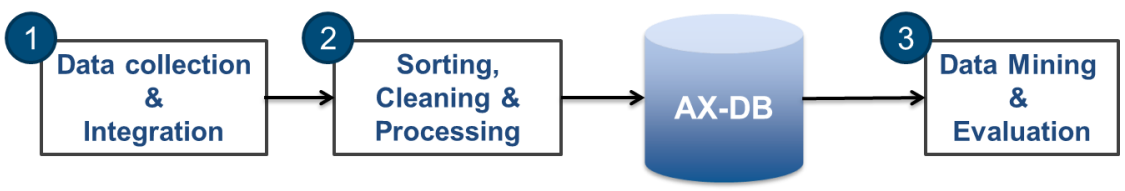
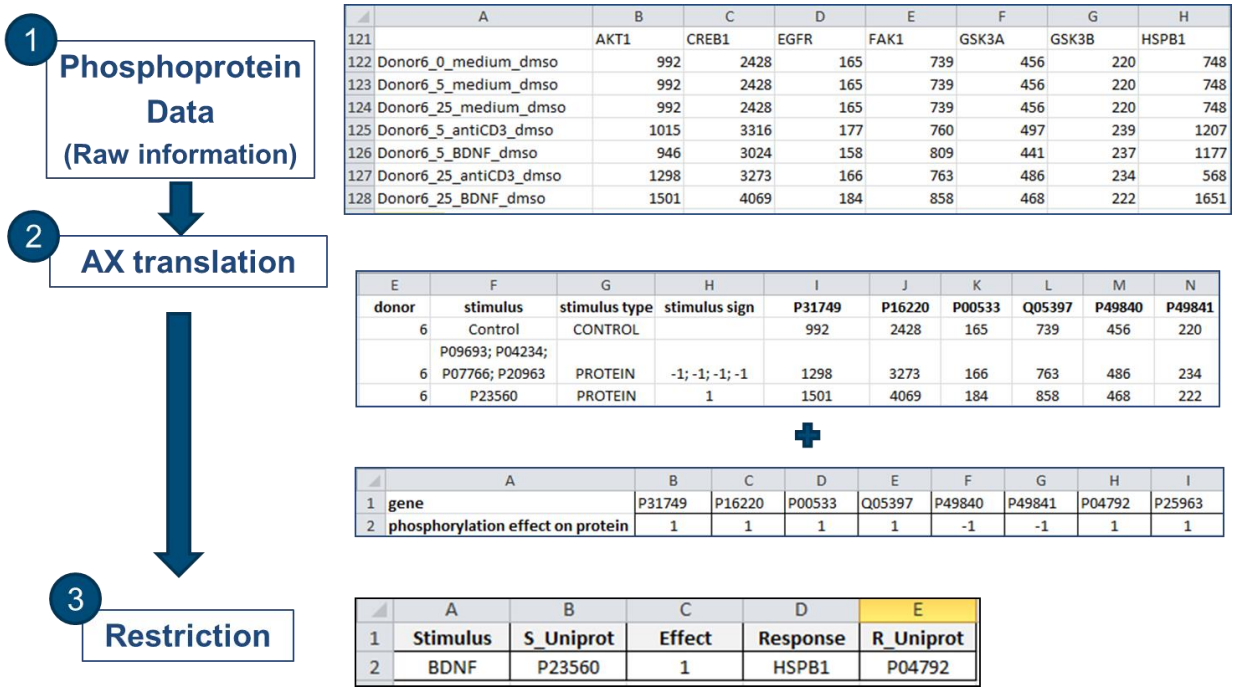


Figure 9. Diagram summarizing the process followed to incorporate phosphoproteomic data into the AX system

The statistical analysis identified proteins that were more or less phosphorylated relative to the control sample and these proteins were then be crosschecked for functional activity, an activity that could then be included in the mathematical model as a biological restriction. Using the biological restrictions extracted from the evaluation of the phosphoproteomic data, different sub-models could be generated for each phenotype and thus, the efficacy of a drug or combination of drugs for the different phenotypes can be evaluated and compared. The clinical data (phosphoproteomic, cytokines and genotyping data) was then used to identify four potential combinations of standard of care MS drugs and non-MS drugs by analysing their influence on the effectors of MS pathology.



	A	B	C	D	E	F	G	H
121		AKT1	CREB1	EGFR	FAK1	GSK3A	GSK3B	HSPB1
122	Donor6_0_medium_dmso	992	2428	165	739	456	220	748
123	Donor6_5_medium_dmso	992	2428	165	739	456	220	748
124	Donor6_25_medium_dmso	992	2428	165	739	456	220	748
125	Donor6_5_antiCD3_dmso	1015	3316	177	760	497	239	1207
126	Donor6_5_BDNF_dmso	946	3024	158	809	441	237	1177
127	Donor6_25_antiCD3_dmso	1298	3273	166	763	486	234	568
128	Donor6_25_BDNF_dmso	1501	4069	184	858	468	222	1651

E	F	G	H	I	J	K	L	M	N
donor	stimulus	stimulus type	stimulus sign	P31749	P16220	P00533	Q05397	P49840	P49841
6	Control	CONTROL		992	2428	165	739	456	220
6	P09693; P04234; P07766; P20963	PROTEIN	-1; -1; -1; -1	1298	3273	166	763	486	234
6	P23560	PROTEIN	1	1501	4069	184	858	468	222

	A	B	C	D	E	F	G	H	I
1	gene	P31749	P16220	P00533	Q05397	P49840	P49841	P04792	P25963
2	phosphorylation effect on protein	1	1	1	1	-1	-1	1	1

	A	B	C	D	E
1	Stimulus	S_Uniprot	Effect	Response	R_Uniprot
2	BDNF	P23560	1	HSPB1	P04792

Figure 10. Summary of the steps followed to integrate phosphoproteomic data from patient samples into the AX workflow.

Identification of potential drug combinations by iterative simulations of the dynamic logic models and analysis of the cell-specific ODE models

The individual patient networks generated previously were used to elucidate drug MoAs and to predict the combination therapy that would restore signalling to a healthy profile. First, interactions were identified for which the difference MS to Healthy is larger than Drug to Healthy (i.e.: the interactions that the drug is expected to revert in the MS signalling phenotype). Then, the interactions that each drug fails to target were identified, on the basis that these disregulated interactions are those that differ the most between healthy and MS patients for each drug used *in vivo.*

The best *in-vitro* stimuli and/or drugs to be combined with the drugs of interest (Fingolimod -Fingo-, Interferon-beta –IFNb-, glatiramer acetate –GA- and epigallocatechin gallate -EGCG) were defined by analysing the interactions that are expected to be successfully and unsuccessfully targeted, and that will revert the network to healthy signalling when accompanying the drugs of interest. Therefore, such combinations, in the case they are drugable, would be candidates for validation. Likewise the cell-specific ODE models were used to propose the best combinatory therapies for each sub-category solely

based on phosphoproteomics data and the statistical analysis. The significance of the biological processes associated with the corresponding combinations was then evaluated.

Identification of the potential side-effects of combination therapy for MS

In view of the relevance of lowering the safety issues related to MS treatments, the safety profile of the combinations of interest was assessed with the models developed to identify the target- and off-target-related predictable safety issues for each combination. Previously reported, unreported and potentially related adverse reactions were identified for each combination, providing a first approximation of the safety profiles and establishing the groundwork for further analysis in animal models.

Testing of the combination therapies identified in vitro to assess their effects on signalling pathways

We performed *ex vivo* assays to test the effect of each stimulus and validate the phosphoproteomic patterns identified as a read-out of the phenotype. For this validation step, we switched from the xMAP technology to flow cytometry assays for two reasons: first, it is important to validate molecular findings with a different technology to that used in the screening; and second, because flow cytometry allows the cell subpopulation responsible for the phosphoprotein signal to be characterized, as well as providing single cell data (information regarding the heterogeneity of cell activation). We selected the set phosphoproteins and stimuli to be validated on PBMCs from MS patients in the cytometry assays based on the phosphoproteome network associated with MS and the different disease phenotypes. Accordingly, we observed large differences in phosphorylation for most of the proteins in the different cell subtypes, and we observed similar phosphoprotein profiles between control and MS groups as in the xMAP experiments. By using flow cytometry in these validation studies, we could also identify the type of immune cells responsible for the differences.

Summary of the most significant results

- After enriching the models with novel data, combinations were identified that are predicted to show synergism, i.e.: increased efficacy with respect to the drugs alone, for all the models tested. One combination in particular was of special interest as it had the highest synergy, a literature-based plausible MoA and there is no previous IP for the combination.
- Other combinations involving the drugs of interest (Fingo, IFNb, GA and EGCG) were also identified and biomarkers identified to be used in the validation experiments.
- Four combinations have been evaluated for safety, assessing their previously reported, previously unreported and potentially related ADRs, a first approximation to define their safety profiles.
- The phosphoproteome signatures were technically and biologically validated *in vitro* assays using PBMCs from MS patients. Thus, these selected combinations therapies could then be validated *in vitro*.

Validation of the model's results and generalization of the experimental-computational pipeline

Validation of the efficacy and predicted side-effects of selected combination therapies in animal models

To evaluate the efficacy of the selected combination therapies identified, initial experiments were performed in an accepted and extensively used animal model of MS, Experimental Autoimmune Encephalomyelitis (EAE), in which inflammatory demyelination is induced in the CNS. The use of suboptimal doses of the two drugs in one combination therapy and of optimal doses of another combination produced an improvement in the clinical score of animals with chronic EAE when compared with the suboptimal or optimal doses of each compound alone. Such differences were higher than the sum of the effect of each drug, suggesting the presence of synergistic effects. Other combination therapies selected by the Consortium are still being assessed and the results are expected to be available by late March/April, 2015.

The safety of these two combination therapies was assessed in the EAE mice and the first was well tolerated by the animals. No immediate adverse events were observed after administration, nor unexpected deaths or changes in the gross pathology. The second combination was tested in the chronic phase of EAE, when animals are severely ill and with significant neurological disability. This clinical stage implies that a given number of animals may die due to the severity of the disease and indeed, some deaths were observed in the mice that received the placebo, or the drugs in isolation. Significantly, no deaths were observed in the mice that received the combination therapy and moreover, these animals did not show significant abnormalities in terms of their gross pathology beyond the changes in the CNS.

Selection of the combination therapies validated in vitro and in vivo for further regulatory development

To perform an appropriate pharmaco-economics study, a general overview of the pathogenesis, epidemiology and current treatment options for MS was first established. The market situation and key facts of the major brands were assessed, allowing a final evaluation of the opportunities for CombiMS combinations to be made. The information for this pharmaco-economics report was gathered from several sources, such as company literature, databases, investment reports, and medical and business journals focused on neurodegenerative diseases research, and in particular, MS research.

The objectives of this task were to:

- assess the potential for new treatments;
- identify optimal R&D development strategies based on market characteristics;
- quantify future size and scope of market and potential for new products;
- understand key drivers and predict the future performance of key compounds.

A preliminary market analysis was performed with the CombiMS-selected drug combinations. Assuming that improvements in the efficacy of individual drugs the combinations could present market advantages, subject to acceptable safety profiles and other variables (route of administration, possibility of treatment of already untreated MS categories…). Only an initial glimpse into the overall environment to determine the likelihood of success is possible at this stage as no concrete experimental data is available on efficacy, synergy or adverse drug reactions.

IP protection for the combinations is difficult as most of the individual drugs are already protected for the indication. There is, however, a niche for protecting drug combinations showing unexpected

synergistic efficacy, or new formulations with fixed-dose combinations. Reformulating drugs that have been proven to be safe and effective into fixed-dose combination (FDC) products represents a strategy for drug development companies to maximise commercial returns. Both patients and physicians could benefit from the potential increase in efficacy, convenience and compliance as well as fewer co-payments.

Packaging the "Drug discovery method" to take it beyond its application for MS

Molecular medicine is presently flooded by terabytes of high-throughput data obtained with different technologies. Machine learning and graph-based methods facilitate our ability to recognize complex patterns. However, the pipeline established and tested here with the phosphoproteomic and genotyping data associated with MS, indicates that the general concept and the tools are adaptable for other complex diseases. Indeed, not only can this pipeline be implemented in other diseases where cell-cell interactions are likely to be the driving force (mediated as here by phosphorylation based signalling) but also, where the phosphoproteomic data can be substituted for any other set of 'omics data.

Summary of the most significant results

- The combination therapies tested to the date have enhanced efficacy in ameliorating the course of EAE when compared to each drug in isolation, coupled to a good safety profile. Hence, they appear to show a synergistic effect, validating our technology for the discovery of combination therapies. Accordingly, new combination therapies have been identified and validated at the preclinical level that can now be considered for testing in patients with MS in clinical trials.
- These results validate our systems biology based drug discovery approach to predict combination therapies based on a phosphoproteomic network analysis.
- Appropriate pharmaco-economics studies have been performed, allowing a final evaluation of the opportunities for CombiMS combinations to be made. Moreover, regulatory requirements have been assessed for the selected combination therapies.
- We have established an integrated experimental – computational modelling methodology to enable the discovery of combination therapies for complex diseases such as MS. The whole pipeline has been tested on the CombiMS data but the general concept and the tools are adoptable for other complex diseases. Indeed, not only can this pipeline be implemented in other diseases where cell-cell interactions are likely to be the driving force (mediated as here by phosphorylation based signalling) but also, where the phosphoproteomic data can be substituted for any other set of 'omics data.

The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

Awareness and Wider Societal Implications

Politicians have been consistently indicating that Europe must shift to an economy based on knowledge and innovation, with biotechnology representing one of the sectors that will play a key role in this process. However, from an economic point of view the current crisis and the innumerable cuts in state funding to the R&D sector threat to delay development and industrial growth, coupled with the increasingly restricted access to private investment (VC, Business Angels, Pharma related family offices).

At the societal level we have also seen how initiatives have been established to spread the knowledge generated by biotechnology, and the effort made by pharmaceutical companies to present a better image is also noticeable. How biotechnology can contribute to social well-being can be seen by the fact that the goods it produces are tightly bound to quality of life. Moreover, this industry may also become an important motor in the creation of stable and quality employment, as clearly demonstrated by the USA biotech.

In terms of the impact of the CombiMS project, we must look beyond the direct health implications to MS patients towards patients with other complex diseases that might benefit from the methodology developed in the course of the CombiMS project. Indeed, the demonstration that such a systems medicine approach may be capable of predicting novel therapeutic approaches for many complex and less complex diseases may leverage the incorporation of this technology into the pharmaceutical industry as a whole, representing an important advance in this field. Such benefits would clearly have important effects on society, speeding up and amplifying drug discovery programmes. This strengthening of drug discovery programmes can only have a beneficial influence on the pharmaceutical industry and on society as a whole.

The practical example of our approach for the drugs being developed by the participant SMEs will give them a significant **competitive advantage and strategic value** to advance their research projects from the preclinical to clinical stage, to raise new investments and agreements with pharmaceutical companies, and to help position the European biotech industry in the global market. Indeed, a major pharmaceutical company has already expressed its interest in following the CombiMS project in order to consider employing this drug discovery pipeline for its own use.

The two main objectives of the CombiMS project have been successfully achieved: the development of at least one novel and more complete therapy for the treatment of at least one sub-group of MS patients; and the validation of a novel systems medicine approach to identify safe and more effective combination therapies to treat complex diseases. The impact of these achievements will be felt by many of the stakeholder groups that may have interest in the project. Not least, it is intended that the foreground arising from the project will be protected and exploited by the 3 SMEs that have participated in CombiMS. These include 3 of the different combination therapies identified, and the sample collection and diagnostic kits produced in the project. In addition, the possibility of developing the phosphoproteome signatures as biomarkers of disease progression and prognosis will also be explored.

Target groups addressed

We specify in the following table the target groups that might benefit from the outputs/outcomes of the CombiMS project and the dissemination tools used to reach each target group.

Table 2: Benefits of CombiMS

Target group	Benefits from the project	Dissemination strategy
Researchers working in the field of MS and systems medicine	A comprehensive understanding of the biological MoA of current MS therapies and potential new therapies. New tools to design combination therapies targeting MS in particular and complex diseases in general. New tools to identify biomarkers of the pathogenesis of MS in particular and complex diseases in general, candidate biomarkers for clinical phenotypes of MS, and biomarkers of response to therapy. A new validated and optimized approach that integrates networks, logic and ODE models, in an 'application' to resolve clinical/biological questions.	Consortium web page Participation at workshops and conferences and courses Scientific publications Promotional leaflet Newsletters Publication of data and models generated in open access repositories and databases
MS patients, MS Patient Associations and Federations	New validated combination therapies for MS at the preclinical level. Possible drug combinations for MS with higher efficacy, better safety profile and wider coverage to address unmet needs, and tailored to the different stages of disease evolution. A basis for the future development of personalized medicine for autoimmune diseases.	Consortium web page Promotional video available on "Youtube" Press releases Promotional pamphlet Contacting directly the patients at the outpatient clinic Future formal contacts with the main MS patient's societies.
The pharmaceutical and biotech industries	Information on a new experimental /computational methodology based on system biology that enables efficient and safe identification of combination therapies and biomarkers for complex diseases A basis for the future development of personalized medicine for autoimmune diseases.	Consortium webpage, Promotional leaflet, Newsletters and Press releases Participation in workshops and conferences, e.g., academic/industry workshops Scientific publications Participation in industrial forums and partnering events Publish data and models in open access repositories and databases

Regulatory authorities, Health Policy makers within the EC, National health authorities	Evidence to make more informed policy decisions on the development of initiatives for the improvement of patient health through systems medicine-based approaches and practice. Data for the development of policies and programmes in MS therapy and services.	Participation in Systems Medicine Open Stakeholder Consultation Conference organized by the EU and other EU consortia Future contacts with the national and EU MS patient's organisations who will translate the information to health policy makers and regulatory authorities Future participation in EU and national policy-making events
Healthcare professionals	New validated combination therapy for a MS at the preclinical level. Potential drug combinations for MS with higher efficacy, better safety profile and wider coverage to address unmet needs (e.g., progressive patients with MS), and tailored to the different stages of disease evolution of the patient. Prediction of the possible side-effects related to proposed combination therapies. Identification of candidate biomarkers for clinical phenotypes of MS and of biomarkers of response to therapy. A basis for the future development of personalized medicine for autoimmune diseases.	Consortium web page Participation in workshops and conferences Scientific publications Promotional leaflet Newsletters Publish data and models generated in open access repositories and databases Collaboration with other EU and international Consortia and initiatives
Other EU consortia and initiatives	Source of information and data to improve the modelling of complex diseases, the identification of biomarkers and the drug discovery process. Knowledge, tools and resources to advance the research and obtain more translational results from systems medicine approaches.	Make the models and data generated available in open access repositories and databases Future formal collaboration with eTRiKS Participation in workshops organised by CASYM and EU/CASYM
General public	Improved awareness and knowledge on MS and system medicine. Demonstration of how European collaboration can achieve scientific excellence, contributing to competitiveness and solving societal and health challenges.	Consortium web page Promotional video Press releases Articles in the popular press Publications via the website of partners' institutions

Dissemination

A Dissemination Plan was drawn up describing the dissemination strategy to be implemented by the consortium partners within the CombiMS project, which involved the preparation of specific material to promote the dissemination of the consortium's activities. All the material bore reference to the funding of the EU and a non-liability clause, as well as the consortium logotype, created in the first months of the project to be used as the image of the project for use in all the materials related to the project.

1) **Website** - The CombiMS website was set up to provide information relating to the main aspects of the project and to promote its activities. It has constituted the consortium main dissemination tool of the goals, activities and results of the project to all target groups. The CombiMS website has been regularly updated with dissemination material (news, newsletters, promotional leaflet, promotional video). The webserver is located at http://www.combiMS.eu/.

2) **Pamphlets -**Information pamphlets have been prepared reflecting the project's scope, approach, objectives and expected outcomes, together with useful contact information. The purpose of these pamphlets was to provide non-electronic material for Partners to distribute at meetings, workshops, and conferences in order to publicize the project and to make all interested parties and the general public aware of the project. In addition, material has been prepared for patients that have or that may be interested in participating in this or other studies.

Accordingly, two different pamphlets have been designed during the project:

- A version of the pamphlet aimed at technically aware audiences (in particular, the scientific community and industrial stakeholders) to be distributed at meetings, workshops and conferences, in order to publicize the project and making all interested parties assisting these events aware of the project's objectives, approach, methodology and expected outcomes, which could lead to future collaborations.
- A version of the pamphlet aimed at less technically aware audiences. This is a brochure to be made available to the general public and that can be circulated among the patients that have participated in the study, as well as other patients that may be interested in participating in other future studies.

Both pamphlets have been made available via the CombiMS website and distributed to the Consortium Partners to be translated into their own language and distributed to interested stakeholders in their respective countries.

3) **Newsletters -** Electronic newsletters were generated to share project related information with stakeholders and other interested parties. The newsletters included a brief update on the progress and achievements of the project, a brief description of the main results obtained to date, information relating to dissemination activities undertaken by members of the CombiMS consortium, and information about project meetings and forthcoming tasks. The CombiMS Newsletters were produced on a more-or-less quarterly basis from February, 2014 and they were published on the CombiMS Website.

4) **Promotional video -** A promotional video was prepared with the general public in mind, in order to present the project in a multimedia format through different media channels. The aim of this communication tool is to provide the wider public with a concise and visual understanding of the significance of the CombiMS research study. The CombiMS video is available publically through the CombiMS website and at YouTube (https://www.youtube.com/watch?v=KnpCnJsp_d4). Partners have been encouraged to ensure that the video receives the best dissemination possible, recommending it where possible and uploading it to sites that might be of interest. The Commission was informed of the availability of the video, which can be posted through servers available by the Commission if interested.

Dissemination activities

Internal dissemination within the Consortium has been carried out through traditional means (phone, e-mail), through monthly teleconferences and via the intranet of the CombiMS website. The Intranet has been used as an internal tool to share confidential material and results related to the project within the Consortium. In addition, consortium meetings were held every 6 months to review progress and to discuss the implication of the results obtained, as well as to plan the activities for the following 6 months.

Dissemination activities beyond the Consortium:

Publications in scientific journals

By the end of the project, six scientific articles based on the methodological approach and results related to the CombiMS project will have been published in peer-reviewed journals:

- Kotelnikova E, Bernardo-Faura M, Silberberg G, Kiani NA, Messinis D, Melas IN, Artigas L, Schwartz E, Mazo I, Masso M, Alexopoulos LG, Mas JM, Olsson T, Tegner J, Martin R, Zamora A, Paul F, Saez-Rodriguez J, Villoslada P. Signaling networks in MS: *A systems based approach to developing new pharmacological therapies*. Mult Scler. 2015 Feb 13; 21(2):138-146.
- Simón Perera, Laura Artigas, Roger Mulet, José M. Mas, Teresa Sardón. *Systems biology applied to non-alcoholic fatty liver disease (NAFLD): treatment selection based on the mechanism of action of nutraceuticals.* Nutrafoods (2014) 13:61-68.
- Iranzo J, Villoslada P. *Autoimmunity and tumor immunology: two facets of a probabilistic immune system.* BMC Syst Biol. 2014 Nov 11;8(1):120.
- Sara Martinez-Pasamar, Elena Abad, Beatriz Moreno, Nieves Velez de Mendizabal, Ivan Martinez-Forero, Jordi Garcia-Ojalvo, Pablo Villoslada. *Dynamic cross-regulation of antigen-specific effector and regulatory T cell subpopulations and microglia in brain autoimmunity.* BMC Syst Biol. 2013 April 26;7(1):34.
- Friedemann Paul, Mike P Wattjes. *Chronic cerebrospinal venous insufficiency in multiple sclerosis: the final curtain.* The Lancet. 2014 Jan 11;383(9912): 106-108.
- Morris MK, Chi A, Melas IN, Alexopoulos LG. *Phosphoproteomics in drug discovery.* Drug Discov Today. 2014 Apr;19(4):425-32.

It is envisaged that other papers will be prepared and published this year, an article presenting the main results of the CombiMS project and an additional one on the phosphoproteomic methods.

Patient Organisations

Apart from having informed the patients recruited at the four specialized MS outpatient clinics, CombiMS promotional leaflets have been made available to the patients that visit outpatient clinics and they have been distributed by email to the patients that participated in the study. The CombiMS project was also presented to the International MS international Federation (MSIF) who endorsed it. The CombiMS consortiums plans to contact in the future National MS societies from each participating country in order to explain their research, findings and the potential benefits for people suffering these diseases.

Presentations at conferences/workshops/courses

The members of the Consortium have participated in conferences and scientific meetings relevant to the field, both to disseminate information on the progress of the project and to seek the opinions of other experts. The consortium has taken every opportunity to present the findings through oral presentations and posters at conferences. Among the most important international conferences that members of the CombiMS Consortium have attended are the International Conference on Systems Biology of Human Disease (SBHD 2014), the International Conference on Systems Biology (ICSB 2014) and the 2014 Joint ACTRIMS-ECTRIMS Meeting.

Participation in Industry Forums and Partnering Events

The methodology and results of the CombiMS project has been presented at several brokerage and partnering events, such as the **BIO-Europe® Conference** (http://www.ebdgroup.com/bioeurope) or the EBD Group's European partnering conference. This allowed contact to be established with some companies, research institutes and other institutions that have shown their interest in the methodology and results of the CombiMS project.

Press releases

A few press releases were prepared at the beginning of the project in which the CombiMS project was promoted and these were published on the webpage of a MS patient associations, pharmaceutical industry and communication portals, and at the website of other partner institution. In addition they have served as the basis for several articles published in economic, medical newspapers, pharmaceutical marketing magazines and public health portals.

Collaboration with European consortia and open databases

The CombiMS Consortium has started formal discussions with the eTRICKS Consortium (http://www.etriks.org) to establish a collaboration for data storage, standardization, harmonization and access. All members of the CombiMS consortium have agreed to transfer a copy of the data obtained in the CombiMS project to the eTriks server as long as it is provided in coded form and free access will be provided once the final results of the CombiMS project have been published.

Members of the CombiMS Consortium (Pablo Villoslada) have participated in two CASyM Systems Medicine Open Stakeholder Consultation Conferences aimed at addressing transversal issues in Systems Medicine, and in the CASyM Academic/Industry interactions 2014 workshop (Ekaterina Kotelnikova) to help bringing about public/private European collaborations in systems medicine

The CombiMS Consortium has participated in an *"EC survey on systems bio-medicine & input to infrastructure needs for implementing systems medicine in Europe*" that was conducted by the CASyM Consortium (https://www.casym.eu) to gather expert opinion to be communicated at the 2014 EC/CASYM workshop.

The CombiMS consortium plans to release data and collaborate with international initiatives aimed to improve the modelling of complex diseases and the drug discovery process. As such, logic models developed by the consortium will be deposited at the Biomodels database (http://www.ebi.ac.uk/biomodels-main/), a curated repository of mathematical models of biological processes. Protein phosphorylation data will be deposited at Phosphosite (http://www.phosphosite.org) a repository of protein modifications maintained by the NIH, and Phosida (http://www.phosida.com/) and PhosphoELM (http://phospho.elm.eu.org/), two phosphorylation site repositories.

Closing workshop

A closing workshop is planned to be held in which the results of the research carried out in CombiMSwill be presented to members of the scientific community and industry that may be interested in the findings. The aim is to organise this workshop as a satellite meeting at the *31st Congress of the European Committee for Treatment and Research in Multiple Sclerosis* that will take place on the 7th-10th of October 2015 in Barcelona, Spain.

Protection and exploitation of the foreground and results of the project

An analysis has been performed of the advances obtained during the project in relation to the state of the art and of the possibilities for further development, and the different stakeholders who might benefit from the outputs/outcomes of the project have been described. In general, the work carried out during the project has increased our knowledge on MS and other neurodegenerative diseases. A novel dataset and knowhow has been generated that is stored by the companies in the consortium according to standard protocols. This expanded knowledge, together with all the proprietary technologies of the different partners, will enhance the services that the CombiMS participants can offer to their clients or stakeholders, increasing market competitiveness. In addition, the results of the CombiMS project will be published in peer reviewed international scientific journals, through at least 5 articles in the next 12 months, one of them hopefully in the top tier publication. Moreover, some partners have identified potential exploitable opportunities.

Combination Therapies

As part of Bionure's commercial programme to develop its proprietary novel BN201 candidate drug, there is clear interest in the company to exploit the combination therapies involving this molecule. Combination therapies mixing 2 drugs could enhance the efficacy of the drugs by targeting different MoAs, while potentially, they may also decrease the side effects as each drug could be administered at a lower dose when prescribed together. Thus, combinations therapies with BN201 could increase Bionure's pipeline and represent a good strategy to bring to the market better and safer drugs. With Bionure's expertise in drug development, this combination will be programmed to pass through all the initial stages of development and should the results prove to be positive in terms of efficacy, safety and toxicology, as would be expected, all efforts will be made to move this therapy towards the market in the forthcoming years. A first draft of a new patent that covers the combination identified will be submitted by Q2 2015, and further research is being carried out and will be needed to determine the most efficacious combination dose.

Anaxomics has defined a potentially useful combination therapy through standard repositioning protocols from a list of combinations of MS drugs + non-MS drugs. The patentability analysis and a

preliminary mechanism of action exploration has identified this combination as interesting for MS treatment.

Diagnostic and prognostic kits

The findings of the CombiMS consortium have opened the opportunity to develop a prognostic and diagnostic kit based on the phosphoproteome of a pool of around 200 patients and controls. ProtATonce (PAO) is a Systems Pharmacology service company that employs a platform based on multi-omics HTS data and computational analysis to provide solid and experimentally proven answers in drug discovery. These include the prediction of efficacy, toxicity, target identification, drug MoA and drug repositioning. The services offered by PAO are based on custom xMAP assays with more than 500 analytes available in their pipeline. The kit developed by PAO for sample collection facilitated the work carried out by systematizing and homogenizing the procedures performed in the clinical centres involved in patient recruitment. Based on the knowledge and expertise acquired during the project, the company is planning to develop the 2 kits outlined below:

Kit for sample collection as part of a diagnostic kit. The kit will be based on the sample collection kit that was developed by PAO during the course of the CombiMS. The kit will be designed to provide everything required for on-site sample collection, including all the supplies necessary to safely collect, label, store and ship patient samples to a desired location where testing of the samples can be performed for diagnostic purposes.

Development of a Multiplex kit that can predict MS responders/non-responders and/or serves as a prognostic for MS. The kit will be based on the sample collection kit that was developed by ProtATonce during the course of the CombiMS study and the prior expertise of PAO in the development of multiplex assays. The kit can be used to predict responders from non-responders and/or it may be predictive of MS.

This new strategy opens possibilities to the company to expand their services and products, and it will improve the company's competitiveness in the biotech sector and in the global market. It is hoped that the development of these kits can be commenced in Q3 of 2015.

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http://www.combims.eu

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