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Molecular Mechanisms of Cancer

Epigenetic Alterations in Sepsis

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Work carried out under the supervision of:
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Declaração de autoria do trabalho

Declaro ser o(a) autor(a) deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(assinatura)

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Resumo

A sepsis representa a resposta inflamatória sistémica do hospedeiro a umainfecção, (Iskander*et al.*, 2013) e é a 15ª causa mais significativa de morbidade e mortalidade em todo o mundo. A sepsis provoca 20 a 30 milhões de casos anuais no mundo inteiro causando uma morte a cada 3-4 segundos (WSD *et al.*, 2013). Os pacientes com sepsis requerem custos substanciais de cuidados de saúde durante a sua hospitalização. Para além disso, os sobreviventes incorrem em custos adicionais devido ao aumento dos requisitos de cuidados de saúde nos anos que se seguem ao episódio da sepsis (Iskander*et al.*, 2013)

A sepsis é um processo complexo que pode ser iniciado por uma ampla gama de infeções, bacterianas, virais ou fúngicas, numa ampla gama de locais corporais. Muitos são os fatores de risco que despoletam um choque séptico ou o desenvolvimento da sepsis severa. Estes incluem, idade (75-85 anos), etnia e sexo (Alexander Melamed *et al.*, 2009), bem como comorbidades como o cancro (linfomas ou leucemias), ou outras doenças imunossupressivas como HIV ou diabetes(Martin GS *et al.*, 2006). As infeções nosocomiais, resultantes da exposição ao ambiente hospitalar, também são uma causa importante de sepsis (Farinas Alvarez *et al.*, 2001).

Na fisiopatologia desta doença heterogénea, uma resposta inflamatória intensa é desencadeada, e seguida por uma cascata de eventos secundários, incluindo disfunção endotelial, coagulopatia, apoptose celular e imunossupressão (Stearns-Kurosawa*et al.*, 2011). Uma infeção localizada torna-se assim uma síndrome sistémica, mediada por fatores solúveis como citoquinas e células imunes circulantes, como neutrófilos e leucócitos (Stearns-Kurosawa*et al.*, 2011). Estes eventos contribuem para a disfunção progressiva dos órgãos e, posteriormente, a morte.

O diagnóstico precoce da sepsis pode ser desafiador devido à existência de outras doenças com apresentações similares. Os "imitadores" de sepsis incluem insuficiência cardíaca, insuficiência renal, pancreatite, anafilaxia, embolia pulmonar, isquemia intestinal, cetoacidose diabética e vasculite, entre muitos outros. Os marcadores de proteínas da sepsis, incluindo a procalcitonina e a proteína C-reativa, não são confiáveis (José Raimundo Araújo

prognóstico.

Este projeto visou estudar esta doença complexa de uma forma inovadora. Com colaboração com um grupo de investigadores no Canadá, criou-se pela primeira vez uma coorte de pacientes sépticos (n=68) e pacientes com terapia intensiva não-séptica (n=66). Um

estudo chamado EPSIS (*EpigeneticProfiling in Severe Sepsis*) visou compreender a importância da regulação epigenética na patogénese, progressão, resposta ao tratamento e resultados de pacientes sépticos críticos.

Esta tese focou-se na hipótese que pacientes com e sem sepsis apresentavam diferentes padrões de metilação de DNA nos genes implicados na patogénese de sepsis. Como primeiro objetivo e fase neste projeto, identificou-se genes-chave na regulação epigenética na sepsis. Identificou-se genes que são diferencialmente regulados na sepsis, e que se correlacionam com diferentes vias e consequências metabólicas importantes, como a coagulopatia, disfunção endotelial, imunossupressão eimunoactivação e apoptose. Assim, após esta revisão bibliográfica extensa criou-se um cluster inicial de 128 genes.

De seguida, com os dados de metilação provenientes do coorteda EPSIS, analisou-se as diferenças de metilação de DNA entre as várias sondas dos 128 genes, potenciado, desta forma, a criação do segundo cluster de genes que se mostraram significativos na distinção de um paciente com sem sepsis e sem sepsis.

Na terceira fase, e como o nosso foco assentava na análise desta doença de uma maneira robusta, analisámos trêsconjunto de dados, provenientes do GeoDatasets, que avaliaram expressão de genes em pacientes com e sem sepsis. Assim, apesar de não podermos fazer uma correlação direta entre os nossos dados de metilação e os dados da transcriptómica, conseguimos entender quais os genes que estão diferencialmente regulados na patogénese desta doença.

Com as análises de metilação e de transcrição criou-se, pela primeira vez, um grupo de 10 genes que se mostraram estar diferencialmente metilados e expressos na sepsis. Entre esses incluem-se HLA-A, HLA-C, HLA-DOB, HLA-DQB1, FADD, APOL3, ITGB2, NLRP12, VARS2 e C3AR1. Este grupo de genes desempenha diferentes funções como inflamação, apoptose, imunidade inata e adaptativa. As análises mostraram que metade destes genes apresentaram um elevado grau de metilação, nomeadamente HLA-DOB, APOL3, FADD, NLRP12 e VARS2, enquanto os outros cinco (HLA-A, HLA-C, HLA-DQB1, C3AR1, ITGB2) se encontravam hipometilados. Analisando as diferenças ao nível de expressão genica a maioria destes genes encontravam-se subregulados.

A última fase deste projeto focou-se na correlação de parâmetros clínicos relevantes com a metilação dos 10 genes que anteriormente se revelaram significativos. Diferentes parâmetros foram analisados para a coortede pacientes, incluindoos dias de permanência da unidade de cuidados intensivos, os dias de sobrevivência, o tipo de cultura bacteriana (gram-

positiva ou gram-negativa); administração de vasopressores, esteróides, ventilação mecânica; e presença de doenças crónicas incluindoinsuficiência cardíaca e doença cardíaca isquémica. Em cada um destes parâmetros analisou-se varias sondas de CpG que apresentaram significância estatística e pudemos estabelecer uma relação entre metilação destes genes eo parâmetro clínico.

Este projeto centrou-se no estudo da sepsis, uma doença altamente complexa, heterogénea, e frequentementefatal. Nós reunimos, para a primeira vez, perfis de metilação de DNA de 66pacientes sépticos e 68pacientes com terapia intensiva não-séptica e compáramos esses dados com informações de transcriptómica de 3coortes diferentes de pacientes com sepsis. Adotamos uma abordagem de bioinformática para identificar e estabelecer assinaturas de metilação de DNA da sepsis em um conjunto focado de genes relacionados a sepsis. Dez desses genes, incluindo HLA-A, ITGB2, FADD, e APOL3, mostraram mudanças significantes de metilação e transcrição, sugerindo regulação epigenética. Além disso, identificamos correlações entre várias sondas CpG e parâmetros clínicos, incluindo infecção gram-positiva versus gram-negativa, sobrevivência global, e necessidade de vasopressores.

Palavras-chave:

Epigenética, Sepsis, Metilação de DNA, Biomarcadores

Abstract

Sepsis is a life-threatening complication of infection. Typically, a localized infection triggers a systemic inflammatory cascade resulting in widespread organ damage, organ failure, and often death. Of the 31.5 million cases of sepsis worldwide annually, it is estimated that there are 5.3 million deaths (Fleischmann *et al.,* 2016). The pathophysiology of sepsis involves widespread reprogramming of gene expression. Bioinformatic approaches have revealed multiple gene pathways that are activated or inhibited in sepsis (DC Angus and Tom Van der Poll *et al.,* 2013). Epigenetic mechanisms, including histone modifications, DNA methylation, and non-coding RNAs (such as microRNAs, siRNAs, andribosomal RNAs) are master regulators of gene expression in both normal and pathological states. Although there is limited data on epigenetic regulation of sepsis, localized epigenetic changes have been identified in individual genes.However no genome-wide data has yet been published. In this studywe define a number of sepsis-related DNA methylation changes in sepsis-associated genes. We correlate these changes with gene expression and with clinically-relevant outcomes. Finally, we will investigate the mechanisms by which DNA methylation regulates individual gene expression in sepsis.

Keywords:

Epigenetic, Sepsis, DNA Methylation, Biomarker

A sepsis é uma complicação potencialmente fatal derivada de uma infecção. Normalmente, uma infecção localizada desencadeia uma cascata inflamatória sistémica, resultando em danos generalizados nos órgãos, falência de órgãos e, muitas vezes, a morte. Dos 31,5 milhões de casos de sepsis reportados em todo o mundo anualmente, estima-se que haja cerca de 5,3 milhões de mortes (Fleischmann *et al.,* 2016). A fisiopatologia da sepsis envolve a reprogramação generalizada da expressão génica. As abordagens bioinformáticas revelaram múltiplas vias genéticas que são ativadas ou inibidas na sepsis (DC Angus e Tom Van der Poll *et al.,* 2013). Os mecanismos epigenéticos, incluindo modificações nas histonas, metilação do DNA e RNAs não codificante (como microRNAs, siRNAs ou RNAs ribossómicos) são os principais reguladores da expressão genica, em estados normais e patológicos. Embora haja dados limitados sobre a regulação epigenética da sepsis, ainda não foram publicados dados *genome-wide*. Neste projecto, definiremos as mudanças de metilação do DNA que caracterizam a sepsis em genes associados a patogénese da doença. Iremos

correlacionar essas mudanças com a expressão génica e com parâmetros clínicos relevantes. Finalmente, investigaremos os mecanismos pelos quais a metilação do DNA está a regular individualmente cada gene envolvido na sepsis.

Abbreviations

3

3´UTR - 3′ untranslated region

5

5´UTR - 5′ untranslated region

A

A2AP - Alpha 2-antiplasmin

Alpha – 1B - Adrenergicreceptor (α1B adrenoreceptor)

ANOVA- Analysis of Variance

APAF-1 - Apoptotic protease activating factor 1

APC – Activated protein C

APCs – Antigen- presenting cells

APOL3 - Apolipoprotein L3

ATP – Adenosine Triphosphate

B

BATF - Basic Leucine Zipper ATF-Like Transcription Factor

BAX - Bcl-2-like protein 4

BCL-2 - B-cell lymphoma 2

BET - Bromodomain and extra-terminal

BID - BH3 Interacting Domain Death Agonist

BIM - BCL-2-like protein 11

BIP - Immunoglobulin protein

BIRC1 - Baculoviral IAP Repeat Containing 1

BIRC2 - Baculoviral IAP Repeat Containing 2

BIRC5 - Baculoviral IAP Repeat Containing 5

BIRC6 - Baculoviral IAP Repeat Containing 6

BIRC8 – Baculoviral IAP Repeat Containing 8

BOK-L - Bcl-2 related ovarian killer

BTN3A2 - Butyrophilin Subfamily 3 Member A2

C

C21ORF56 - Spermatogenesis And Centriole Associated 1 Like

C3AR1 - Complement C3a Receptor 1

C6ORF15 - Chromosome 6 Open Reading Frame 15

C9ORF95 - Nicotinamide Riboside Kinase 1

CARS- Compensatory Anti-inflammatory response syndrome

CCL-4 - Chemokine (C-C motif) ligand 4

CD14 - Cluster of differentiation 14

CD2 - Cluster of differentiation 2

CD28 - Cluster of differentiation 28

CD4 - Cluster of differentiation 4

CD80 - Cluster of differentiation 80

CD86 - Cluster of differentiation 86

CEACAM1 - Carcinoembryonic Antigen Related Cell Adhesion Molecule 1

CHN2 - Chimerin 2

CLDN1 - Claudin 1

CNX – Calnexin

CRT - Calreticulin

C-SMAC - Central-SMAC

D

DEFB1 - Defensin Beta 1

DIC - Disseminated intravascular coagulation

DNA - DesoxiriboNucleicAcid

DNMT - DNA methyltransferaseenzymes

DNMT 1 - DNA Methyltransferase 1

DNMT 3a - DNA Methyltransferase 3a

DNMT 3b - DNA Methyltransferase 3b

DNMT b - DNA Methyltransferase b

DPP4 - Dipeptidyl Peptidase 4

D-SMAC - Distal-SMAC

dsRNA - Double stranded RNA

DYNAMICS - DNA as a Prognostic Marker in ICU patients

E

EPSIS - Epigenetic Profiling in Severe Sepsis study

EWAS – Epigenome-wide association study

F

FADD - Fas-associated protein with death domain

FDR - False Discovery Rate

FGA - Fibrinogen Alpha Chain

FGB - Fibrinogen Beta Chain

FGG - Fibrinogen Gamma Chain

G

G-CSF - Granulocyte colony-stimulating factor

GM-CSF - Granulocyte macrophage colony-stimulating factor

GNA12 -G Protein Subunit Alpha 12

GRP79 - Glucose related protein 78

GSTM3C - Glutathione S-Transferase Mu 3

H

H3K9me2 - Dimethylation of histone H3 at lysine 9

HAS-2 - Hyaluronan Synthase 2

HLA - Human leukocyte antigen

HLA-DQA2 - HLA Class II Histocompatibility Antigen, DQ(6) Alpha Chain

HMWK - High-molecular-weight kininogen

HP - Heparin

Hsp100 - 100 Kilodalton heat shock protein

Hsp60 - 60 Kilodalton heat shock protein

Hsp70 - 70 Kilodalton heat shock protein

Hsp90 - 90 Kilodalton heat shock protein

HSPG2 - Heparan Sulfate Proteoglycan 2

I

ICAM1 - Intercellular Adhesion Molecule 1

ICAM2 - Intercellular Adhesion Molecule 2

ICU – Intensive care unit

IL-1 – Interleukin-1

IL-1 RA - IL-1 receptor antagonist

IL-10 – Interleukin-10

IL-12 – Interleukin-12

IL-4 – Interleukin-4

IL-6 – Interleukin-6

IL-8 – Interleukin-8

INO80 - INO80 Complex Subunit

ITGB2 - Integrin Subunit Beta 2

K

KDM6b - Lysine Demethylase 6B

L

LPB - Lipopolysaccharide-binding protein

LPS – Lipopolysaccharides

M

MHC-I - Major histocompatibility complex type I

MHC-II - Major histocompatibility complex type II

miRNA – MicroRNA

ML-IAP - Melanoma IAP

MODS – Multiple organ dysfunction syndrome

MTCH1 - Mitochondrial carrier homolog 1

MyD88 - Myeloid differentiation primary response 88

N

ncRNA – Non-coding RNA

NF-κB - nuclear factor kappa B

NK – Natural killer cell

NLRP12 - NLR Family Pyrin Domain Containing 12

NMP - Nuclear matrix protein

NOD-1 - Nucleotide Binding Oligomerization Domain Containing protein 1

O

OCLN – Occludin

P

PAMPs - Pathogen-associated molecular patterns

PaO2 -Arterial partial pressure of oxygen

PCLN - Paracellin-1

PDI - Protein disulfide isomerase

PF4 - Platelet Factor 4

piRNA - Piwi-interacting RNAs

POL1RD -RNA Polymerase I Subunit D

PRRs - Pattern recognition receptors

P-SMAC - Peripheral SMAC

R

RAGE - Receptor for advanced glycation end products

RFX1 - Regulatory Factor X1

RNA - Ribonucleic acid

RPGRIP1 - Retinitis Pigmentosa GTPase Regulator Interacting Protein 1

Runx3- Runt Related Transcription Factor 3

S

SESN-2 - Sestrin 2

sFas - Soluble Fas receptor

siRNA - Short interfering RNAs

SIRS – Systemic inflammatory response syndrome

SLE - Systemic lupus erythematosus

SNPs - Single nucleotide polymorphisms

SPAKs - Stress-activatedprotein kinases

SRNX1 - Sulfiredoxin 1

SSBP1 - Single Stranded DNA Binding Protein 1

ssRNA - Single Stranded RNA

T

TET- Ten eleven translocation

TLR-10 – Toll-like receptor 10

TLR-2 – Toll-like receptor 2

TLR-4 – Toll-like receptor 4

TLR-6 – Toll-like receptor 6

TLRs - Toll-like receptors

TNF-α - Tumor necrosis factor α

TPA -Tissue plasminogen activator

TSS – Transcription start site

TSS1500 - Transcription start site to 1500 nucleotides upstream of TSS

TSS200 - Transcription start site to 200 nucleotides upstream of TSS

V

VARS2 - Valyl-TRNA Synthetase 2

VWF - Von Willebrand factor

W

WBSCR27 - Williams Beuren Syndrome Chromosome Region 27

Y

YKL-40 - Human Cartilage Glycoprotein-39

Índice

1. Introduction

1.1- Sepsis

Sepsis represents the host´s systemic inflammatory response to a severe infection (Iskander*et al.,* 2013). It is the 15th most significant cause of morbidity and mortality worldwide. In 2016 it was estimated there were 31.5 million cases of sepsis and 19.4 million cases of severe sepsis worldwide, with over 5 million deaths (Fleischamann *et al.,* 2016). Sepsis patients incur substantial health care costs during hospitalization and survivors incurs additional costs due to increased health care requirements in the years following their sepsis episode (Iskander *et al.,* 2013). Although sepsis episodes are short, lasting only days to weeks (as opposed to cancer or other chronic diseases), the associated costs are very high accounting for more than 5% of total US hospital costs ((KN Iskander*et al.,* 2013, Torio CM *et al.,* 2011). Mortality from severe sepsis is also extremely high, with over 40% of patients admitted to the ICU ultimately dying, despite receiving all necessary care.

1.2- Infection and Immunity

Sepsis is a complex process that can be initiated by a wide range of infections, including bacterial, viral and fungal, in almost any body site including the lungs, kidney, abdomen, brain, soft tissues, and blood stream. Risk factors for sepsis include age, ethnicity and sex (Alexander Melamed *et al.,* 2009) as well as comorbidities such as cancer, HIV, and diabetes (Martin GS *et al.,* 2006; Kang *et al.,* 2011). Nosocomial infections, resulting from exposure to the hospital environment, are also an important cause of sepsis (Farinas Alvarez *et al.,* 2001). In US studies the groups with the worst outcomes from sepsis are African-Americans, elderly males between age 75 and 84, and those with nosocomial infections (Alexander Melamed *et al.,* 2009).

1.2.1- Infection

The immune system consists of a network of organs, cells and molecules thataims to maintain homeostasis of the body during an infection (Cruvinel*et al.,* 2010). The non-specific response is immediate and maximum, independent of the antigen and does not result in immunological memory. It is represented by physical, chemical and biological barriers and

can recognize structures shared by microorganisms, named pathogen-associated molecular patterns (PAMPs). Among them are nucleic acids (e.g. single stranded RNAor double stranded RNA present in viruses), proteins, cell wall lipids (LPS in gram negative bacteria) and carbohydrates (e.g. beta-glucan, present in fungi). Innate immune cells, namely macrophages, defend against pathogensby means of surface receptors that recognize and bind to microbial components (https://www.ncbi.nlm.nih.gov/books/NBK27090/). The receptors are known as pattern recognition receptors (PRRs), among which the family of toll-like receptors (TLRs) is the most studied. TLRs play a central role in binding to pathogens and triggering the initiation of aninflammatory response (Takeda *et al.,* 2005). TLR-4, for examplebinds to the gram-negative cell wall component LPS, while TLR-2 recognizes specific structures on the membrane of gram-positive bacteria (Cohen *et al.,* 2002; Opal *et al.,* 2002).

1.2.2- Innate Immunity

This first line of defence involves physicial barriers, including the epitheliumand endothelium at entry points such as the skin,respiratory and gastrointestinal systems. These are also the sites where the main effector cells of innate immunity act, including macrophages, neutrophils, dendritic cells and Natural Killer (NK) cells. Phagocytosis the release of inflammatory mediators, and activation of the complement system constitute the main defence mechanisms of innate immunity. The innate immune response also triggers the release of cytokines and chemokines, secreted proteins involved in the immune response and management of immune cell trafficking. These proteins have growth, differentiation and activation functions (Borish*et. al.,* 2003) and their production is induced by an immune insult. Cytokines may have both pro and anti-inflammatory potential; their ultimateactivity dependingon the immune cells present and their state of responsiveness to the cytokine (Mark D. Turner et. al., 2014). Early recognition of bacterial substances is important for the immune response and for overall survival. The role of the innate immune system is to detect microbialproducts in the tissues and to trigger an effector function to the site of infection (Martin *et al.,* 2000).

1.2.3- Adaptive Immunity

Unlike theinnate response, the adaptive immune response depends on the activation of specialized cells, lymphocytes and soluble molecules. Itis more targeted and specialized to fight an infection. The adaptive immune response is characterized by specificity and diversity of pathogen recognition, response expertise, tolerance to the body's own components, and the creation of immunological memory. In addition to lymphocytes, antigen presenting cells (APCs) play a key role in the activation of the adaptive response by presenting antigens to T lymphocytes.

This secondphase of the immune response begins when a pathogen is ingested by an immature dendritic cell in the infected tissue. These immune cells are derived from the same bone marrow precursors as macrophages.After ingestion of a potential pathogen, the dendritic cell migrates to the lymph nodes where it “presents” the microbial antigens to circulating lymphocytes. Lymphocytes are white blood cells that provide the effector function of the adaptive immune system. They are divided into two classes - B cells, that mediate antibody responses, and T cells, that provide cell-mediated immune responses.
Illustration 1 provides a brief overview of immune system activation in response to a viral infection:

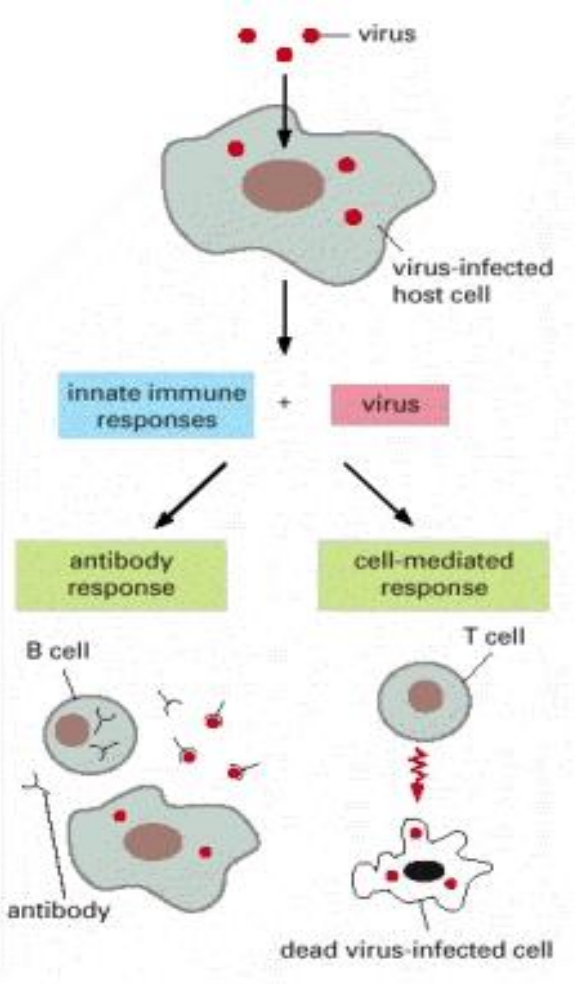


Illustration 1: Adaptive immunity: the two classes of lymphocytes. B cells secrete antibodies that neutralize the virus. And T cells that act primarily on inducing the death of cells that were previously infected by a virus.(Adapted from Alberts B *et al.*, 2002)

1.3- Pathophysiology of Sepsis

1.3.1- Immunoactivation and Immunosuppression

The pathophysiology of sepsis is complex and only partially understood. In response to infection, an intense inflammatory response is mounted. A set of inflammatory mediators are produced and released into the bloodstream, including TNF-α, IL-1, IL-6, and IL-8. These mediators have the capacity to bind to target cells and trigger signal transduction and activate secondary response pathways (Rubinstein *et al.,* 1998).). The immune response is complex and includes simultaneous immune activation and immunosuppression(Hotchkiss *et al.,* 2003).

As a result of increased osmotic pressure extravascularly and increased hydrostatic pressure intravascularly, fluid, proteins, red blood cells and white blood cells escape from the intravascular space. This facilitatesthe flow of chemical mediators and inflammatory cells towards the stimulus but also causes tissue edema and damage (Hunter *et al.,* 2012). Other secondary events in sepsis includeendothelial dysfunction, coagulopathy, and cellular apoptosis, all of which contribute toorgan failure (Stearns-Kurosawa *et al.,* 2011), A localized infection thus becomes a systemic syndrome (Stearns-Kurosawa *et al.,* 2011). These events contribute to progressive dysfunction of organs and subsequently death.

1.3.2- Apoptosis

Apoptosis, or “programmed cell death”, is also dysfunctional in sepsis. Widespread cell death among immune cells (dendritic cells, monocytes, macrophages and lymphocytes) contributes to a state of secondary immunosuppression (Hotchkiss *et al.,* 2003). This compromises the ability of the patient to eradicate the initial infection and predisposes them to secondary infections, such as nosocomial infections (Wesche *et al.,* 2005).

1.3.3- Endothelial Integrity

In normal conditions, fibrinolysis is strongly regulated and kept in balance by the endothelium, such that it allows blood to flow freely without systemic bleeding or clotting (Levi M *et al.,* 2013). However, in an hyperinflammation situation during severe sepsis

drives hemostasis toward a prothrombotic and antifibrinolytic state, which can lead to disseminated microvascular thrombosis, organ ischemia, and MODS (Levi M *et al.*, 2013). Inflammatory meditors like IL-6, vWF, protein C, plasma-free hemoglobin, are some of the essential mediators for the disseminated platelet, activating the coagulation products or activating the fibrinolytic pathway (Can Ince *et al.*, 2016). These mediators are directly correlated with coagulation and fibrinolysis. For example, protein C is one of the molecules produced by endothelial cells in cases of intact endothelium. These endothelial cells serve as a barrier between blood products and procoagulant molecules, such as heparin, residing in the extracellular matrix.

Under normal conditions, large numbers of peripheral blood neutrophils, induced by the colony stimulating factors granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF), enter sites of bacterial infection by first adhering to activated endothelial cells and then migrating along a gradient of chemotactic factors. These chemotactic factors are produced at the local site of infection. Neutrophils use toll-like receptors (TLR-2 or TLR-4) to interact with pathogen-associated molecular patterns (PAMPs) on bacteria to phagocytize and eliminate the pathogens. However, neutrophils from septic patients have increased expression of surface integrins, which promote firm adhesion to endothelial cells. As a consequence, the neutrophils remain bound more tightly to the endothelial cells and fail to migrate appropriately into the site of the bacterial infection, which result in a continuous bacterial infection and an accumulation of neutrophils on the endothelial wall.

This endothelial disruption comes begins briefly this way because of increased expression of adhesion molecules on the endothelial cells, resulting in attachment of white blood cells. Also become increasingly the cross talk exists between the coagulation system and the inflammation system in sepsis (Abraham *et al.*, 2000).

Due to its importance in the pathophysiology of sepsis, endothelium function has been one of the routes that have been chosen to create new therapeutics, namely inducing the release of proinflammatory cytokines such as IL-6 (Cianferoni S *et al.*, 2013).

1.3.4- Coagulopathy

The coagulation system, through complex interactions, also plays an important role in the sepsis-induced inflammatory cascade. A balance that normally exists between

anticoagulant mechanisms and the procoagulant response is altered in sepsis with the balance tipped towards coagulation. Activated protein C (APC), an endogenous vitamin K-dependent anticoagulant, plays a major role in the down-regulation of the procoagulant arm and was briefly used as a treatment for sepsis although it ultimately proved ineffective (Robert Satran *et al.,* 2003).

In sepsis there is a generalized activation of the clottingcascade leading to the formation of clots in small vessels along with tissue hypoperfusion and a cytopathic hypoxia (Fink *et al.,* 1997; Remick *et al.,* 2007). Cytopathic hypoxia refers to conditions in which mitochondria have reduced ability to perform aerobic respiration and oxidative phosphorylation due to relative lack of oxygen.

1.4- Epigenetics

Epigenetic is the "science of change" (Bob Weinhold *et al.*,2006). The word epigenetic literally means "additional changes that occur in the genetic sequence". This term encompasses any process that alters gene expression without changing the DNA sequence. These changes are heritable and are therefore passed on to daughter cells. Many diseases have some level of evidence linking them with epigenetic mechanisms, including cancer of almost all types, cognitive and cardiorespiratory disorders, such as cardiac hypertrophy (Charbel Khalil *et al.,* 2014); reproductive disorders, namely obesity and several cancers of the reproductive system, such as endometrial, ovarian, breast, testicular and prostate cancers (Crujeiras *et al.,* 2015(, and also autoimmune diseases, including SLE (Systemic lupus erythematosus), autoimmune thyroid disease, inflammatory bowel disease, psoriasis (Judith Greer *et al.,* 2012) and neuro-degenerative diseases, such asHuntington's disease (Luca Lovrečić *et al.,* 2015,Weinhold et. al.,2006). Common epigenetic modifications include DNA methylation; histone modifications such as methylation, acetylation, phosphorylation and ubiquitination; and non-coding RNAs, including microRNAs.

Of all the epigeneticprocesses, the best-known and most studied is DNA methylation. This change occurs at cytosine bases, which are converted into 5-methylcytosine by DNA methyltransferase enzymes (DNMT). The modified cytosine residues are almost always associated with a guanine residue and are known as CpG sites.

1.4.1- DNA Methylation

DNA methylation is the most studied of the epigenetic changes. It involves addition of a methyl group to a cytosine residue in a cytosine-guanine pair. In mammals, there are 3 major DNA methyl transferases: DNMT1, DNMT3a and DNMT3b. DNMT1 is a maintenance methyltransferase, while DNMT3a and 3b are de novo methyltransferases (Phillips *et al.*,2008). DNMT1 is the most abundant DNMT in adult cells (Robertson *et al.*,1999). It binds to hemimethylated DNA (DNA with only one strand methylated), at CpG sites. After DNA replication, although the parent strand remains methylated the newly synthesized strand is not. DNMT1 binds to these hemimethylated CpG sites and methylate the cytosines on the newly synthesized strand. The de novo DNA methyltransferases DNMT3a and DNMT3b do not require hemimethylated DNA to bind: they show an equal affinity for hemimethylated and non-methylated DNA (Okano *et al.*,1998). Both DNMT3a and DMNTb are essential for early development, and the loss of either is lethal. It should be noted that the role of these enzymes is not restricted and absolute, DNMT1 can function as a de novo DNMT whileoverexpression of DNMT1 leads to de novo methylation of CpG islands (Vertino *et al.*,1996).

A new class of ezymes,ten eleven translocation (TET) enzymes oxidize 5-methylcytosines (5mCs) and promote locus-specific reversal of DNA methylation. The TET family comprises three main proteins, namely, TET1, TET2, and TET3—that catalyze the successive oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (He et al., 2011). Disruption of epigenetic landscapes, including DNA methylation patterns, is a hallmark of different diseases, including cancer. When theses enzymes are muted a disruption of crucial proteins happens (Kasper Dindler Rasmussen *et al.*, 2016).

1.4.2- Histone Modifications

Histone modifications are covalent post-translational modifications (PTM) of the histone proteins, which bind to DNA and give chromatin its structure. Histones can be post-translationally modified to restructure chromatin in many ways, including by phosphorylation, ubiquitination, acetylation, and methylation (Kouzarides *et al.*,2007). Histone modifications can impact gene expression by altering chromatin structure or by

recruiting histone modifiers. Among these different modifications, histone acetylation, at the ε-amino group of lysine residues in H3 and H4 tails, is the one most consistently associated with promoting transcription (Diane Handy *et al.*,2011). Histone proteins form histone cores, consisting of 8 histone proteins, and act to package DNA, which wraps around the histone cores into chromosomes. With respect to histone modification, histone acetylation seems to be important for the regulation of proinflammatory gene expression in lung epithelial cells (Mélanie Anne Hamon *et al.*,2008). Expression of H3-acetyl, H3K4me2 and H3K9me2 are associated with modulation of IL-8 transcriptional activity in response to LPS exposure (T.Angrisano *et al.*,2010). In monocytes, chromatin remodelling occurs at the promoters of pro-inflammatory and anti-inflammatory genes after LPS stimulation, increasinglevels of H3K9me2 in pro-inflammatory genes like IL-6, IL-12 and TNF-α. The expression of H3K9me2 in monocytes increases the capacity to produce anti-inflammatory cytokines, such as the IL-1 receptor antagonist (IL-1RA) and IL-10 (Cavaillon*et al.*,2006; Biswas *et al.*,2009).

1.4.3- Non-coding RNAs

A non-coding RNA (ncRNA) is a functional RNA molecule that is transcribed from DNA but not translated into protein. ncRNAs function to regulate gene expression at the transcriptional and post-transcriptional level. The three major classes of short non-coding RNAs are microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). MicroRNAs (miRNAs) are endogenous 22 nt RNAs that play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression (Fazli Wahid *et al.*,2010). Both major groups – miRNAs and siRNAs- are shown to play a role in heterochromatin formation, histone modification, DNA methylation targeting, and gene silencing (David Bartel *et al.*,2004). miRNAsplay an important role in the maturation, development and regulation of B cells, as well the maturation of the antibody response (Danger *et al.*,2014). Expression of the miR-17-92 cluster (miR-155 and miR-181a) participates in B cell proliferation and apoptosis. This cluster functions by targeting BCL-2-like protein 11 (BIM) transcripts, which belong to a family of apoptosis regulators. Expression of another miRNA, miR-34a, blocks B cell development, which leads to aberrant antibody responses to foreign antigen (Danger *et al.*,2014; Rao *et al.*, 2010).

1.4.4- Epigenetic changes in sepsis

Although prior studies of epigenetic changes in sepsis are limited, there are some data to suggest a role for epigenetics in sepsis pathophysiology. For instance, clinical studies show CD4+ T cell loss in septic patients accompanied by histone methylation and chromatin remodelling in the promoters of the T-bet and GATA-3 genes (Javier Cabrera-Perez *et al.*,2014). This contributes to anergy of the CD4+ T cells, which are crucial players in the humoral immune response andare essential for formation of functional CD8+ T cell memory (Green *et al.,* 2013; Weinstein *et al.*,2012).

As mentioned above, LPS exposure affects histone methylation in a number of pro-inflammatory genes including IL-6, IL-8, IL-12 and TNF-α (T.Angrisano *et al.,* 2010; Cavaillon*et al.,* 2006; Biswas *et al.,* 2009).

DNA methylation changes may also occur in direct response to the pathogen. The tumour suppressor gene, runx3, is methylated in gastric epithelial cells in response to Helicobacter pylori infection resulting in its downregulation (Y. Katayama *et al.*,2009). This may play a role in the connection between H.pylori infection and gastric cancer (Y. Katayama *et al.,* 2009).

1.5- Summary

The present project aims to study sepsis, a highly complex disease, in an innovative way. We have assembled, for the first time, DNA methylation profiles from 66septic and 68non-septic intensive care patients. Using these samples, we will adopt a bioinformatics approach to identifying DNA methylation signatures associated withsepsis. We will also correlate epigenetic changes with important clinical outcomes, such as survival and organ failure and will look for markerswith prognostic and diagnostic potential. In this way, we hope to clarify the role of DNA methylation in the pathogenesis of sepsis and to identify new diagnostic tools that will improve our ability to diagnose and manage septic patients.

2- Materials and Methods

2.1- DYNAMICS

The data used in this study were derived from human DNA samples collected as part of the DYNAMICS trial (DNA as a Prognostic Marker in ICU patients study) (NCT01355042). DYNAMICS was a multi-center prospective observational clinical trial of the prognostic value of cell frees DNA (cfDNA) in sepsis. The aim of this proposal is to analyze DNA methylation patternsvalidate the prognostic utility of cell free DNA (cfDNA) in critically ill septic and non-septic patientsand to use this data to establish two different epigenetic signatures that correlate with the septic state as well as with severe disease and mortality.

The DYNAMICS cohort comprises 1000 patients (400 septic patients and 600 non-septic critically ill patients) enrolled at 9hospital sites in Canada (Hamilton General Hospital; St. Joseph's Hospital, Hamilton; Ottawa Civic Hospital; and Ottawa General Hospital. Four of these hospital sites collected whole blood samples, resulting in a total of 481 available DNA samples. For the purposes of the DNA methylation analysis a cohort of 72 septic and 69 non-septic critically ill patients was selected from the patients enrolled in DYNAMICS.Four groups of patients were specifically created:

1. Septic patients with low severity of illness (MODS ≤ 8) – 34 patients
2. Septic patients with high severity of illness (MODS ≥ 9) – 32 patients
3. Non-septic patients with high MODS (≥ 9) at ICU admission – 32 patients
4. Non-septic patients with low MODS (< 9) at ICU admission – 36 patients

Groups were balanced in terms of male-female ratio and age. Relevant clinical parameters such as survival, presence of septic shock, days to ICU discharge, days to hospital discharge, and the presence of co-morbidities (including liver disease, diabetes, chronic heart failure, ischemic heart failure, chronic lung disease, AIDS, cancer, chronic renal insufficiency, and chronic dialysis) were also recordedfrom the DYNAMICS database. Details of the patient's treatment plan were also available including the presence or absence of mechanical ventilation, vasopressors, and steroids and the results of any positive cultures.

On Day 1 of ICU admission, a whole blood sample was collected from each enrolled patient. Depending on the hospital site, the sample was collected in either a DNA PaxGene

tube or in a tube containing anti-coagulant and then centrifuged to obtain a buffy coat. DNA extraction was performed using the DNA QiaQuick kit (Qiagen) andthe samples were subjected to bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA), according to the manufacturer's specifications. DNA methylation analysis was performed by hybridization to the Illumina HumanMethylation 450k beadchip at The Centre for Applie Genomics in Toronto, Canada.

2.2- Data Extraction and Normalization

The power calculation was based on a case-control design comparing septic patients (N=64) and non-septic critically ill patients (N=64). The log transformed DNA methylation level measure (beta value) was assumed to benormally distributed and the Bonferroni-corrected significance level was set at0.05. It was assumed thatapproximately 400K of the 450K CpG probes would pass quality control for the final analysis. A two tail T-test was used to evaluate the power given the available sample sizes under different effect sizes. The sample size was selected to achieve 80% power given an effect size equal to or larger than 1.15.

Data cleaning and normalization was performed by our collaborators in Canada. In brief, the ChAMP analysis pipeline, which integrates a number of Bioconductor packages, loaded raw data (IDAT files) and performed quality control, normalization, and batch correction as described below. Patient samples were excluded if 1) > 20% of sample probes had detection p-value > 0.01 or 2) median bisulfite conversion efficiency (BSCE) control probe signal < 4000 in the green channel. Seven patient samples were removed. A total of 134 high quality patient samples were included in the final analysis. Probes with the following criteria were excluded: 1) detection p-value < 0.01 in at least 10% of samples, 2) probes mapping to sex chromosomes, 3) probes with non-unique genomic mapping (cross-reactive probes), and 4) probes containing single nucleotide polymorphism (SNP)-introduced artifacts. A total of 414,826 probes passed quality control and were used for further analysis.

Three replicate samples from the same patient were processed on 3 separate batches. Correlation between replicates was 0.998 after batch correction. White blood cell-type proportions were estimated from the methylation data using the reference-based model proposed by Housemanusing the function champ.refbase.

2.3- Phase 1: Literature Search

Sepsis is a complex disease and many proteins are involved in its pathogenesis. To identify genes associated with sepsis we conducted an extensive literature search. Our focus was on identifying proteins involved in 5 key sepsis pathways – namely coagulopathy, apoptosis, immune activation, immunosuppression and endothelial dysfunction. Relevant citations were identified in Pubmed using the key word "sepsis" in combination with one of these pathways. In-depth review of papers published in the most recent 5 years resulted in a set of target genes. The genes of interest were subsequently converted to a set of CpG sites using the Illumina documentation provided with the HumanMethylation 450k BeadChip array.

2.4- Phase 2: Methylation Arrays

The methylation data obtained from the 450k arrays is represented as beta values. These values estimate the site-specific methylation level using the ratio between methylated and unmethylated alleles. β values therefore range from 0 and 1, with 0 being unmethylated and 1 fully methylated.

From the genome-wide data we extracted the beta-values for our CpG sites of interest from each of our 134patients. We averaged the beta values across the septic and non-septic groups and then used the T-test correction for multiple comparison testing, using Bonferroni comparisons to determine which CpG sites were significantly differentially-methylated. To identify the most significant methylation changes we then applied a Δbeta value cut-off of 0.02 ($\Delta\beta \geq 0.02$) as very small methylation differences are unlikely to be of biological significance.

2.5- Characterization of the differentially-methylated CpG sits

Annotation of individual CpG sites is available from Illumina including gene location (1st exon, gene body, 5'UTR or 3'UTR, TSS1500, TSS200) and CpG Island location (open sea, S-shore, S-shelf, N-shore, N-shelf, island).

2.6- Phase 3: Transcriptomic data analysis

Three datasets relevant to sepsis were identified and analyzed. These include:

1. Hector Wong - pediatric sepsis (GSE26440)
2. Mihali Natea- neonatal sepsis (GSE4273)
3. Brian Scicluna and Tom Van der Poll- pneumonia versus noninfectious respiratory failure (GSE74224)

Analysis of expression data was performed in GEO2R by allocating the patients as either septic or non-septic and then using the built-in R script to identify differentially-expressed genes. We also imposed a minimum expression change ratio of 1.5 fold. This ratio was chosen as it suggests biological significance.

2.7- Phase 4: Correlating DNA methylation changes with clinical parameters

Clinical data including survival, length of ICU and hospital stay, culture results, use of vasopressors, mechanical ventilation and steroids, was available for our cohort. Clinical criteria were used to divide septic patients into groups, which were then compared to methylation status at specific CpG sites. Correlations were assessed using a two tailed T-test with adjustment for multiple comparison testing, using FDR.

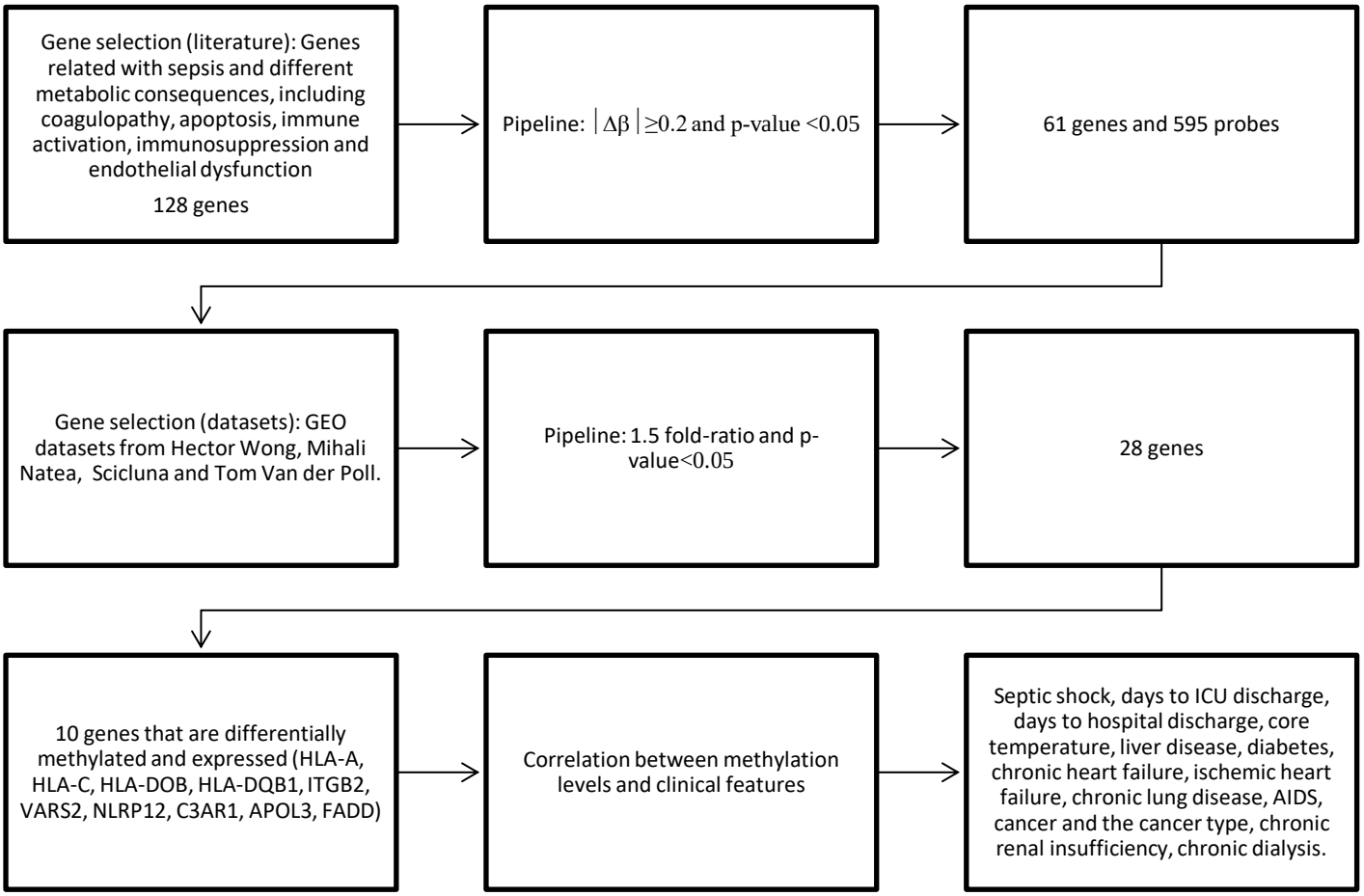
2.8- Statistical analysis

Statistical analyses wereperformed using the GraphPad Prism 5.0 software. Since the methylation values were normalized we were able to perform unpaired t-tests to determine statistical significance.

Correction for multiple comparisons was performed via adjusted p-values using the Bonferroni and FDR. Both these methods attempt to control the expected proportion of false discoveries. These corrections are based in an adjustment made to P- values when several dependent or independent statistical tests are being performed simultaneously on a single data

set. Do to these comparisons the p-value must be divide by the number of comparisons being made.

2.9- Study pipeline



3- Results

3.1- EPSIS Study

The current study is a nested case-control study of DNA methylation changes in sepsis. It utilizes genomic DNA samples obtained from a subset of patients in the DYNAMICS (DNA as a Prognostic Marker in ICU patients (clinicaltrials.gov ID NCT01355042) cohort to investigatethe importance of epigenetic regulation in the pathogenesis, progression, treatment response and outcomes of critically ill septic patients.

DYNAMICS was an observational multi-site study of critically patients in Canada for which 1000 ICU patients were enrolled, of whom 400 had severe sepsis and 600 had non-septic critical illness. Four of the nine sites involved in DYNAMICS (Hamilton General Hospital; St. Joseph's Hospital, Hamilton; Ottawa Civic Hospital; and Ottawa General Hospital) also consented patients for whole blood DNA samples drawn on day 1 of study enrolment. A total of 260 septic and 221 non-septic patients consented to provide whole blood samples.

For the present study, a total of 141 patient samples from DYNAMICS were selected for genomic DNA methylation analysis. Samples were selected to provide equal numbers of patients in 4 distinct groups based on sepsis status and severity of illness, as indicated by the Multi Organ Dysfunction Score (MODS):

Group 1: Septic patients with high MODS (≥ 9) at ICU admission – 32 patients
Group 2: Septic patients with low MODS (< 9) at ICU admission – 34 patients

Group 3: Non-septic patients with high MODS (≥ 9) at ICU admission – 32 patients

Group 4: Non-septic patients with low MODS (< 9) at ICU admission – 36 patients

Genomic DNA was extracted by our collaborators in Toronto and bisulfite-converted (see methods). Hybridization to the Infinium HumanMethylation450 BeadChip array, comprising over 450,000 individual CpG sites, was performed at The Centre for Applied Genomics in Toronto, Canada. The Human Methylation450 array interrogates methylation at >485,000 CpG sites, providing coverage to >99% of RefSeq genes, targeted across gene regions including the promoter, 5'UTR, first exon, gene body, and 3'UTR (Sandoval *et al.*, 2011).

Bisulfite-converted gDNA (500 ng) was hybridized to Infinium Human Methylation 450K (HM450K) BeadChips (Illumina, San Diego, CA) and scanned with iScan (Illumina) in accordance with manufacturer's protocol. From the initial cohort of 141 patients, only 134 high quality patient samples were included in the final analysis (samples were excluded if >20% of sample probes had detection p-value >0.01 or median bisulfite conversion efficiency (BSCE) control probe signal <4000 in the green channel).A total of 414,826 probes passed quality control and were used for further analysis.

Inclusion Criteria for Sepsis: Patient must have A or B and C	Inclusion Criteria for Non-Sepsis: Patient must have A or B or C
A. Severe Sepsis 1. ≥ 3 SIRS criteria (systemic inflammatory response syndrome) 2. Infection (suspected or confirmed) 3. ≥ 1 acute organ dysfunction	A. 1. Multiple trauma with episode of shock on presentation 2. Patient expected to remain in ICU for ≥ 72 hours
B. Septic Shock 1. All of the above in "A" and currently on vasopressors	B. 1. Critically Ill Severe Sepsis 2. ≥ three SIRS criteria (systemic inflammatory response syndrome) 3. Infection (suspected or confirmed) 4. ≥ 1 acute organ dysfunction
C. Patient expected to remain in ICU for ≥ 72 hours	C. i.e.: - Intracranial hemorrhage, subarachnoid hemorrhage, subdural hemorrhage - No shock - No organ failure 2. Patient expected to remain in ICU for ≥ 72 hours

	C. 1. Non-Septic Shock i.e.: - Cardiogenic shock - Heat shock - Burns - Hypovolemia - Pulmonary embolism - Abdominal aortic aneurysm rupture 2. Patient expected to remain in ICU for ≥ 72 hours

Patient data collection for DYNAMICS study

A. Demographic data

1. Baseline
   - Date of birth; gender, height and weight
   - Admission diagnosis; admission type – medical vs surgical
   - Severity of illness measurement: APACHE II
2. Presence of SIRS criteria
   - Fever/hypothermia, tachycardia, tachypnea, leukocytosis/leucopenia
3. Presence of organ dysfunction at the time of enrolment
   - Cardiac – systolic blood pressure
   - Respiratory – PaO2/FiO2 ratio
   - Renal – creatinine
   - Hematological – platelet
4. Chronic health variables

B. Daily data

1. Interventions and therapies – dialysis, central lines, arterial lines, pulmonary artery catheters, intra aortic balloon

pumps, mechanical ventilation, insulin, therapeutic anticoagulation, therapeutic steroids, inotropes 2. Specimen acquisition for cell free DNA 3. New sepsis (suspected or confirmed) 4. Laboratory results relevant to sepsis (white blood cell count, lactate, P/F ratio etc) 5. ICU specific scoring systems: MODS and SOFA 6. Vital signs – including temperature, blood pressure, respiratory rate 7. Fluid status – quantity of fluid intake and output
C. Sepsis specific data 1. Allmicrobiology culture data – including organisms, and sites of specimen All antimicrobials (antibiotics, antifungals, antivirals) agents administered

Table 1: Inclusion and exclusion criteria for the DYNAMICS study and patient data collection summary.

The goal of the current study is to identify sepsis-related genes that are both epigenetically modified AND regulated in sepsis. Our collaborators in Canada have undertaken an epigenome-wide association study (EPSIS) looking for DNA methylation changes that correlate with the presence of sepsis anywhere in the genome. The disadvantage of this approach is that with a highly heterogeneous patient population and a large number of candidate CpG sites it may miss DNA methylation changes that are biologically relevant to sepsis. The objective of our study is therefore to look at a subset of genes that we believe to be important in the pathophysiology of sepsis. By limiting our analysis to this small group of genes we hoped to minimizefalse positives and maximize the chances of identifying methylation changes that are important for the pathogenesis and prognosis of sepsis.

3.2- Phase 1: Literature Search

The first step in our pipeline was to perform an extensive bibliographic investigation of the metabolic pathways and genes involved in the pathophysiology of sepsis. As such, dozens of articles and journals were read in order to understand which proteins are implicated in the pathogenesis and progression of this heterogeneous disease and its systemic effects.

Five specific pathways were identified as central to the pathogenesis of sepsis: immunoactivation, immunosuppression, coagulopathy, apoptosis and endothelial dysfunction.

Our literature review resulted in a list of 128 genes that were highly relevant to the pathogenesis and systemic effects of sepsis (table 3). Genes are divided into 12 groups based on their association with the 5 pathways described above.

Genes relevant to immunoactivation include those involved in innate immunity, adaptive immunity, and inflammation. The innate immune system consists of cells and proteins that are always active and ready to mobilize and fight microbes at the site of infection. The main components of the innate immune system are 1) physical epithelial barriers, 2) phagocytic leukocytes, 3) dendritic cells, 4) natural killer (NK) cells, and 5) circulating plasma proteins (Alberts *et al.*, 2002). Genes involved in this pathway include TLR-2, TLR-4, TLR-6, TLR-10, CX3AR1, DEFB1, YKL-40. The toll-like receptor genes (TLR) are important mediators of innate immunity because they recognize bacterial antigens on entry into the body (Kawai *et al.*, 2010). CX3AR1, DEFB1, and YKL-40 are also important toinnate immunity because they are associated with neutrophil activity and inducing apoptosis.

Adaptive immunity is called into action against pathogens that are able to evade or overcome the innate immune defense measures. Machinery of the adaptive immune system are normally silent; however, when stimulus occurs and is activated, this machinery have the capacity to "adapt" to the presence of infectious agents by activating, proliferating, and creating potent mechanisms for neutralizing or eliminating the microbes. There are two types of adaptive immune responses: humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity, mediated by T lymphocytes (Alberts *et al.*, 2002). Genes important for the adaptive immune response include CHN2, CD4, CCL-4, BTN3A2, C9ORF95, RFX1, C21ORF56, C6ORF15, BATF, CD14, CD40, CD80, CD86, C-SMAC [CD2, CD4, CD8, CD28], P-SMAC [ICAM-1, ICAM-2], and D-SMAC. The supramolecular activation cluster, or immune synapse, is formed by the P-SMAC, D-SMAC, and C-SMAC proteins in response to the tight apposition of a T cell with an antigen-presenting cell (APC) (Balbino Alarco´n *et al.*, 2011). At the centre of the immune synapse is the MHC complex, which presents the antigen to the T cell. MHC class I proteins are found on the surface of all nucleated cells and comprise the HLA-A, HLA-B, and HLA-C proteins while MCH class II proteins are found only on dedicated antigen presenting cells (eg dendritic cells, phagocytes)

and comprise the HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DR, HLA-DQB1, and HLA-DQ proteins (Sung Yoon Choo *et al.*, 2017).

Another crucial featureof the innate and adaptive immune responses is the release of humoral mediators, namely cytokines and chemokines, which modulate the immune response. Cytokines play a crucial role in the pathogenesis of sepsis. They are secreted proteins with many effects including promoting cell proliferation, cell differentiation, and cell activation. These proteins also control immune cell trafficking and the cellular arrangement of immune organs. Chemokines have the "power" to decide whether an immune response develops and subsequently whether that response is cytotoxic, humoral, cell-mediated, or allergic. Chemokine receptor antagonism may represent a novel therapeutic approach against sepsis in the future (Raina Davi Ramnath *et al.*, 2006;Borreli *et al.*, 1996).

Pro-inflammatory cytokines such as TNF-α and IL-6 contribute to the immune response through the promotion and stimulation of the inflammatory response. These pro-inflammatory mediators facilitate inflammation and trigger pathological pain (Jun-Ming Zhang *et al.*, 2007). IL-6 has been shown to play a central role in the neuronal reaction and is also involved in microglial and astrocytic activation in the central nervous system (MA Klein *et al.*, 1997). TNF-α acts in several different signaling pathways, as well as apoptotic pathways, NF-kB activates inflammation as well as stress-activated protein kinases (SAPKs) (by NCBI).

Heat shock proteins are a crucial and highly conserved family of proteins across species. They act as chaperones for the folding and unfolding of proteins. They also play a role in cell-cycle control and signaling, and the protection of cells against stress/apoptosis (Li Z *et al.*,2004). During periods of stress, such as sepsis, there is overproduction of heat shock proteins. These molecular chaperones act to ensure that proteins are properly folded under periods of stress and elevated temperature. They include Hsp40, Hsp60, Hsp70 (Li Z *et al.*, 2004).

Although sepsis is commonly associated with overwhelming inflammation, it is also accompanied by systemic immunosupression. The signs of immunosuppression in patients with sepsis include apoptosis-induced loss of cells of the innate and adaptive immune system including CD4+ and CD8+ T cells, B cells, and dendritic cells (Richard Hotchkiss *et al.*, 2009)and a reduction in the expression of MHC-II complex on macrophages and APCs (James Faix *et al.*, 2013). CARS or immune-paralysis, as it was originally described, is

mediated by a predominance of a Th2 T cell response, increased T regulatory cells, apoptosis of lymphocytes and decreased MHC class II molecules on monocytes and macrophages (Opal *et al.,* 2011). It is accompanied by overexpression of anti-inflammatory cytokines, such as IL-4 and IL-10 (Jiang *et al.*, 2006.

Humoral mediators such as IL-4 and IL-10, play an important role in immunosuppression by inhibiting TNF-α, thereby augmenting acute-phase reactants and immunoglobulins and inhibiting T-lymphocyte and macrocyte functions (Evan *et al.*, 1996).

Apoptosis, or programmed cell death, is one of the factors that contributes to sepsis being such a heterogeneous, aggressive and dysregulated syndrome. "Apoptosis is an evolutionarily conserved, energy-dependent mode of cell death requiring the initiation and regulation of complex genetic programs" (Mahidhara *et al.,* 2000). Apoptosis has been linked to MODS. Septic patients with MODS have elevated levels of soluble Fas (sFas), an important apoptosis mediator, which decreases when MODS improves (S Endo *et al.*, 1996). The nuclear matrix protein (NMP), a marker of cell death, has also been shown to correlate with MODS-scores and disease severity in sepsis (Y Yamada *et al.*, 1998). Additional proteins including BIRC1, BIRC2, ML-IAP (anti-apoptotic proteins), BID, BAX, BCL-2, FADD, and caspases (pro-apoptotic proteins) are all critical to apoptosis. Cells have the capacity for remarkable regeneration in response to injury or trauma. This is also true of sepsis-related organ injury, although there is an imbalance between anabolism and catabolism that leads to cellular wasting (Rocheteau *et al.,* 2015).

The final sepsis-related pathway that was explored was coagulopathy. A large number of proteins are involved in regulating coagulation as both pro- and anti-coagulants. These include platelet and coagulation proteins (FGA, FGB, FGG, PF4, Heparin, vWF, thrombin, thrombomodulin, collagen, tissue plasminogen activator, platelets antithrombin, protein C and S, plasminogen) as well as the coagulation factors [II (prothrombin), V, VII, VIII, IX, X, XI, XII, XIII, IV]) and proteins related to the complement system (C1q, C1r, C1s, C4b, C4aC2b, C2a, C3b, C3a). The complement system is a key player in the defense against infections. It comprises a group of plasma membrane and circulating proteins that opsonize pathogens and promote their phagocytosis. [REF]

Sepsis has profound effects on coagulation, ranging from mild alterations up to severe disseminated intravascular coagulation (DIC) (Paola Saracco *et al.*, 2013). DIC is characterized by widespread microvascular thrombosis, which contributes to multiple organ dysfunction/failure, and subsequent consumption of platelets and coagulation factors, eventually causing simultaneous bleeding and clotting (Cheng-MingTsao*et al.*, 2015). "The pathogenesis of coagulopathy in sepsis is driven by an up-regulation of procoagulant mechanisms and simultaneous down-regulation of natural anticoagulants" (Simmons *et al.*, 2017).

128 genes across the different pathways involved in sepsis	
Innate immunity	TLR-2, TLR-4 TLR-6, TLR-10, CX3CR1, DEFB1, YKL-40
Adaptive immunity	CHN2, CD4+, CCL-4, BTN3A2, C9ORF95, RFX1, C21ORF56, C6ORF15, BATF, CD5, CD14, CD40, CD80, CD86, C-SMAC (CD2, CD4, CD8, CD28), P-SMAC (ICAM-1 and ICAM-2), D-SMAC
Proinflammatory pathway	NF-KB, TNF-α, IL-1, IL-12, IL-6, IL-8, IL-1β, IL-18, IL1R2, MyD88, RAGE, HMGB1, NOD-1, Alpha- 1B glycoprotein, Haptoglobin, Dipeptidyl peptidase-4
Cell replication and proliferation	GSTM3C, BET, INO80, HAS-2, WBSCR27, SRNX1, RPGRIP1, C3AR1, CEACAM1, SESN-2, HSPG2, A2AP, MTCH1
Membrane proteins	Lipocalin (transporter), Lysosome-associated membrane proteins-1, GNA12, OCLN, CLDN1
Protein folding	HSP100, HSP90, HSP70, HSP60
Anti-apoptotic proteins	BIRC1, BIRC2, BIRC5, BIRC6, BIRC8, Livin, ML-IAP
Pro-apoptotic proteins	Bid, Bax, Cytochrome C, Apaf-1, Bcl-2, Diva, Bok-L, BoK-s, FADD, SMAC,

	Caspase (1, 2,3,7,8,9, 10, 14)
Complement system	C1q, C1r, C1s, C4b, C4a, C2b, C2a, C3b, C3a
MHC-I proteins	HLA-A, HLA-B, HLA-C
MHC-II proteins	HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, HLA-DR, HLA-DPB1
Platelet and coagulation proteins	Fibrinogen (FGA, FGB, FGG), Platelet factor 4 (PF4), Factor Xa, Thrombin, Heparin, Thrombomodulin, Collagen, Tissue plasminogen activator, Platelets Antithrombin, Platelet activating factor, Protein C and S, Clotting factor, Plasminogen, Prothrombin Fibrinogen, Vwf, Factor II Factor (XI, V, VII, XII, VIII, IX, XIII, XIV, X), HMWK

Table 2: Reorganization of the 128 genes according to the metabolic pathway in which they are inserted and according to their function.

3.3- Phase 2: Methylation Arrays

The EPSIS methylation dataset is, to our knowledge, the first database of genome-wide DNA methylation data from critically-ill patients and from septic patients of any kind. The current study utilizes the EPSIS dataset to perform a targeted analysis of DNA methylation changes in sepsis-related genes.

In Phase 2 of our pipeline we used the EPSIS dataset to look for sepsis-associated DNA methylation changes in the 128 genes identified in our literature search. Using the documentation provided by the manufacturer, we identified a total of 705 CpG sites located in our target genes that are also present in the llumina HumanBeadchip 450k array.

We next analyzed the EPSIS database to identify which of these 702 CpG sites showed significant methylation differences between the septic and non-septic critically-ill patients. Using a beta-value difference cutoff of > 2% and a false discovery rate of 5%, we identified a total of 595 CpG sites that were differentially methylated in the EPSIS samples. The beta-

value difference cutoff was selected to ensure biological relevance as values below 2% are unlikely to be important regulators of gene expression (Pan Du *et al.*, 2010).

Initially, we hoped to pair our EPSIS data with an additional database of non-critically ill patients as a "negative" control. Our plan was to compare the DNA methylation status of our septic and non-septic critically ill patients with that of "healthy" patients. Unfortunately, we were unable to locate an appropriate comparison group and realized that we would be unable to control adequately for batch effects between the EPSIS samples and any external database.

3.4- Heatmap analysis

To provide a visual representation of the methylation analysis data we constructed heatmaps displaying the beta-value difference and p-value of our CpG sites. Two examples are shown in Figures 1 and 2. CpG sites indicated in red are hypermethylated in the septic samples relative to the non-septic samples, while those shown in blue are hypomethylated. The location on the Y axis indicates the p-value, with those located closest to the bottom of the heatmap having the smallest p-value, and therefore the greatest statistical (but not necessarily biological) significance. As is apparent from the heatmaps, a large percentage of CpG sites were significantly differentially methylated between septic and non-septic patients. Certain genes including IL-10, NFKB1 and NFKB2 (Figure 1), as well as NLRP2, VARS2 and ITBG2 (Figure 2) showed a high number of differentially-methylated CpG sites with similar direction of methylation change. Other genes, including HMGB1 (Figure 1) and HLA-DRB1 and HLA-DQB2 showed almost no changes in methylation status. The TNF gene was unique in showing a large number of methylation changes with a mixture of hyper- and hypo-methylated sites.

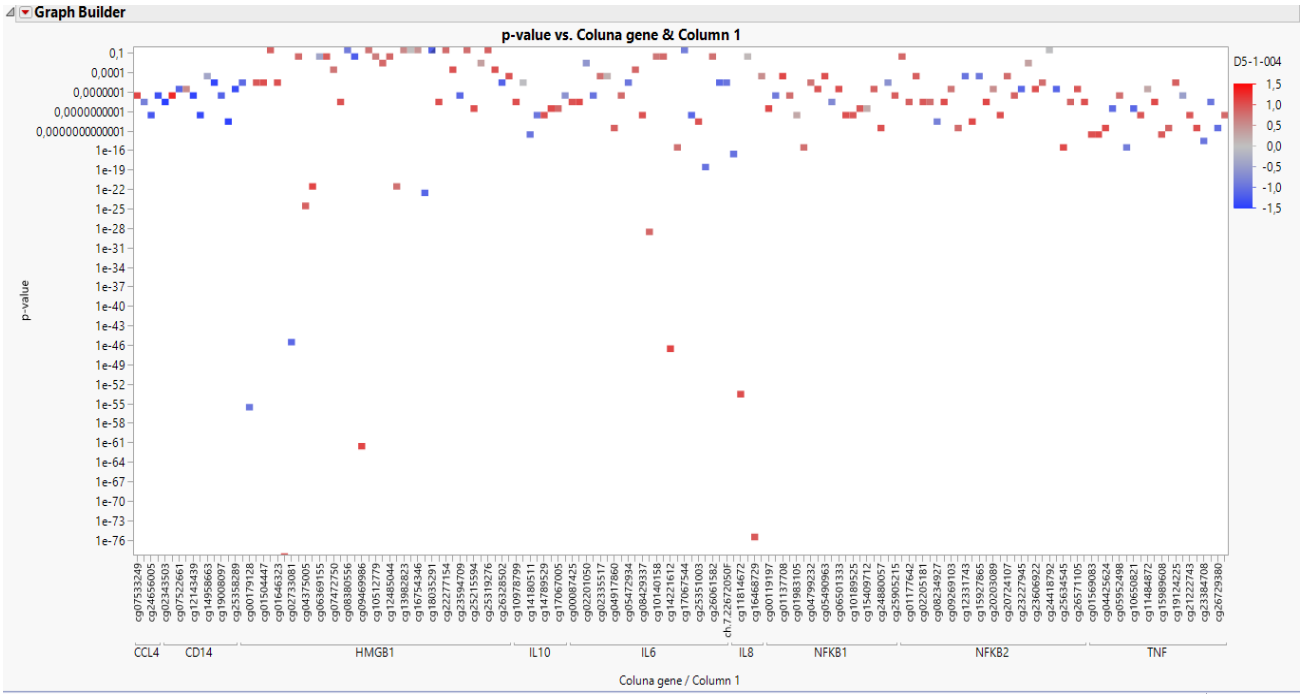
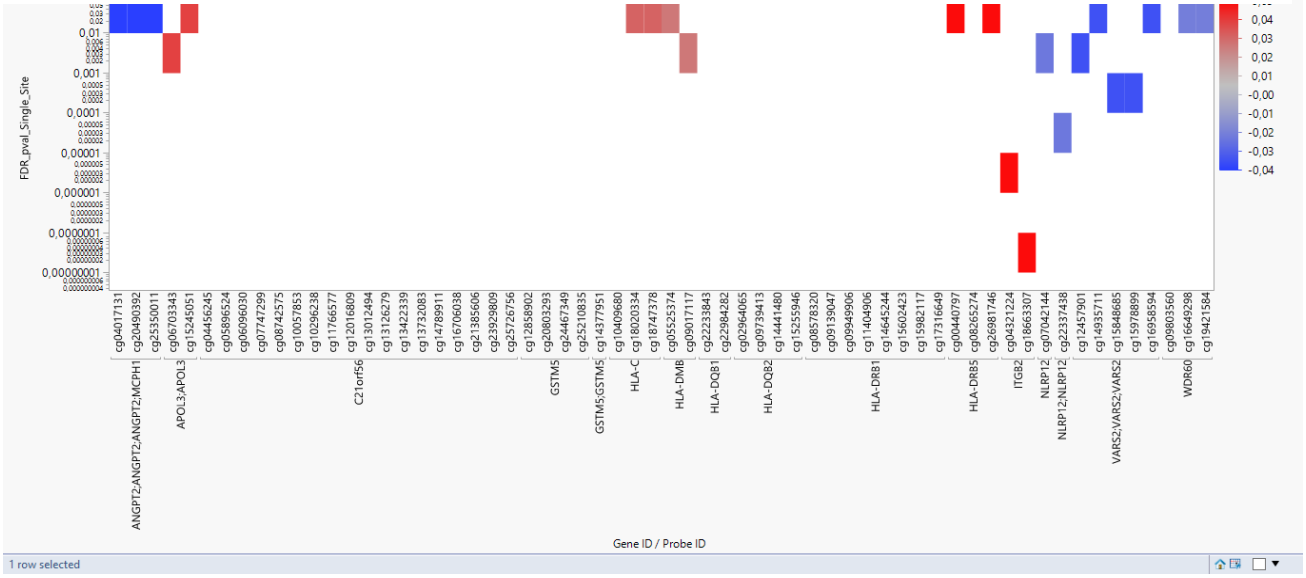


Figure 1: Heatmap analyzing the differences in the methylation state between a specific cluster of genes, between patients with and without sepsis. In the Y axis it indicates the p-value and in the axis of the X the different genes and CpG associated sites. In this heatmap the following cluster of genes is analyzed: CLL4, CD14, NFKB1, NFKB2, HMGB1, IL10, IL6, IL8 and TNF-α



Of the 128 genes identified in our literature search, a total of 61 genes contained at least 1 differentially-methylated CpG in our methylation analysis. Table 3 shows the list of 61 genes along with their “average” methylation status.

Gene ID	Methylated CpG sites / All CpG sites	Methylation Change
HLA-DOB	20/44	
HLA-DQB1	20/27	
HLA-C	22/44	
HLA-A	13/22	
FADD	13/20	
C3AR1	3/7	
NLRP12	3/8	
ITGB2	13/33	
C21ORF56	7/19	
VARS2	21/61	
APOL3	11/21	
FGB	1/5	
FGG	7/15	
PFA	14/17	
F12A1	7	
BIRC5	5/16	
TPA	12/15	
APC	28/40	
VWF	11/33	
F11R	11/22	
HLA-DOA	7/73	
HLA-DRA	7/41	
HLA-B	15/20	
ICAM1	15/17	
ICAM2	8/18	
HMGB1	21/40	
HP	2/3	
HSPG2	11/46	
IL18	6/8	
IL1B	5/14	
BID	8/18	
BAX	9/12	
APAF1	20/20	
BCL-2	37/47	
BIRC2	8/13	
BIRC6	14/18	
OCLN	10/14	
CLDN1	7/20	
NOD1	14/24	
TLR-2	7/13	

TLR-4	10/14
TLR-6	5/10
TGFB1	6/24
SESN2	14/22
CD40	12/15
POLR1D	13/21
C6ORF15	5/126
C9ORF95	5/6
DPP4	11/20
SSBP1	10/12
ORM	3/3
LCLN1	4/7
LAMP-1	7/34
GNA15	10/21
BATF	8/20
KIAA1370	7/12
MTCH1	8/13
IL-6	6/23
HLA-DPB1	10/43
TNF	8/27
ZDHHC19	12/20

Table 3: Scheme that analyzes the methylation differences among the 61 genes. In the first column the identity of the gene is shown; In the second column it analyzes the number of CpG methylated sites on the total number of probes for each gene. In the third column the state of change of methylation in each gene is presented; red, hypermethylated genes, blue

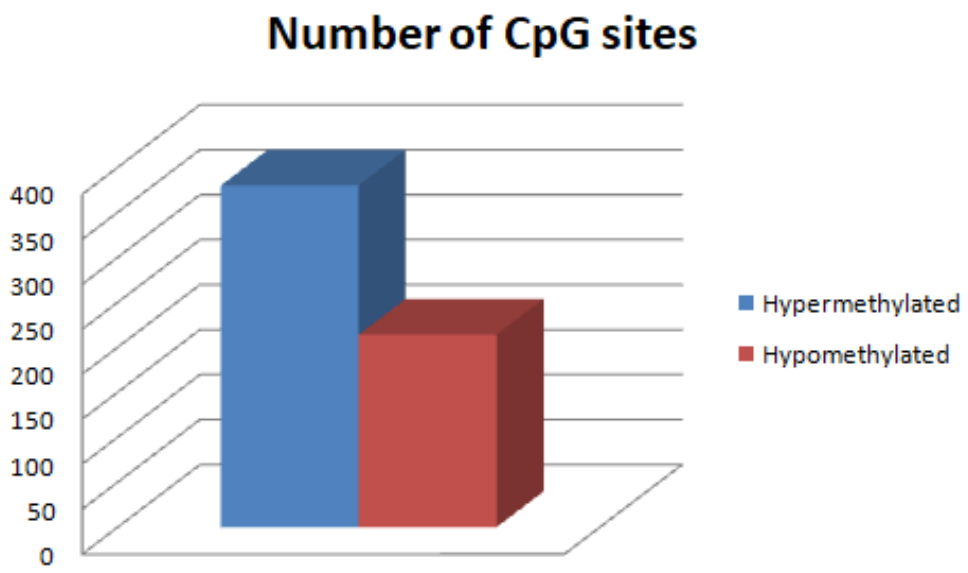
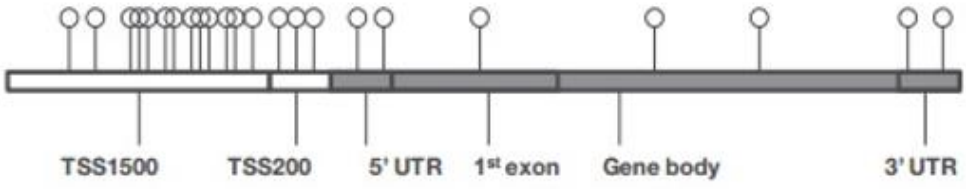


Figure 3: Graph showing the number of CpG sites that are hypermethylated (n=380) and hypomethylated (n=214) within 61 genes

3.5- CpG Localization

We next analyzed of the localition of the differentially-methylated CpG sites relative to our genes of interest. The Illumina HumanBeadchip 450k array documentation provides basic information about CpG localization. Sites are identified as being in one of 6 locations: TSS1500, TSS200, 5'UTR, 1st exon, Body, and 3'UTR. The schematic below illustrates these locations relative to the gene structure (Figure 3).



TSS1500: from -200 to -1,500 nt upstream of the transcription start site (TSS)

TSS200: from -200 nt upstream to the TSS itself

5´UTR: downstream of the TSS and upstream of the initiation codon

1st exon: the first part of the coding sequence prior to the 1st intron

Gene body: from the 1st intron to the translation termination codon (TTC)

3´UTR: between the TTC and the polyadenylation site

Adapted from: K Look *et al.*, 2014

Figure 4: Localization of CpG sites along the structure of a gene, including TSS1500, TSS200, 5'UTR, 1st exon, gene body and 3'UTR

The distribution of CpG sites within genes is important in determining their effect on gene regulation. Hypermethylation of CpG sites in the gene promoter is associated with inhibition of gene expression while hypermethylation of CpG sites in the gene body is associated with increased gene expression (Shimrat Mamrut *et al.*, 2013). Thus, a CpG site that is hypermethylated in the promoter will likely have a different impact on transcription of the associated gene than a CpG site that is hypermethylated in the 3'UTR.

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We first analyzed all of our differentially methylated CpG sites to determine their localization relative to the associated genes. Figure 5 reveals that 73,5% of the differentially-methylated sites are located upstream of the transcription start site or in the 5'UTR and 1st exon. This compares to 73% amongst the 702 CpG sites that we analysed.

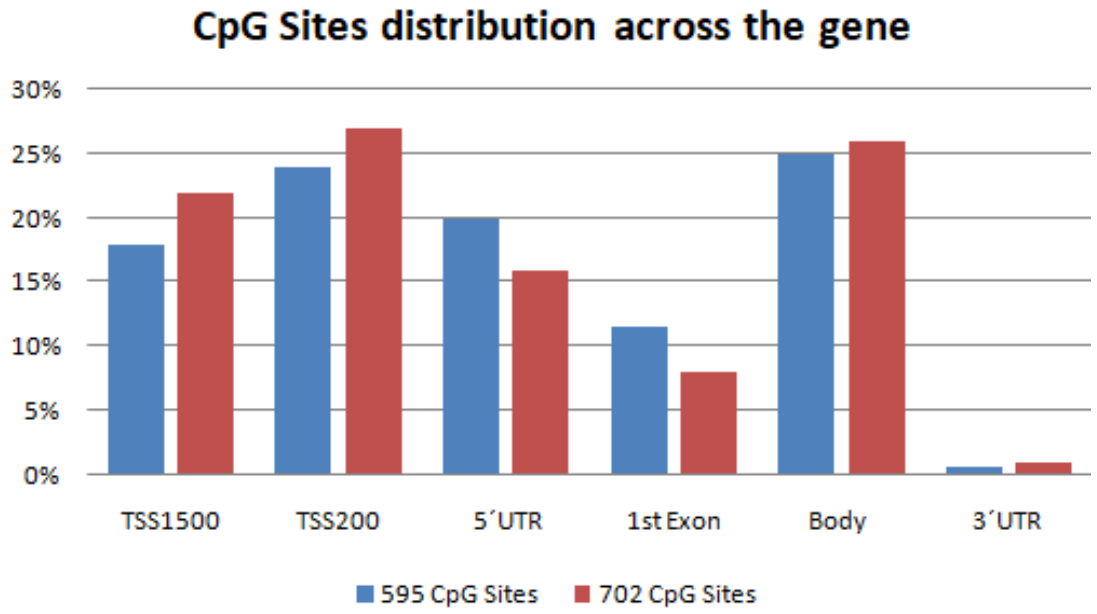


Figure 5: Graph analyzing the distribution of the 595 and 702 significant CpG sites across the gene.

We next divided our differentially-methylated probes according to their methylation status – hyper and hypomethylated. We then plotted these two groups of probes individually to determine whether there were any differences in their relative localization. Figure 5 shows that the hypomethylated probes were much more likely to be localized in the TSS200 or 5'UTR regions – key gene regulatory regions. As mentioned above, hypomethylation of upstream regulatory sequences is usually associated with increased gene expression.

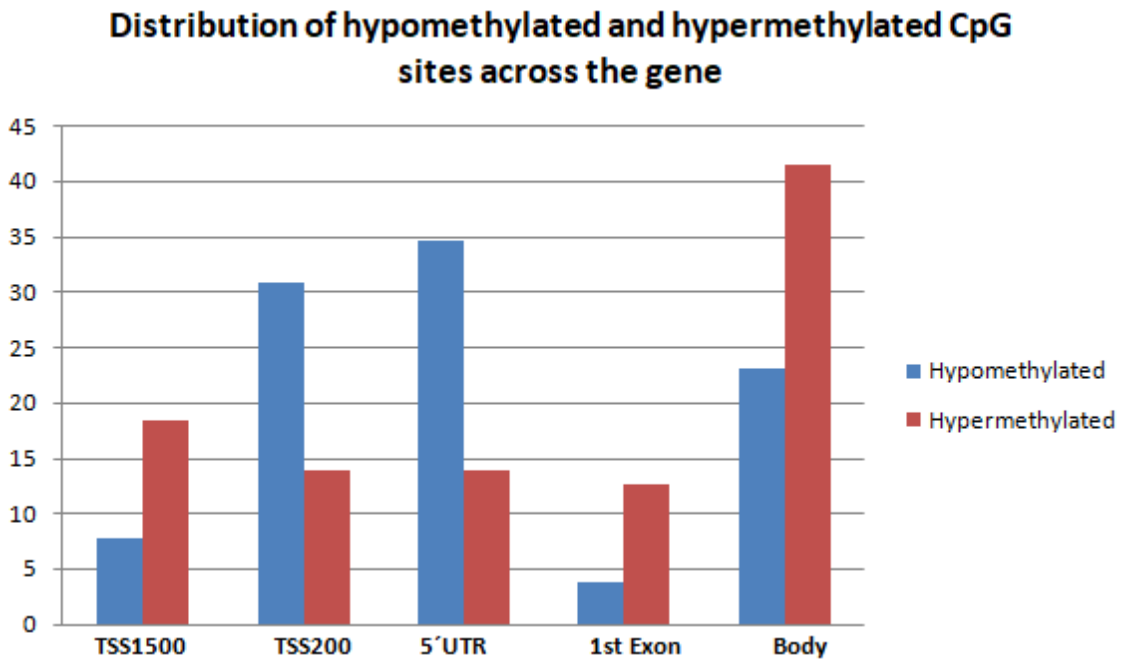


Figure 6: Graph analyzing the overall distribution of the hypermethylated and hypomethylated significant probes across the gene

We next analysed the location of our CpG sites relative to CpG islands. CpG islands are short interspersed DNA sequences. These regions are GC-rich, CpG-rich, and predominantly nonmethylated. CpG islands are commonly known as sites of transcription initiation, including thousands that are remote from currently annotated promoters (Aimée M. Deaton et al., 2011). Using the documentation from the manufacturer our CpG sites were classified as being in the CpG island itself ("island"), within 2kb of a CpG island ("shore"), within 2-4kb of a CpG island ("shelf"), or at least 4kb away from a CpG island ("open sea.").

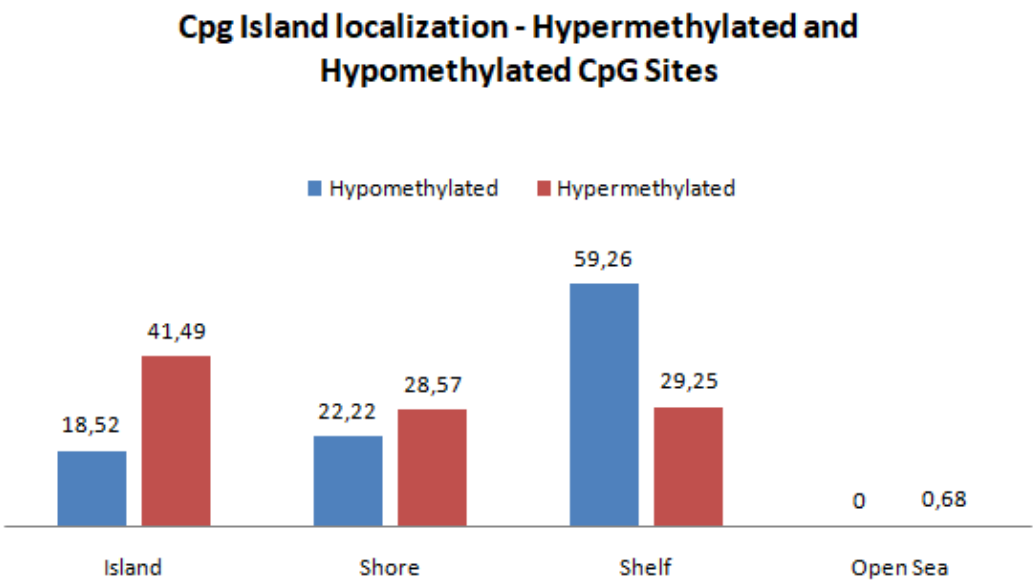


Figure 7: Graph that analyzes the general distribution of the hypermethylated and hypomethylated probes according to the CpG islands.

Almost all of the differentially-methylated probes were located within 4kb of a CpG island. Interestingly, hypermethylated sites were found primarily on the CpG island and CpG shore while hypomethylated sites were found predominantly on the CpG shelf, up to 4kb from the CpG island. The presence of hypermethylated CpG sites in the CpG islands is an important mechanism for gene inactivation. Other immunosuppressive diseases, such as AIDS and lymphoma, also show hypermethylation of the CpG islands (Cheah *et al.*, 1984) whereas in normal tissue the majority of CpG islands are completely unmethylated (Bird *et al.*, 1986).

3.6- Phase 3: Transcriptomic data analysis

Epigenetic changes are most relevant when they impact gene regulation. Thus, epigenetics is fundamentally the study of gene expression. Although the EPSIS database does not include information on gene expression we were able to identify three datasets in the Gene Expression Omnibus (GEO) that contained both septic and non-septic patients and that we felt were reasonable comparators for the EPSIS data.

The transcription data sets were as follows:

- GSE26440 [REF] is a dataset from Hector Wong and co-workers comparing pediatric septic vs. non-septic ICU patients. Hector Wong has published several articles (Hector Wong *et al.,* 2012: "Genetics and genomics in pediatric septic shock"; "The pediatric sepsis biomarker risk model") on the subject of the intrinsic heterogeneity of clinical septic shock;
- GSE4273 is a dataset contributed by Mihali Natea comprising neonatal sepsis patients. Early-onset sepsis remains a common and serious problem for neonates, especially preterm infants (Kari A. Simonsen et al., 2014).
- GSE74224 is a dataset contributed by Brian Scicluna and Tom van der Poll comprising adult critically-ill patients with pneumonia versus noninfectious respiratory failure. Sepsis and septic shock can result from an infection anywhere in the body, including pneumonia. Furthermore, pneumococcus remains the most common cause of community-acquired pneumonia worldwide (Tom van der Poll *et al.*, 2009).

Geo Dataset	Cohort	
GSE26440- Hector Wong	N=130	Analysis of whole blood from children (up to 10 years old) within 24hrs of PICU admission for septic shock.
GSE4273 - Mihali Natea	N=65	Biomarkers have shown great promise in diagnosis of sepsis and guiding appropriate treatment of neonates.
GSE74224 - Brian Scicluna and Tom van der Poll	N=105	Molecular classifier based on a small number of RNAs expressed in peripheral blood

Table 4: Table that summarizes the characteristics of the different GeoDatasets analyzed for the differences in the level of gene expression. In the first column it is observed the identity of each dataset and the respective author; in the second column the number of patients that compose each of the cohorts and in the third column a succinct explanation of the type of comparisons that were performed.

Although these transcriptomic datasets are not a perfect match for our EPSIS cohort, they all contain a mixture of septic and non-septic critically-ill patients. By using 3 different databases we hoped to maximize the chances of identifying genes that are differentially-expressed in sepsis.

Genes were considered differentially-expressed in sepsis if they showed a 1.5 fold change in expression and an adjusted p-value <0.05 in any of the 3 datasets.

Of the 61 genes identified in phase 2, a total of 10 genes showed differential expression in the GEO datasets (figure 8).

- HLA-A
- HLA-C
- HLA-DQB1
- HLA-DOB
- ITGB2
- FADD
- NLRP12
- APOL3
- VARS2
- C3AR1

Figure 8: List of 10 genes that after analysis of the methylation data and the analysis of the different datasets of the transcriptomics, were presented as differentially methylated and expressed between patients with and without sepsis.

These 10 genes are both differentially methylated and differentially expressed in sepsis. They include 4 members of the human leukocyte antigen genes, which form the class II major histocompatibility complex or MHCII. These are HLA-DQB1, and HLA-DOB. MHC class II molecules are critical for the initiation of the antigen-specific immune response (Holling *et al.*, 2004). Two of the 10 proteins, FADD and APOL3, are involved in apoptosis. Apoptosis represents the execution of an ATP-dependent death program that is triggered following death receptor activation (Sundar *et al.*, 2017). FADD is relevant in sepsis because it recruits and activates caspase-8 and thereby the downstream executioner caspases 6 and 7 (Hotchkiss *et al.*, 2009). APOL3 is a member of the apolipoprotein L gene family, a group of proteins that can initiate apoptosis, or programmed cell death, by responding to stimuli such as DNA damage and cell detachment [Smith and Malik, Genome Res, 2009]. It may play a role in sepsis due to its involvement in the neutrophil apoptotic program (Akl *et al.*, 2015).

NLRP12 is a cytoplasmic sensor thought to be a negative regulator of inflammation (Gurung *et al.*, 2015). It has been proposed as a positive regulator of MHC-I molecule expression (Marcela Hernandez *et al.*,2013) but is a crucial negative regulator of the NF-κB signaling pathway (Gurung *et al., 2015*).

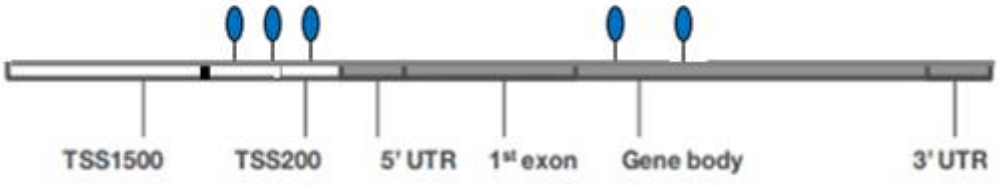
The ITGB2 gene belongs to a group of integrins, integral cell-surface proteins that participate in cell adhesion as well as cell-surface mediated signaling (by Gene Cards-ITGB2). It is important for endothelial integrity (Wilson ZS *et al.*, 2017).

Finally, the VARS2 gene is involved in mitochondrial metabolism. Mutations in this gene cause combined oxidative phosphorylation deficiency (Diodato *et al.*, 2014). Mitochondrial metabolism is impaired in sepsis leading to relative depletion of cellular energy levels (Daria Diodato *et al.*, 2014).

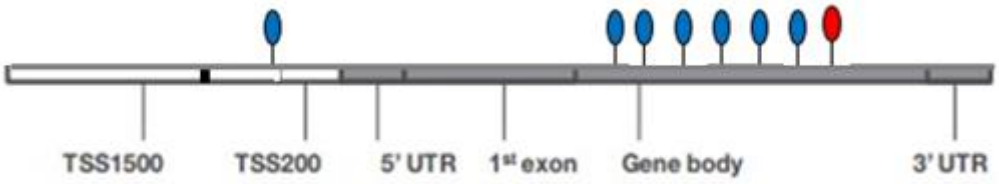
Gene ID	CpG sites	Methylation change	Transcriptional change
HLA-A	5	Hypomethylated	Downregulated
HLA-C	22	Hypomethylated	Downregulated
HLA-DOB	19	Hypermethylated	Downregulated
HLA-DQB1	22	Hypomethylated	Downregulated
NLRP12	3	Hypermethylated	Upregulated
ITGB2	13	Hypomethylated	Upregulated
C3AR1	3	Hypomethylated	Upregulated
FADD	13	Hypermethylated	Upregulated
APOL3	11	Hypermethylated	Downregulated
VARS2	21	Hypermethylated	Downregulated

Table 5 shows the direction of methylation and expression change for each of the 10 genes identified in Phase 3. Three of the genes are hypomethylated and downregulated – all of these are members of the HLA family. Two genes are hypomethylated and upregulated. Two are hypermethylated and upregulated and three genes are hypermethylated and downregulated.

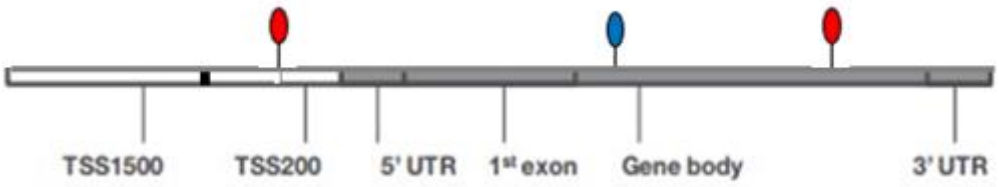
- HLA-A (Downregulated)



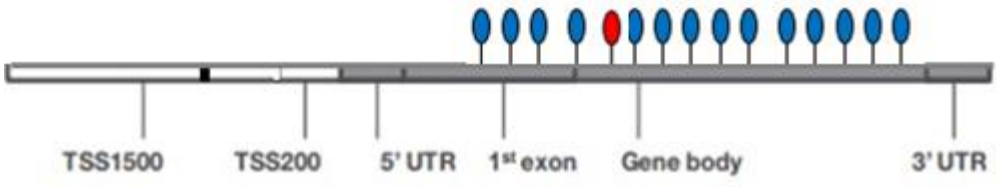
- HLA-C (Downregulated)



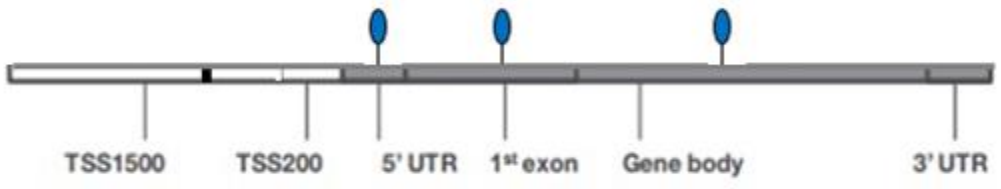
- HLA-DOB(Downregulated)



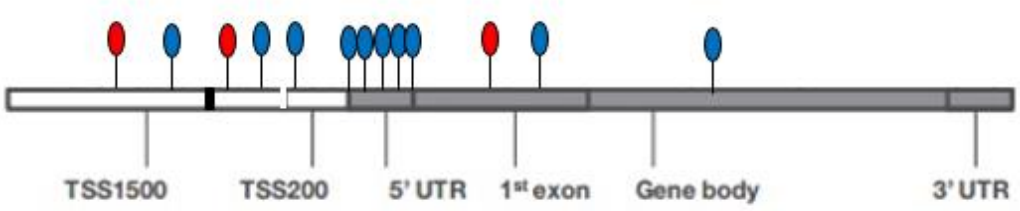
- HLA-DQB1 (Downregulated)



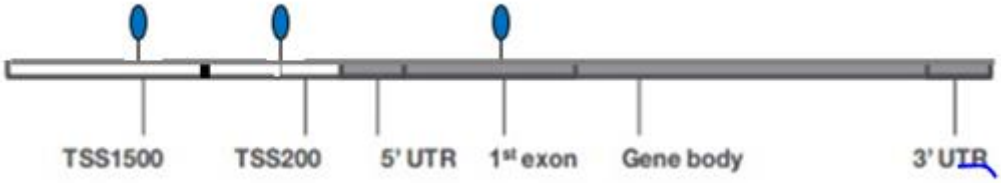
- NLRP12 (Upregulated)



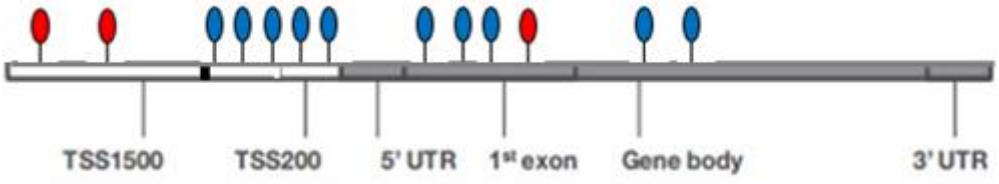
- ITGB2 (Upregulated)



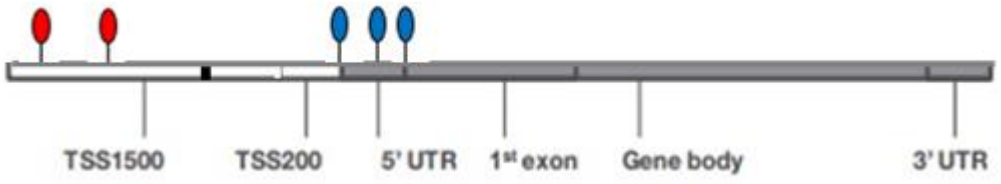
- C3AR1 (Upregulated)



- FADD (Upregulated)



- APOL3 (Downregulated)



- VARS2 (Downregulated)

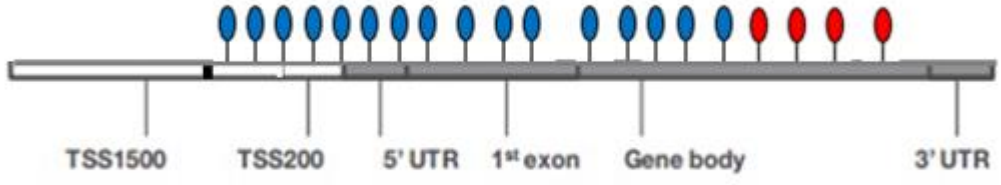


Figure 10: Multiple schemes that show the 10 individual genes along with the location of the differentially-methylated CpG sites. All of the genes contain at least 3 differentially-methylated sites, suggesting the existence of a differentially-methylated region (DMR). No correlation is apparent between the location and direction of methylation change and the direction of expression change.

The last group of analyzes made on these 10 differentially methylated and differentially expressed genes was analyzed between the CpG sites, which were significant (Fig. 9), their location along the gene and their methylation state, when sepsis occurs. It should be noted that, at the very least, there are 3 CpG sites that presented themselves as differentially methylated. The different schemes, in figure 10, visually show these methylated probes (hypomethylated probes in blue and hypermethylated probes are shown in red).

The location of these probes was analyzed using data provided by Illumina when performing methylation arrays with 450k of probes. Unfortunately, as can be seen in three gene situations, namely HLA-C, HLA-DOB and APOL3, it was not possible to present all the significant probes. As can be seen from the different schemes, most of the CpG sites are located in TSS200 or in the body of the gene. When most probes are located in the TSS200, the genes are usually upregulated, whereas genes that are downregulated, most of the CpG sites are distributed by the other regions of the gene, specifically the body of the gene. Other analyzes would have to be made to draw a direct correlation between the location of the probes, their methylation status and the change in direction of expression. However, these

schemes show for the first time the relationship between methylation and the expression of these genes in sepsis

Amongst the non-HLA genes presented in Figure 10, FADD, VARS2, and ITGB2 are notable for the very large number of differentially-methylated CpG sites. There is also some clustering of methylation sites, such as in VARS2 where the hypomethylated sites are all located upstream and the hypermethylated sites are all located downstream.

3.7- Phase 4: Correlating DNA methylation changes with clinical parameters

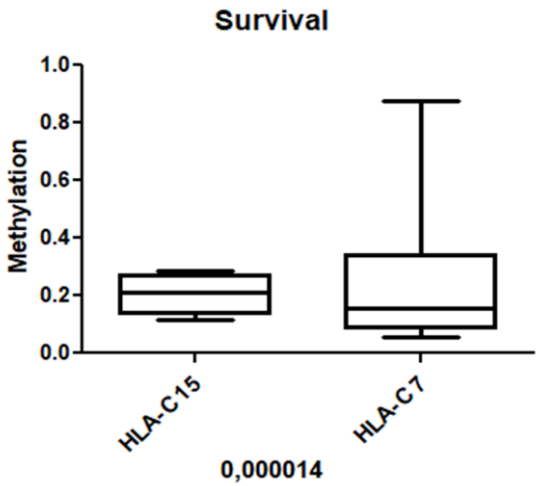
The EPSIS dataset includes clinical data relating to bacterial culture results, the presence or absence of septic shock, the use of mechanical ventilation, vasopressors and steroids, as well as the presence of pre-existing chronic diseases including cancer and ischemic heart failure.

In Phase 4 we used the 133 differentially-methylated CpG sites located in the 10 genes identified in Phase 3 to explore whether any of these sites provided diagnostic or prognostic information for our patients. We compared different parameters such as presence / absence of septic shock, mechanical ventilation, vasopressors, use of steroids, culture results (gram-positive vs gram-negative bacteria), and overall survival (less than 7 days s more than two weeks) as the dependent variable for the analysis.

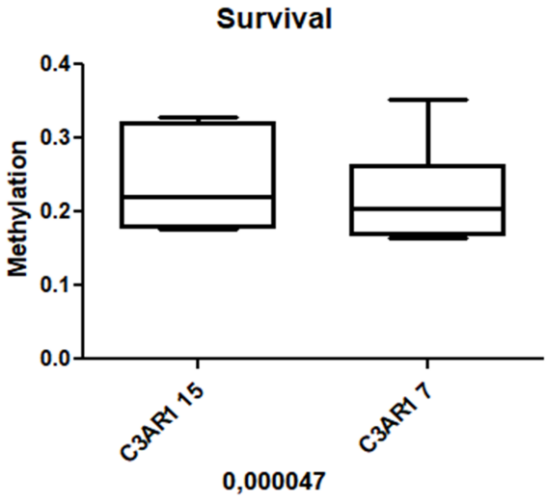
We then divided the septic group of patients (n=66) into two groups based on each of these criteria and used a T-test and adjusted p-value to determine if there were any associations between methylation status at these CpG sites and clinical outcomes.

We first compared methylation profiles in septic patients with survival of less than 7 days (early mortality, n=28) vs those with survival of at least 15 days or longer (early survivors, n=14). The results for two CpG sites with differential methylation are shown in Figure 11. Site cg22993154 in the HLA-C gene showed a wide range of methylation values amongst the early mortality group compared to consistently low methylation levels amongst early survivors. This was also true of cg23463743 in HLA-DQB1. A total of 39 CpG sites showed differential methylation between the early mortality and early survival groups.

cg22993154 – HLA-C



cg27423177- C3AR1



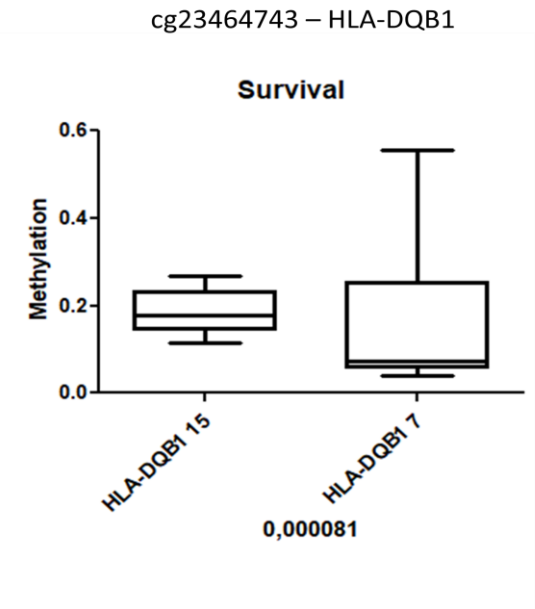
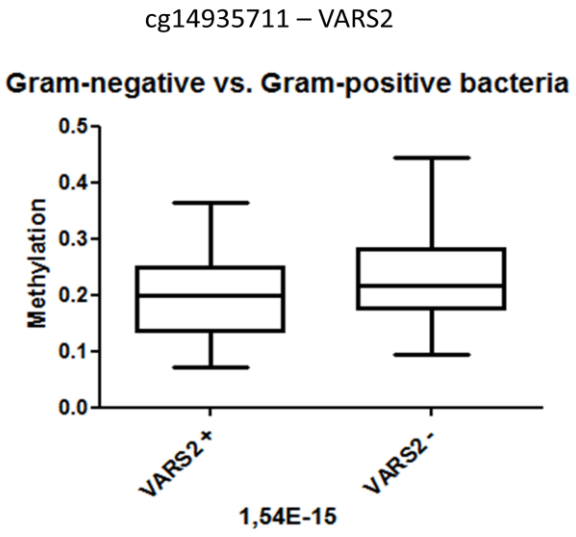


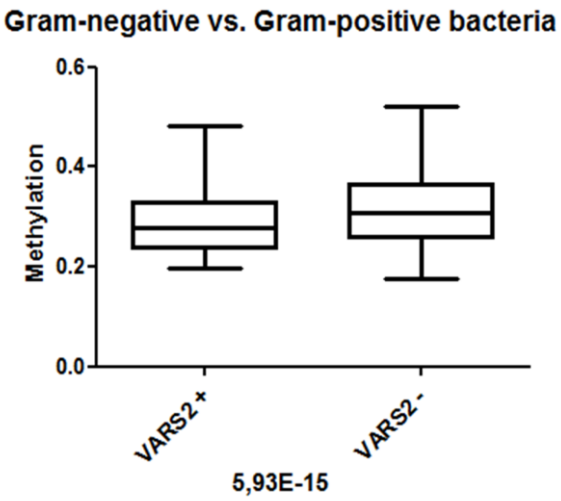
Figure 11: Graphs that analyze which gees and associated CpG sites relate survival (patients who survived 7 days less and patients who survived longer than 2 weeks) among the group of patients with sepsis (n = 64). Genes presented were A) HLA-C with CpG site 22993154; B) C3AR1 with CpG site 27423177 and C) HLA-DQB1 with CpG site 23464743

- Type of culture (gram-negative / gram-positive bacteria)

Only a subset of septic patients had positive cultures available for analysis. Using only the septic patients with positive culture results from within 3 days of admission we subdivided them into those with gram-positive infections (n=30) and those with gram-negative infections (n=30). Two sites in the VARS2 gene (cg14935711, cg12457901) correlated with the presence of gram positive vs gram negative sepsis. In both cases the CpG sites wre relatively hypomethylated amongst patients with gram-positive sepsis. Amongst septic patients generally, VARS2 is relatively hypomethylated and downregulated, suggesting that this effect is more pronounced in gram-positive infections. Differentially-methylated CpG sites were also identified in APOL3 and HLA-DQB1. In total, 55 CpG sites were differentially methylated between patients with gram-positive and gram-negative sepsis.



cg12457901– VARS2



cg23464743– HLA-DQB1

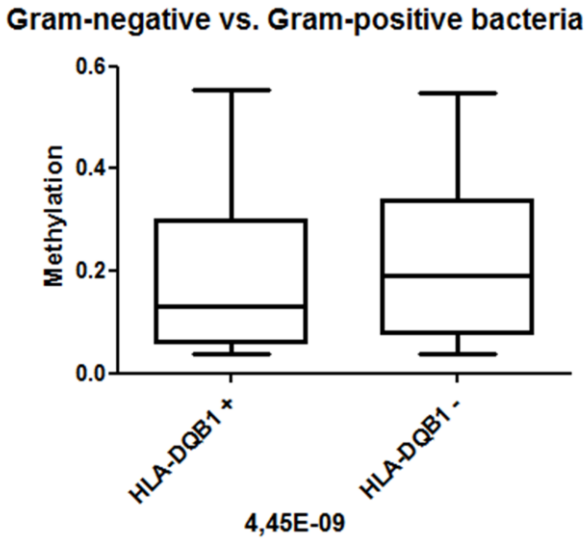


Figure 12: Graphs that analyze which genes and CpG associated sites relate the type of bacterial infection (patients who were infected by gram-negative bacteria and patients who were infected by gram-positive bacteria) among the group of patients with sepsis (n = 64) . Genes presented were A) VARS2 with CpG site14935711; B) VARS2 with CpG site12457901; C) APOL3 with CpG site 10257263; D) HLA-DQB1 with CpG site 23464743

- Presence/absence of septic shock

Septic shock is clinical defined by persisting hypotension requiring vasopressors to maintain a mean arterial pressure (Mervyn Singer *et al.*, 2016). Is also known as a subset of sepsis with significantly increased mortality due to severe abnormalities of circulation and/or cellular metabolism. On average, about 30 to 40% of people with septic shock die (Paul M. Maggio). A total of 65 CpG sites were found to be differentially methylated in cases of septic shock.

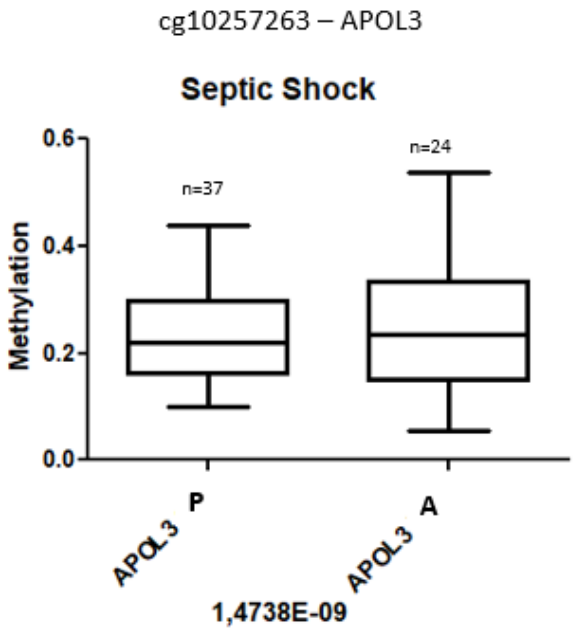


Figure 13: Graphs that analyze which genes and associated CpG sites relate the presence or absence of septic shock among the group of patients with sepsis (n = 64). Genes presented were A) APOL3 with CpG site10257263;

- Vasopressors

Septic shock is a severe form of sepsis in which blood pressure is no longer maintained due to loss of endothelial integrity and sepsis-induced cardiac dysfunction. Vasopressors are medications used to reverse sepsis-induced hypotension (RC Bone *et al.*, 1992). They have the potential to increase the systemic arterial pressure and to augment cardiac contractility (Leeanne Stratton*et al.*, 2017). The septic patient cohort was subdivided into those who did and did not require vasopressors on admission to the ICU.

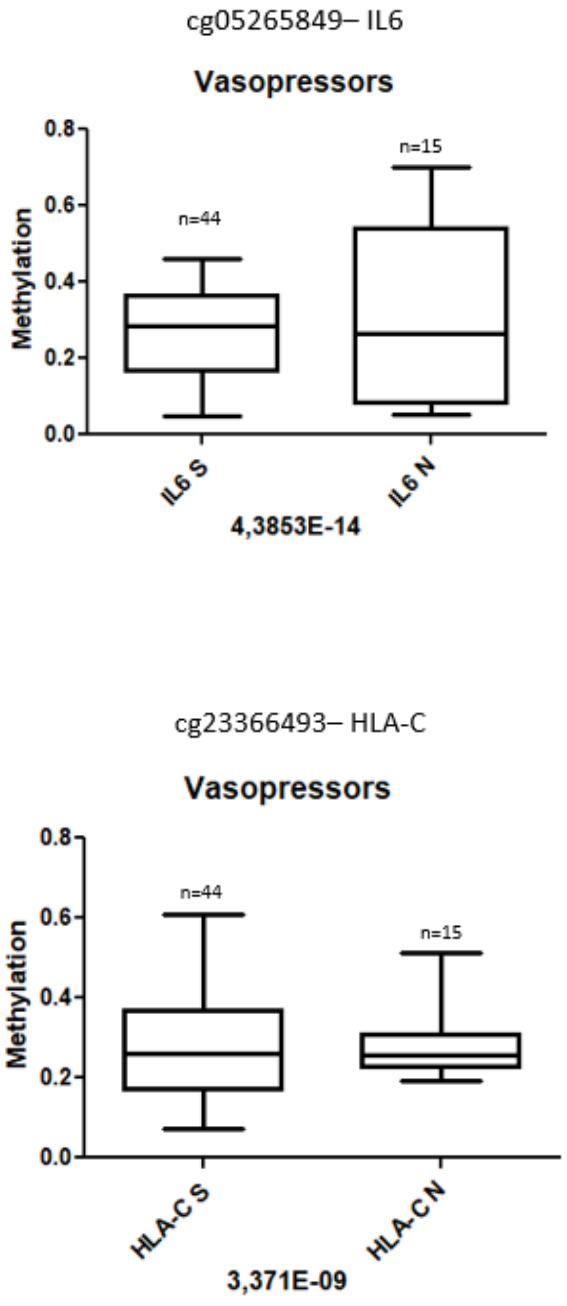


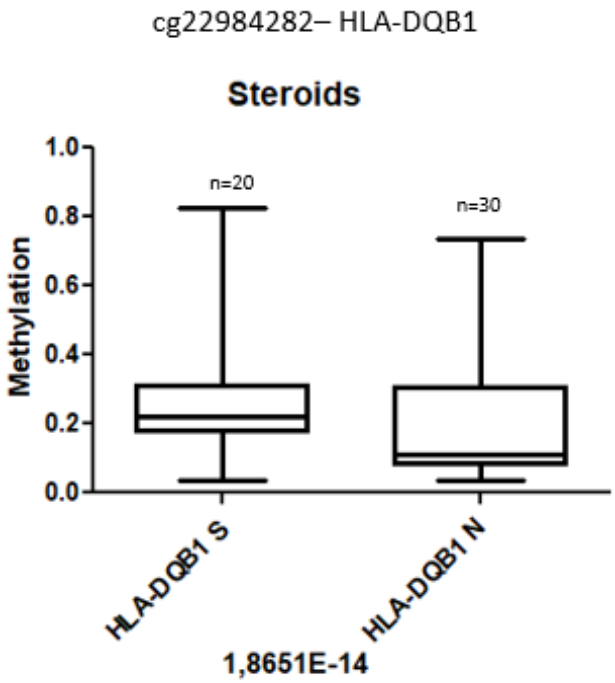
Figure 14: Graphs that analyze which genes and associated CpG sites relate the presence or absence of administration of vasopressors among the group of patients with sepsis (n = 64). Genes presented were A) IL6 with CpG site 05265849; B) HLA-C with CpG site 23366493

- Mechanical ventilation

Only two of the septic patients were not mechanically ventilated. The sample size was therefore insufficient to provide any meaningful data.

- Steroids

Corticosteroids are often used as a treatment of septic shock although the evidence is controversial. Three main indications for linked exist for corticosteroids in sepsis: 1) as immunosuppressants; 2) to treat relative glucocorticoid deficiency during critical illness and 3) for patients who are unresponsive to high-dose vasopressors (PE Marik et al., 2008). Our sepsis cohort contained a sub-group of patients who were treated with corticosteroids at the discretion of the admitting physician.



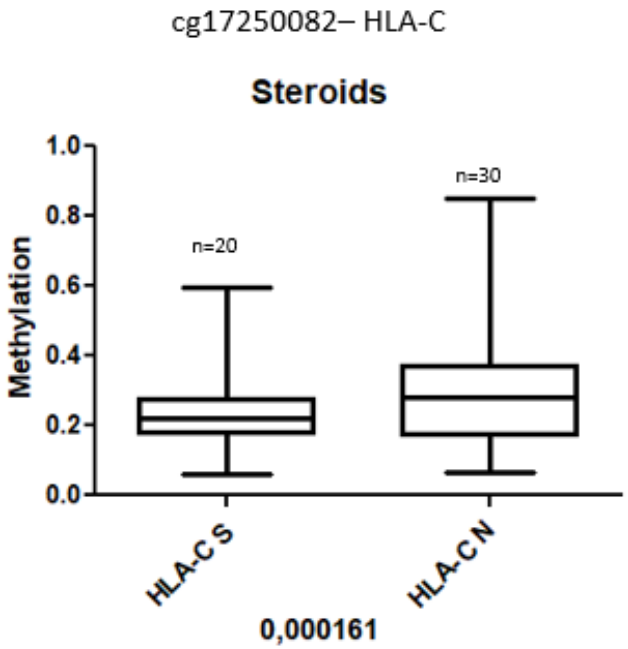


Figure 15: Graphs that analyze which genes and CpG associated sites relate the presence or absence of steroids administration among the group of patients with sepsis (n = 64). Genes presented were A) HLA-DQB1 with CpG site 22984282; B) HLA-C with CpG site 17250082;

In these last analyzes was made, for the first time for our knowledge, direct relations between clinical parameters and the 10 genes differentially methylated and differentially expressed in sepsis. Different genes and CpG sites showed crucial for this relationship. Some of them deserve attention.

HLA haplotypes are one of the genetic features that most strongly associate with autoimmunity (Cho *et al.*, 2011). HLA-C is one of the MHC-I proteins, who plays a role as a ligand for killer immunoglobulin receptors expressed on NK cells (Maria Eve Blais *et al.*, 2011). The three main CpG sites (CpG22993154, CpG23366493 and CpG17250082) were found to be important in different parameters, thus constituting a good future marker of diagnosis in sepsis. On the other hand, HLA-DQB1 plays a central role in the immune system by presenting peptides derived from extracellular proteins (U.S National Library of Medicine). Associated with this gene are also different probes, CpG23464743 and CpG22984282.

4- DISCUSSION

Sepsis is the result of a massive immune response to bacterial infection that gets into the blood. A disease that is not heterogeneous and aggressive, such as this one, has taken a special emphasis over the years, mainly due to the total lack of knowledge of its pathophysiology, as well as the exorbitant expenses to keep these patients in intensive care units. The present project aimed to understand the changes in transcriptome and methylome of septic and non-septic patients, in order to distinguish and establish two distinct epigenetic assemblages. For such, bibliographic reviews of genes important for sepsis, analysis of methylation differences, in the first cohort of septic patients done; study of transcript differences in transcriptomic datasets previously made and the final ratio of differentially methylated and expressed genes with different clinical parameters.

4.1- Phase 1: Literature screen

The starting point of our study was an intensive bibliographic investigation of the metabolic pathways and genes involved in the pathophysiology of sepsis. Five specific pathways were identified as being of critical importance: immunoactivation, immunosuppression, coagulopathy, apoptosis and endothelial dysfunction. After researching and reading dozens of articles on this subject a list of 128 genes was generated, all of which are highly relevant to the pathogenesis and consequences of sepsis.

These 128 genes fall into a number of functional categories: innate immunity, adaptive immunity, proinflammatory mediators, pro- and anti-apoptotic proteins, cell replication and proliferation proteins, membrane proteins, protein chaperones, complement proteins, MHC-I and MCH-II proteins, and platelet and coagulation proteins.

Genes involved in immunoactivation, either as part of the innate or adaptive immune systems included the toll-like receptor genes, the MHC-I and MCH-II class proteins, and the supramolecular activation cluster genes (P-SMAC, D-SMAC, and C-SMAC). Genes involved in immunosuppression included IL-10, TGF-β or IL1B. Apoptosis proteins included BIRC1, BID, BAX, and BCL-2. Genes involved in coagulopathy included FGB, FGG, HP or vWF. Finally, genes involved in inflammation included NF-KB, TNF-α, RAGE or HMGB1.

Although this is not an exhaustive list of all the proteins involved in the pathogenesis of sepsis, it includes many of the most important players. Our objective was not

to itemize every possible protein involved in sepsis. Instead our goal was to generate a list of proteins to serve as the basis for a "focused" analysis of DNA methylation in sepsis.

This bibliographic review has become essential, as the basis of this whole project.

4.2- Phase 2: Methylation arrays

Our study is a nested case-control study utilizing genomic DNA samples from the DYNAMICS cohort of critically-ill septic and non-septic patients. DYNAMICS was an observational multi-site study of 1000 ICU critically patients in Canada (400 septic and 600 non-septic critical illness patients). From DYNAMICS, 141 patient samples were selected for hybridization to the Infinium HumanMethylation450 BeadChip arrays. This represents the first cohort of septic patients with genome-wide DNA methylation data, as well as the first cohort of critically ill patients. We refer to this cohort as the EPSIS (Epigenetic Profiling in Severe Sepsis) cohort.

Using the 128 genes identified in phase 1 of our study we identified 705 CpG sites contained within these genes and also assayed in the HumanMethylation450 Beadchip Array. We then analysed the DNA methylation data to identify which of these CpG sites showed a significant difference between septic and non-septic patients. Using a beta-value difference cutoff of > 2% and a false discovery rate of 5%, we identified a total of 595 CpG sites that were differentially methylated. These CpG sites represented 61 of our original 128 gene list, or approximately 48% of our target genes. When looking at "average" methylation status of these genes we found 64% were hypermethylated while 36% were hypomethylated.

In parallel to our study, our collaborator Dr. Chris Walsh has been using the same genomic methylation data to perform an epigenome-wide association study (EWAS). This unbiased approach entails analyzing all of the CpG sites on the beadchip for DNA methylation differences associated with sepsis. The EWAS approach has the advantage of interrogating all of the available CpG sites in the genome but the disadvantage of identifying many false positives as well as many differences that may not be relevant to the pathogenesis of sepsis. Our "focused" approach has the benefit of focusing on a smaller number of genes and therefore having greater sensitivity for small DNA methylation changes. It also ensures that the DNA methylation changes we find are likely to be biologically relevant to sepsis. However we may miss important DNA methylation changes in genes that are not as strongly associated with sepsis in the current literature.

After we had induced a filter for the beta values ($\Delta\beta \geq .02$) and by calculating the p-value<0.05, we had arrived at a list of 61 genes. In this list of 61 genes analyzed within all of the probes that appeared in the methylation arrays, we counted which CpG sites were differentially methylated. And thus understand the average change in methylation for each of these genes. In a total of 595 probes, about 64% were hypermethylated (n = 380), while 34% of these CpG sites (n = 215) were hypomethylated. These results are important to understand, for the first time, the type of alterations, at the level of the methylome that the sepsis patients suffer.

We next analyzed the localization of the differentially methylated CpG sites relative to their associated genes and relative to the CpG islands. The Illumina HumanBeadchip 450k array documentation provides basic information about gene localization (TSS1500, TSS200, 5'UTR, 1st exon, Body, and 3'UTR). Hypermethylation of CpG sites in the gene promoter is often associated with inhibition of gene expression while hypermethylation of CpG sites in the gene body is associated with increased gene expression.

When we analyzed the distribution of these probes from the 61 genes (595 CpG sites) and the probes from the initial 128 genes (702 CpG sites), it shows that the distribution of these probes accompanies a similar distribution.

About 25% of all of 595 probes are found in the gene body, while while the highest percentage of the 702 probes are in the TSS200. Similarly, 3'UTR is the site in the gene that receives the least number of significant probes. Regarding the methylation status of these probes, more than 40% of all CpG sites are hypermethylated in the body of the gene, rather than about 35% of these are hypomethylated in the 5'UTR. Regarding the methylation status of these probes, more than 40% of all CpG sites are hypermethylated in the body of the gene, rather than about 35% of these are hypomethylated in the 5'UTR.

CpG islands (CGIs) are short interspersed DNA sequences that are often sites of transcription initiation. Hypermethylation of CpG islands located in the promoter regions of crucial genes, such as tumor suppressor genes, causes suppression of gene expression. Looking at our differentially-methylated CpG sites we first note that 99% our differentially-methylated probes are located in or adjacent to a CpG island. Amongst the hypermethylated probes, 42% localize to CpG islands, while 29% localize to CpG shore (0-2kb from the CpG island) and 29% localize to the shelf (2-4kb from the CpG island). Conversely, amongst hypomethylated probes, 60% of the probes localize to the CpG island ”shelf” and only 19% and 22% to the CpG island and shore respectively. This represents a significant difference between our hypermethylated and hypomethylated probes.

Hypermethylation of important genes, like oncogenes, tumor suppressor genes or involved in cell proliferation and apoptosis, are linked to DNA methylation. And most of all DNA methylation changes are located in CpG islands (Rafael Irizarry *et al.*,2009) like we saw on our analysis.

As a last step, we compared our results with the results from the EWAS study. In this comparison only 2 targets of our 10 differentially methylated and differentially expressed genes do not appear in the EWAS results, namely HLA-DOB and HLA-DQB1. These two genes participate in adaptive immunity, belonging to class II of the MHC complex. These results prove to be quite satisfactory, because although in both studies they adopted opposite working guidelines, the same targets appeared in both analyzes.

These targets thus constitute a cluster of genes important to undergo future validation analyzes, in order to create a group of prognostic biomarkers for sepsis.

4.3- Phase 3: Transcriptomic data

One of the goals of our study was to correlate epigenetic and transcriptional changes in sepsis for the first time. Although no transcriptional data is available for our cohort we were able to identify three publicly-available datasets containing both septic and non-septic patients. These include Hector Wong's cohort pediatric septic vs. non-septic ICU patients (Hector Wong *et al.,* 2012); Mihali Natea's cohort of neonatal septic and non-septic patients (Kari A. Simonsen *et al.*, 2014). The last dataset was from Brian Scicluna and Tom van der Poll's cohort of adult critically-ill patients with pneumonia versus noninfectious respiratory failure (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74224. Although these transcriptomic datasets are not a perfect match for our cohort, they provided us with an indication of which genes are differentially expressed in whole blood samples from septic and non-septic patients.

Of the 61 genes with significant methylation changes identified in phase 2, a total of 10 genes showed differential expression in at least one of these data sets. These genes included: HLA-A, HLA-C, HLA-DOB, HLA-DQB1, FADD, C3AR1, ITGB2, VARS2, APOL3, NLRP12. We can group these 10 genes according to their roles in sepsis: the HLA proteins, HLA-A and HLA-C belongs to MHC-I complex and HLA-DOB, HLA-DQB1

belong to the MHC-II group of proteins, which present antigens on antigen presenting cells; ITGB2 is a mediator of adaptive immunity that participate in cell adhesion as well as cell-surface mediated signalling (Wilson ZS *et al.*, 2017). This protein is involved in the endothelial integrity. Lack of ITGB2 leads to low leukocyte extravasation from blood into tissues; FADD is a pro-apoptotic protein (Sundar *et al.* 2017), NLRP12 and C3AR1 are associated with inflammation. Regarding the NLRP12 gene, this is implicated in the activation of proinflammatory caspases (Gurung *et al.*, 2015). Namely to C3AR1 is an important factor in the activation of the complement response cascade; and finally APOL3 and VARS2 are involved in the regulation of cell proliferation, namely in the movement of lipids or allow the binding of lipids to organelles (Diodato *et al.*, 2014).

With respect to the relationship between DNA methylation and gene expression, we don't see a clear correlation in our 10 genes. There are several possible reasons for this. Firstly, the relationship between methylation status and gene expression is not always consistent [REF]. Althought promoter methylation may prevent binding of transcription factors leading to decreased gene expression, it may also prevent binding of transcriptional inhibitors leading to increased gene expression. Secondly, our transcriptomic data comes from unrelated datasets. We therefore don't know whether the gene expression changes seen in these datasets reflect our patient population. Finally, there may be some false positives in our data. In the absence of another cohort we cannot validate our findings.

4.4- Phase 4: Correlating DNA methylation changes with clinical parameters

Clinical data for the EPSIS cohort includes bacterial culture results, the presence or absence of septic shock, the use of mechanical ventilation, vasopressors and steroids, as well as the presence of chronic diseases including cancer and ischemic heart failure. Using the 133 differentially-methylated CpG sites located in the 10 genes identified in Phase 3 we next explored whether any of these sites provided diagnostic or prognostic information for our patients. The clinical parameters selected were patients' overall survival (less than 7 days vs more than two weeks), presence or absence of septic shock, administration of steroids, vasopressors and mechanical ventilation, and bacterial culture results (gram-positive vs gram-negative bacteria).Using only the patients in the sepsis cohort (n=66) we subdivided the patients according to the presence or absence of each criteria and then performed direct correlations using a T-test and adjusted p-value calculation, using GraphPad software.

In our analysis of patients with "early mortality" (mortality in < 7 days) vs "early survivors" (survival > 15 days) we found that CpG site 22993154 in HLA-C was able to distinguish the two groups. HLA-C is reported to be important for cell survival, especially hematopoietic cells, in addition to its role in antigen presentation in the immune system (Ho VT et al., 2006). With respect to gram positive vs gram negative infections, CpG site 23464743 in HLA-DQB1 was able to distinguish the two culture types.

When we analysed patients for the presence or absence of septic shock we found that CpG site 10257263 in the APOL3 gene strongly correlates with septic shock. Septic shock occurs when cardiac dysfunction, vasodilation, and leakage of fluid out of the bloodstream lead to dangerously low blood pressures and poor organ perfusion (Mervyn Singer et al., 2016). APOL3 is a positive regulator of NF-kB and may also be involved in apoptosis (B. Vanhollebeke *et al.,* 2006).

4.5- Strengths and weaknesses of this study

As with any project, there are always aspects that need further discussion and critical analysis. The EPSIS cohort is the first cohort of septic and non-septic patients with genome-wide DNA methylation data. Although the data is extremely novel, it is important to address some specific weaknesses of our study: a) the use of whole blood samples; b) the use of Day 1 samples; c) the use of GEO datasets for transcriptomic validation and d) the relevance of the correlations between our data and the clinical parameters.

a) Whole blood samples: Whole blood is easy to collect but it contains a mixed cell population. In our patient cohort approximately 80% of the nucleated blood cells are neutrophils while the rest are lymphocytes, monocytes, eosinophils and basophils (unpublished data). This reduces the chances that we will detect significant methylation changes in our cohort. We also don't know whether any methylation changes we find are occurring in neutrophils or in one of the other cell types. This could only be resolved by cell sorting prior to methylation analysis.
b) Day 1 samples: Genomic DNA was collected on Day 1 of ICU admission. This is very early in the patient's course and we may have missed important DNA methylation changes that occur later on in sepsis.

c) GEO datasets: There is no transcriptomic data available for the EPSIS cohort. We therefore used data from unrelated datasets of septic and non-septic patients in GEO. This data does not necessarily represent gene expression in our patient cohort.
d) Correlations with clinical parameters: We have identified a number of DNA methylation changes that correlate with important clinical parameters, such as survival and gram negative vs gram positive infection. These are candidate biomarkers but will need to be validated in a separate cohort.

4.6- Comparison with EWAS study

Our collaborators have used the EPSIS dataset to perform an epigenome-wide association study. The approach taken in this study was more directed, namely to focus on genes directly involved in sepsis. One disadvantage to our approach is that by focusing on specific genes, we may have missed other, less obvious, targets. However, the opposite may also have happened. By analysing a relatively small group of genes involved in the pathogenesis of sepsis, we have improved the sensitivity of our analysis. Thus two of the 10 genes we found that are both differentially methylated and expressed in sepsis were not identified in the EWAS analysis.

4.7- Future work

The most important next step in this project is to create a validation cohort so that we can confirm our results. Additionally, we would like to create a cohort with both DNA and RNA samples, that would allow us to compare DNA methylation and gene expression in the same patients. Furthermore, we would like to confirm that correlations between DNA methylation changes and clinical parameters that we have identified. DNA methylation changes have great potential as clinical biomarkers – we hope that the creation of a set of epigenetic-based biomarkers will provide clinicians with a tool that allows them to better diagnose sepsis and help guide their treatment decisions.

5- Conclusion

This project focused on the study of sepsis, a highly complex and heterogeneous disease, in an innovative and novel way. We have assembled, for the first time, DNA methylation profiles from 66 septic and 68 non-septic intensive care patients and we have compared these data with transcriptomic information from 3 cohorts of patients with and without sepsis. We adopted a bioinformatics approach to identifying and establish DNA methylation signatures of sepsis in a focused set of sepsis-related genes. Ten of these genes - HLA-A, HLA-C, HLA-DOB, HLA-DQB1, ITGB2, FADD, APOL3, C3AR1, FADD, VARS2 and NLRP12, showed significant methylation and transcription changes, suggesting epigenetic regulation. Furthermore, we identified correlations between several of the CpG sites and clinical parameters including gram-positive vs gram-negative infection, septic shock, vasopressors, and early mortality vs early survival.

The role of epigenetics in sepsis is only just beginning to be explored. Our group has established a collaboration with a group in the United States that has a cohort of septic and non-septic intensive care patients with correlated RNA and DNA samples. These samples will enable us to further explore the relationship between DNA methylation and gene expression, and to validate our initial findings. In this way, we hope to clarify the role of DNA methylation in the pathogenesis of sepsis and to identify new diagnostic tools that will improve our ability to diagnose and manage septic patients.

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