Point-Of-Care Monitoring and Diagnostics for Autoimmune Diseases

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Abstract—In this paper we present the POCEMON platform, a platform aiming to the early prognosis and diagnosis of autoimmune diseases at any point of care, even the primary. The objective of the POCEMON platform is the development of a diagnostic lab-on-chip device based on genomic microarrays of HLA-typing. The POCEMON is going to advance and promote the primary health care across Europe by supporting a) point-of-care diagnostics, b) monitoring of immune system status and c) management of the chronic multiple sclerosis (MS) and rheumatoid arthritis (RA) autoimmune diseases. The platform combines high-end Information and Communication Technologies based on microfluidics, microelectronics, microarrays and intelligent diagnosis algorithms.

I. INTRODUCTION

Personalized diagnosis and treatment is driven by the rapid development of information and communication technologies concerning advances on both hardware and software development. These efforts promote new diagnostic procedures based on Lab-on-a-chip (LOC) technologies [1]. The LOC technologies are capable of performing a wide range of proteomic and genomic tests by using a sample of blood or other body fluid such as saliva. These tests aim to facilitating healthcare at preferred environments and point of care disease diagnosis at primary healthcare level. The LOC technologies expose a tremendous potential to improve the health of people worldwide. Ever since the modern inception of LOC and microfluidic technologies around 1990 their use in remote settings has been perceived as potentially one of the most powerful applications of the technology by taking advantage of its small size, low volume requirement for samples, and rapid analysis. Indeed, portable LOC devices are now beginning to be used in remote settings as a result of developments in integrating fluid actuation, sample pre-treatment, sample separation, signal amplification and signal detection into a single device. These advances place the field of LOC research in a prime position to tackle the profound issue of global health where the challenges in device design are arguably the most demanding and the need for new health technologies the greatest.

Autoimmune disorders [2] develop when the immune system destroys normal body tissues. This is caused by a hypersensitivity reaction similar to allergies, where the immune system reacts to a substance that normally would ignore. The disorder may affect only one organ or tissue type or may affect multiple organs and tissues. Organs and tissues commonly affected by autoimmune disorders include blood components such as red blood cells, blood vessels, connective tissues, endocrine glands such as the thyroid or pancreas, muscles, joints, and skin.

There are many diseases which are believed to be autoimmune. These include Multiple Sclerosis (MS) [3], Rheumatoid Arthritis (RA) [4], Addison's Disease, Ankylosing Spondylitis, Autoimmune Haemolytic Anaemia, Autoimmune Hepatitis, Coeliac Disease, Chronic Fatigue Syndrome (CFS, CFIDS), Chronic Inflammatory Polyneuropathy, Crohn's Disease, Dermatomyositis, Grave's Disease, Hashimoto's Thyroiditis, Type 1 Diabetes, Polymyalgia Rheumatica, Systemic Lupus Erythematosus (SLE) and many others. More specific, there are over 80 autoimmune diseases.

The HLA (human leukocyte antigens) are proteins found

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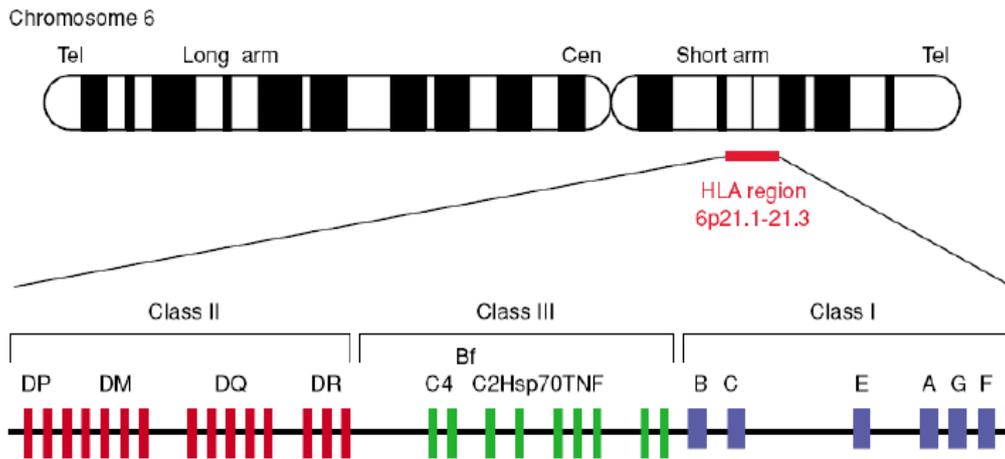
Gene map of the human leukocyte antigen (HLA) region

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Fig. 1. Gene map of the human leukocyte antigen (HLA) region. The HLA region spans 4×10^6 nucleotides on chromosome 6p21.1 to p21.3, with class II, class III and class I genes located from the centromeric (Cen) to the telomeric (Tel) end. HLA class I molecules restrict CD8+ cytotoxic T lymphocyte function and mediate immune responses against 'endogenous' antigens and virally infected targets, whereas HLA class II molecules are involved in the presentation of 'exogenous' antigens to T helper cells. The HLA class III region contains many genes encoding proteins which are unrelated to cell-mediated immunity but that nevertheless modulate or regulate immune responses in some way, including tumour necrosis factor (TNF), heat shock proteins (Hsps) and complement proteins (C2, C4).

in the membranes (outer coating) of nearly every cell in the body (all cells that have a nucleus) (Fig. 1). HLA antigens are the major determinants used by the body's immune system for recognition and differentiation of self from non-self (foreign substances). There are many different major histocompatibility (HLA) proteins and individuals possess only a small, relatively unique set that is inherited by their parents. It is unlikely that 2 unrelated people will have the same HLA make-up. Many HLA molecules exist but some are of special interest because they are more common in certain autoimmune diseases. For example, HLA-B27 antigen is found in 80-90% of people with ankylosing spondylitis and Reiter's syndrome and can aid in the diagnosis of these diseases. HLA-B27 is also present in 5-7% of people without autoimmune disease. Thus, the mere presence of this HLA molecule is not indicative of a disease. HLAs detectable in blood provide clues to immune conditions.

There have been some reports on HLA genotyping by microarray [5,6]. Most of these studies focused on tissue transplantation and association between HLA genotypes and disease or pathological process [7,8]. A genotyping microarray approach can analyze polymorphisms at multiple-sites in a single gene or in multiple genes rapidly and in a parallel way [9]. The genomic HLA microarray scanning data can also be computerized and shared with a Personal Digital Assistant (PDA) device which will be hosted in the diagnostic POCEMON platform. Although, the feasibility of microcantilever based DNA biosensors has been demonstrated [10,11], as well as, the integration of a electronic interface realized with a CMOS technology

[12,13], a complete system including the detection module and the microfluidic structures for the sample handling is still not available in the market for specific applications in the genomic field.

In this paper, we describe the methodology which is applied to the huge amount of data produced by the gene discovery phase (a Whole Genome Association Study) of the POCEMON platform to select the most informative SNPs which are directly related with the RA & MS autoimmune disorders. Finally, we present the lab-on-chip technology which will be used to develop the innovative diagnostic LOC platform based on HLA-typing microarrays.

II. MATERIAL AND METHODS

A major milestone of the POCEMON platform is the development of specific HLA-typing microarrays capable to diagnose the MS & RA autoimmune disorders. To complete this task the following stepwise procedure is applied:

a) Genome-wide association study. Genotyping of 750 control and case DNA samples both from MS & RA patients and production of a huge amount of raw data. This process is based on the iLLumina technology [14].

b) Selection of the most informative SNPs by using an approach based on data mining methods and genetic algorithms.

c) Development of a diagnostic lab-on-chip device based on genomic microarrays of HLA-typing to allow the early prognosis of diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA).

A. Genome-Wide Association Study

It is estimated that more than 10 million SNPs exist in the human genome. SNPs are known to contribute to population diversity and phenotypic differences between individuals, and cause predispositions to diseases. Whole-genome association studies hold the promise of identifying SNPs associated with a certain phenotype of interest as well as those that can serve as diagnostic markers. Genes in the vicinity of SNPs that appear to cause the phenotype could be qualified as new drug targets for pharmaceutical companies. To successfully identify candidate SNPs using whole genome association analysis, the researcher needs to consider sample size, multiple testing correction, SNP selection and genotyping quality.

A genome-wide association study (GWA study) - also known as whole genome association study (WGA study) - is an examination of genetic variation across the human genome, designed to identify genetic associations with observable traits, such as blood pressure or weight, or why some people get a disease or condition.

These studies require two groups of participants: people with the disease (cases) and sex- and age-matched unaffected individuals (controls). After obtaining samples from an individual, the set of markers such as SNPs are scanned into computers. The computers survey each participant's genome for markers of genetic variation.

If genetic variations are more frequent in people with the disease, the variations are said to be "associated" with the disease. The associated genetic variations are then considered pointers to the region of the human genome where the disease-causing problem resides. Since the entire genome is analysed for the genetic associations of a particular disease, this technique allows the genetics of a disease to be investigated in a non-hypothesis-driven manner.

A genome-wide association study is an approach that involves rapidly scanning markers across the complete sets of DNA, or genomes, of many people to find genetic variations associated with a particular disease. Once new genetic associations are identified, researchers can use the information to develop better strategies to detect, treat and prevent the disease. Such studies are particularly useful in finding genetic variations that contribute to common, complex diseases, such as asthma, cancer, diabetes, heart disease and mental illnesses.

Illumina Infinium II technology allows the fast and reliable genotyping of up to 1.000.000 SNPs per individual at once. Whole Genome Genotyping (WGG) is based on the Sentrix BeadChip platform [15] on which 13 million DNA-immobilized Beads are randomly dispersed and assembled into wells created on a single slide. The location and identification of each bead is obtained through a decoding process. On average a redundancy of around 20 beads per bead type is obtained. The first step of Infinium WGG is a Whole Genome Amplification of genomic DNA (WGA). WGA is based on Multiple Displacement Amplification (MDA) which uses the φ29 DNA polymerase and random primers to amplify the entire genome generating hundreds of micrograms of amplified DNA starting from an initial input of several hundred nanograms (currently, 750 ng). WGA gives a uniform locus representation that enables the access to almost all the SNPs in the genome. The amplified DNA is then fragmented to an average size of around 300 bp using an enzymatic fragmentation protocol and locus-specifically hybridized to each individual target on the beads. The specificity of the hybridization is assured by the use of long full-length 50-mer oligonucleotides. After hybridization, each SNP is characterized through a single-base enzymatic extension assay using two-color labeled nucleotides. After extension, the labels are visualized by staining with a sandwich-based immunohistochemistry assay that increases the overall sensitivity of the assay. BeadChips are imaged using a two-color confocal laser system with 0.8μm resolution. The Bead intensities are extracted and genotypes are calculated with the BeadStudio 3.2.29 software using a "cluster" file, based on a set of reference samples supplied by Illumina.

B. Selection of the most informative SNPs

Genetic profiles of the MS & RA individuals are constructed using the data produced by the gene discovery phase using the iLLumina technology. The analysis of the produced huge amount of data, leads to genes/SNP patterns which are responsible for the RA & MS diseases as well as genetic risks. In order to handle such a dataset there is a need to select the most informative genes/SNPs for further analysis.

While the target of POCEMON aims to the early prognosis and diagnosis of the RA & MS autoimmune diseases the selection of the most informative SNPs is necessitated. The approach which is used in the POCEMON platform is based on data mining and genetic algorithms. Artificial intelligent methods such as global search and weighted decision trees are employed for the selection of the most significant SNPs.

The SNPs selection approach is applied to datasets to provide the early prognosis and diagnosis of the RA & MS autoimmune disorders. It is expected that the number of the features is significantly reduced while the quality of knowledge is enhanced. The selection approach is going to provide the most significant SNPs.

Data mining methods support all the processes of discovering interesting and previously unknown patterns in the genotyped MS & RA data sets. A main advantage of the data mining concerns the study of an individual rather than the population, providing routes for personalization in early prognosis and diagnosis. The goal of the selection is to identify the minimum set of non-redundant features (e.g., SNPs, genes) which are useful in the classification phase.

C. LOC platform development and integration

The integration of LOC technology with PDA - using microelectronics - is going to provide new diagnostic tools at the primary care level (Fig. 2). The implementation and the development of the portable POCEMON diagnostic LOC is achieved by following a modular approach of the various sub-parts of the integrated platform. The different components are developed according to the following steps:
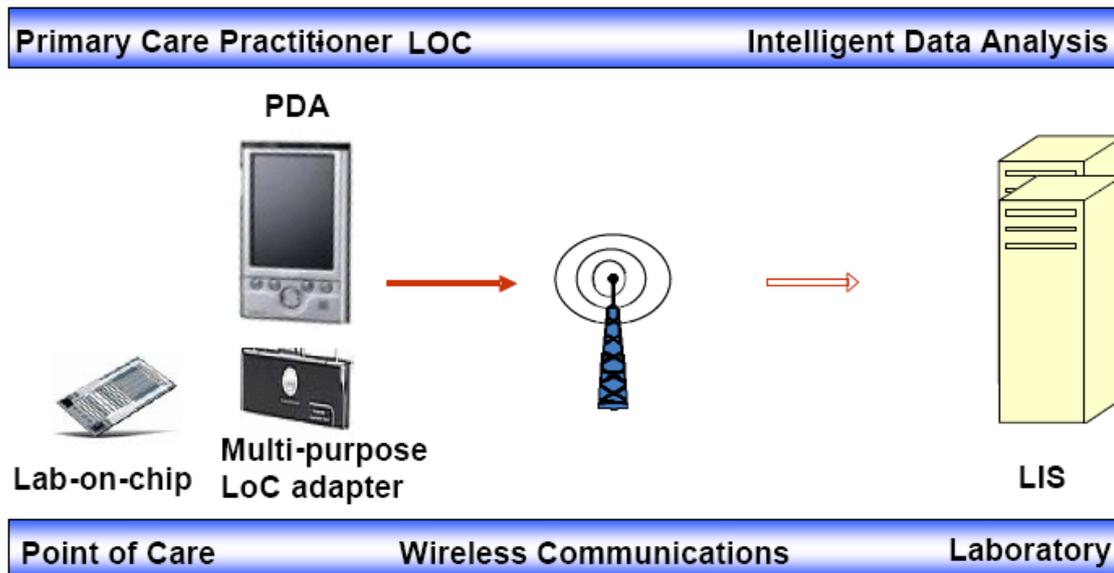
Fig. 2. The integration of all POCEMON parts via wireless communication

- Identification of the major relationship between HLA alleles and the specific multiple sclerosis and rheumatoid arthritis autoimmune diseases.
- Design of the HLA microarray probes based on the technology of oligonucleotide microarrays for autoimmune diseases.
- Implementation of the HLA genotyping microarrays on appropriate materials.
- Design of the microelectronics LOC with mechanisms to handle blood and/or saliva samples.
- Incorporation of LOC techniques to automatically analyze oligonucleotide microarrays through hybridization inside the diagnostic platform using microelectronics.
- Employment of portable and mobile technology in the diagnostic LOC using PDAs.
- Development of a large data repository (database) which is hosted on a central laboratory or medical centre. The PDA device communicates and interchanges data with the database server by tracking and updating medical records concerning the patient immune system.
- Development of the oligonucleotide Microarray computerized scanning mechanism in the PDA of the diagnostic platform.
- Implementation of appropriate desktop PDA software for signal intensity analysis of the microarray.
- Extraction of HLA probe's results in the PDA screen. In this phase the LOC platform provides a preliminary diagnosis for autoimmune diseases.
- Wireless communication with centralized database to provide detailed diagnostic analysis of scanned microarray probes using comprehensive and intelligent algorithms.
- Integration of all of the above in the final diagnostic

product, an autonomous portable diagnostic LOC platform.

D. Development of the Diagnostic HLA Lab-on-Chip

Starting from the state-of-the-art in literature a suitable configuration for the LOC is studied in order to provide the handling of DNA samples, PCR amplification and HLA typing with a labelfree approach based on a microcantilever array of appropriate size functionalized with oligonucleotide probes (Fig. 3). The microfluidics includes sample ports, channels for sample delivering and reservoirs with temperature control for PCR amplification. The selected technology for the realisation of the LOC fluidics involves bulk micromachining techniques (wet and dry etching steps) and wafer-to-wafer bonding to create channels and reservoirs, deposition of thin film metallization to implement heaters and thermometers for the thermal control of the PCR reactor. The label-free detector is realised with a microcantilever array working in the bending mode (i.e. in the stress-detection mode) which is more suitable for operating in liquid phase. The beam surface is provided with a gold electrode in order to address and bond the specific DNA probes based on thiol chemistry. The signal conditioning electronics, including a switching matrix, signal amplification and ADC is integrated on-chip with a CMOS technology. The technologies involved deals with both ad hoc Silicon-On-Insulator (SOI) approaches for the realisation of thin suspended Silicon beams with tight thickness control and the implementation of thin film cantilever compatible with CMOS processes. In the framework of this system implementation, the design of single system modules (microfluidic module for sample handling and PCR amplification, detector module and signal conditioning electronic module) is studied as well as a technological approach suitable for the realisation of a single module and system integration. At first, each module is
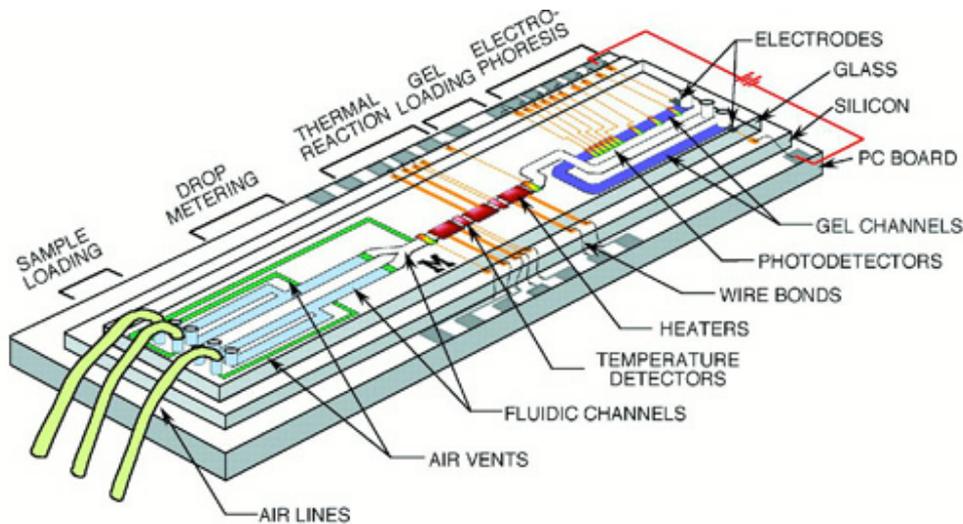
Fig. 3. Example of a Lab-on-Chip device. Obtained from Burns, et al., Science, vol 282, pp. 484-487, 1998 [16].

developed as stand-alone device for validation of the selected technological processes and preliminary efficiency testing. In a second step a definitive design is realized in order to implement the final modules in a monolithic chip. The signal conditioning interface and device specifications will be addressed to the development on a PDA platform of the software tools needed to control the LOC operation and data acquisition and analysis.

Summarizing, the approach aimed to develop a diagnostic HLA Lab-on-Chip is based on:

- The development of the microfluidics components for DNA sample handling, PCR amplification and reservoirs.
- The development of detector modules based on cantilevers array for preliminary studies of the sensitivity to specific DNA chains.
- The development of optimized detector modules based on a high density cantilevers array on CMOS technology. Related to the ultra-high expected sensitivity of the sensors, the PCR amplification module could be substituted with a less sophisticated microfluidics pre-concentrator.
- The development of an integrated control unit (electronic circuit) for sensor read out and signals conditioning.

III. CONCLUSIONS

The integration of LOC technology with genomic microarrays of HLA-typing is going to provide new diagnostic tools at the primary care level. Also, the most informative selected SNPs are used to design and produce new microarray chips containing the appropriate probes to diagnose the RA & MS autoimmune diseases. The application of the algorithms and methods to datasets produced by other similar genome wide association studies is going to enhance the scientific and research community with innovative tools for the early prognosis and diagnosis of almost all autoimmune disorders.

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