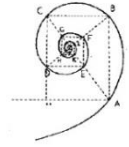Università degli Studi di Milano

PhD in Molecular and Translational Medicine

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PhD Thesis

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The role of cellular and exosomal neural-derived long non-coding RNA (lncRNA) in multiple sclerosis: potential biomarkers of disease susceptibility and progression

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ABSTRACT

Long non-coding RNAs (lncRNAs) are a novel class of transcripts that are pervasively transcribed in the genome. Several lines of evidence correlate dysregulation of different lncRNAs to human diseases including neurological and autoimmune disorders, but their expression has not been exhaustively investigated in MS so far.

The main aim of this study was to identify a specific signature of cellular and neural-derived exosomal lncRNA expression.

Regarding lncRNA expression levels from Peripheral Blood Mononuclear Cells (PBMC), we studied a discovery cohort of MS patients who were compared against controls. Results were validated in a larger cohort and further replicated in an independent Belgian population.

LncRNA PCR arrays from System Bioscience (SBI) containing 90 common lncRNAs were used to screen lncRNA expression levels in PBMC from 5 patients with Relapsing Remitting (RR)-MS, 5 with Primary Progressive (PP)-MS and 5 age-matched controls. Results were validated by real time PCR in a further independent Italian cohort consisting of 30 PBMC samples from MS patients and 30 controls. Best hits were replicated using droplet digital PCR in a Belgian cohort consisting of 24 MS patients and 23 controls.

In particular, in the Italian validation cohort ANRIL, TUG1, XIST (p<0.0001) and SOX2OT (p<0.001) were strongly down-regulated in RR-MS versus controls, while GOMAFU, HULC (p<0.0001) and BACE-1AS (p<0.001) showed a robust down-regulation both in RR and Progressive MS in comparison with controls. NRON and TUG1 downregulation in MS patients, compared with controls (p<0.05 and p<0.0001 respectively), was confirmed in the Belgian population.

In addition, a protocol for the extraction and characterisation of neural-derived exosomes has been developed in order to investigate exosomal lncRNA expression levels. Using two types of commercial arrays, the human RT² lncFinder array (QIAGEN) and the human RT² lncRNA inflammation response and autoimmunity array (QIAGEN), generalised deregulation in exosomal lncRNA was observed. Moreover, the expression pattern of these molecules was different in RR-MS and in PP-MS.

Precisely, results from the human RT² lncFinder array (QIAGEN) analysis led to the identification of 7 most significantly deregulated lncRNAs, precisely AIRN (5.30-fold increase over controls, p=0.04); FAS-AS1 (4.76-fold increase over controls, p=0.02); HOTAIR (4.47-fold increase over controls, p=0.03); NAMA (13.24-fold increase over controls, p=0.01); TRERNA1 (5.84-fold increase over controls, p=0.01) and HOXA-AS2 (0.56-fold increase over controls, p=0.04).

Six lncRNA were significantly deregulated in the RR-MS subgroup, precisely AIRN (10.77-fold increase over controls, p=0.04); DLX6-AS1 (46.95-fold increase over controls, p=0.01); FAS-AS1 (11.37-fold increase over controls, p=0.001); HOTAIR

(9.31-fold increase over controls; p=0.02); and TRERNA1 (6.61-fold increase over controls, p=0.003).

In PP-MS only SOX-2OT showed a significant upregulation (8.95-fold increase over controls, p=0.02).

When we used the array containing lncRNA linked with inflammation and autoimmunity, MZF-AS1 (0.47-fold decrease over controls, p=0.03), CEP83-AS1 (0.15-fold decrease over controls, p=0.02), RP11-282O18.3 (0.27-fold decrease over controls, p=0.02), RP11-84C13.1 (0.28-fold decrease over controls, p=0.04), SNHG7 (0.064-fold decrease over controls, p=0.04) and TP73-AS1 (0.48-fold decrease over controls, p=0.04) were significantly downregulated in MS, regardless of the subtype, while RP11-38P22.2 (19.5-fold increase over controls, p=0.04) showed an upregulation.

Considering the disease subgroups, RR-MS patients showed a significant downregulation in RP11-363G2.4 (0.07-fold decrease over controls, p=0.008) and in TP73-AS1 (0.76-fold decrease over controls, p=0.02), while RP11-38P22.2 levels were upregulated (22.32-fold increase over controls, p=0.04). We found a general downregulation in lncRNA expression analysed in PP-MS, in particular FGF14-IT1 (0.08-fold decrease over controls, p=0.007) and RP11-282O18.3 (0.14-fold decrease over controls, p=0.04) were significantly altered.

Some important forms of dysregulation were observed, considering the expression levels of lncRNAs known to be involved in brain function and in neurological and autoimmune disorders. The rationale of this study might then be used to set up a future study with the purpose of selecting potential biomarkers for disease aggressiveness and possible response to therapy.

RIASSUNTO

I long non-coding RNA (lncRNA) rappresentano una nuova classe di trascritti, che vengono prodotti a partire dal genoma. Diverse evidenze mostrano una correlazione tra un'alterata espressione dei lncRNA e differenti malattie dell'uomo, tra cui i disturbi autoimmuni e neurologici. Tuttavia, il loro ruolo nella sclerosi multipla (MS) non è ancora stato delucidato.

Lo scopo principale di questo studio è stato quello di identificare una specifico pattern di espressione dei lncRNA, derivanti da cellule e contenuti negli esosomi di origine neuronale.

In primis abbiamo studiato i lncRNA espressi dalle cellule mononucleate del sangue periferico (PBMC) in una coorte esplorativa di pazienti con MS e li abbiamo confrontati con dei soggetti di controllo. I risultati sono poi stati validati in una coorte più numerosa e ulteriormente replicati in una popolazione belga indipendente. Grazie a piastre contenenti 90 sonde complementari ai più studiati LncRNA (System Bioscience (SBI) abbiamo condotto uno screening in RT-PCR dei livelli di espressione dei lncRNA espressi nelle PBMC provenienti da 5 pazienti con la forma recidivante-remittente (RR-MS), 5 con la forma primariamente progressiva (PP-MS) e 5 controlli paragonabili per età. I risultati sono stati quindi validati in un'ulteriore coorte indipendente italiana composta da PBMC isolate da 30 pazienti con MS e da 30 controlli, usando sonde Taqman in RT-PCR. I risultati migliori sono stati successivamente replicati utilizzando la tecnica di droplet digital PCR (ddPCR), in una coorte belga composta da 24 pazienti con MS e 23 controlli. In particolare, nella coorte di validazione italiana, i livelli di espressione di ANRIL, TUG1, XIST ($p < 0,0001$) e SOX2OT ($p < 0,001$) sono risultati fortemente ridotti nel gruppo RR-MS rispetto ai controlli, mentre GOMAFU, HULC ($p < 0,0001$) e BACE-1AS ($p < 0,001$) hanno mostrato una robusta alterazione sia nel gruppo RR-MS che in quello dei PP-MS rispetto ai controlli. La riduzione dei livelli di espressione di NRON e TUG1 nei pazienti con MS rispetto ai controlli ($p < 0,05$ e $p < 0,0001$ rispettivamente) è stata confermata nella popolazione belga.

Parallelamente, abbiamo messo a punto un protocollo per l'estrazione e la caratterizzazione di esosomi derivanti da neuroni, con il fine di indagare i livelli di espressione dei lncRNA contenuti in queste vescicole. Utilizzando due tipi di array commerciali, l'array human RT2 lncFinder (QIAGEN) e lo human RT^2 lncRNA Inflammation response and autoimmunity (QIAGEN), è stata osservata una de-regolazione generalizzata nei livelli di espressione dei lncRNA esosomali. Inoltre, il pattern di espressione di queste molecole era diverso nei sottogruppi dei RR-MS e dei PP-MS.

Precisamente, i risultati dell'analisi dell'array human RT^2 lncFinder (QIAGEN) hanno

portato all'identificazione di 7 lncRNA più significativamente alterati: AIRN (aumentato di 5,30 volte rispetto ai controlli, p = 0,04); FAS-AS1 (aumentato di 4,76 volte rispetto ai controlli, p = 0,02); HOTAIR (aumentato di 4.47 volte rispetto ai controlli, p = 0,03); NAMA (aumentato di 13.24 volte rispetto ai controlli, p = 0,01); TRERNA1 (aumentato di 5,84 volte rispetto ai controlli, p = 0,01) e HOXA-AS2 (aumentato di 0.56 volte rispetto ai controlli, p = 0,04). Sei lncRNA sono stati trovati significativamente alterati nel sottogruppo RR-MS: AIRN (aumentato di 10.77 volte rispetto ai controlli, p = 0,04); DLX6-AS1 (aumentato di 46.95 volte rispetto ai controlli, p = 0,01); FAS-AS1 (aumentato di 11.37 volte rispetto ai controlli, p = 0,001); HOTAIR (aumentato di 9.31 volte rispetto ai controlli; p = 0,02); e TRERNA1 (aumentato di 6.61 volte rispetto ai controlli, p = 0,003). Nel gruppo dei PP-MS solo SOX-2OT ha mostrato una espressione maggiore significativa (aumentato di 8.95 volte rispetto ai controlli, p = 0,02). Utilizzando l'array human RT² lncRNA Inflammation response and autoimmunity (QIAGEN), MZF-AS1 (ridotto di 0.47 volte rispetto ai controlli, p = 0,03), CEP83-AS1 (ridotto di 0.15 volte rispetto ai controlli, p = 0,02), RP11-282O18,3 (0,27 ridotto di 0.27 volte rispetto ai controlli, p = 0.02), RP11-84C13.1 (ridotto di 0.28 volte rispetto ai controlli, p = 0,04), SNHG7 (ridotto di 0.0064 volte rispetto ai controlli, p = 0,04) e TP73-AS1 (ridotto di 0.48 volte rispetto ai controlli p = 0,04) hanno mostrato livelli significativamente ridotti in MS, indipendentemente dal sottotipo di malattia, mentre RP11-38P22.2 (aumentato di 19.5 volte rispetto ai controlli, p = 0,04) ha mostrato livelli di espressione maggiori se confrontato con soggetti di controllo. Considerando i sottogruppi di malattia, i pazienti RR-MS hanno mostrato livelli di espressione ridotti in RP11-363G2.4 (ridotto di 0.07 volte rispetto ai controlli, p = 0,008) e in TP73-AS1 (ridotto di 0.76 volte rispetto ai controlli, p = 0,02). I livelli di RP11-38P22.2 sono, invece, aumentati (aumentato di 22.32 volte rispetto ai controlli, p = 0,04). Inoltre, abbiamo trovato un generale de-regolazione nell'espressione dei lncRNA analizzati nel sottogruppo dei PP-MS, in particolare FGF14-IT1 (ridotto di 0.08 volte rispetto ai controlli, p = 0,007) e RP11-282O18.3 (ridotto di 0.014 volte rispetto ai controlli, p = 0,04) erano significativamente alterati.

Considerando i livelli di espressione dei lncRNA notoriamente coinvolti nei disturbi neurologici e autoimmuni, sono emerse alcune importanti de-regolazioni. La logica di questo studio potrebbe quindi essere utilizzata per crearne uno futuro, allo scopo di selezionare potenziali biomarcatori di progressione della malattia e forse indici per valutare la risposta alla terapia.

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SYMBOL INDEX

A2ML1-AS1: A2ML1 antisense RNA 1
Ab: antibody
ABCA11P: ATP-binding cassette, sub-family A (ABC1), member 11, pseudogene
ACTB: actin, beta
AIRN: antisense of IGF2R non-protein coding RNA
APC: antigen presenting cells
ATXN8OS: ATXN8 opposite strand (non-protein coding)
B2M: beta-2-microglobulin
BAFF: B-Cell Activating Factor
BANCR: BRAF-activated non-protein coding RNA
BBB: blood brain barrier
BCYRN1: brain cytoplasmic RNA 1
BDNF-AS: BDNF antisense RNA
BMI: Body Mass Index
BOK-AS1: BOK antisense RNA 1
CCAT1: colon cancer-associated transcript 1 (non-protein coding)
CCAT2: colon cancer-associated transcript 2 (non-protein coding)
CDKN2B-AS1: CDKN2B antisense RNA 1
CEP83-AS1: CEP83 antisense RNA 1 (head-to-head)
CHI3L1: chitinase-3-like protein 1
CIS-MS: clinically isolated syndrome
CNS: central nervous system
COX2: cyclooxygenase 2
CROCCP2: ciliary rootlet coiled-coil, rootlet in pseudogene 2
CSF: cerebrospinal fluid
DANCR: KIAA0114
DC: dendritic cells
DHFR: dihydrofolate reductase
DIO3OS: DIO3 opposite strand (non-protein coding)
DISC2: disrupted in schizophrenia 2 (non-protein coding)
DLEU2: deleted in lymphocytic leukaemia 2 (non-protein coding)
DLX6-AS1: DLX6 antisense RNA 1
EAE: experimental autoimmune encephalomyelitis
EBV: Epstein Barr virus

EDSS: Expanded Disability Status Scale
EGOT: eosinophil granule ontogeny transcript (non-protein coding)
EMX2OS: EMX2 opposite strand (non-protein coding)
EPB41L4A-AS1: non-protein coding RNA 219
ER: endoplasmic reticulum
ERICH1-AS1: ERICH1 antisense RNA 1
eRNA: enhancer RNA
ESCRT: endosomal sorting complexes required for transport
FACS: fluorescence-activated cell sorting
FALEC: long intergenic non-protein coding RNA 568
FAS-AS1: FAS antisense RNA 1
FENDRR: hypothetical LOC400550
FGD5-AS1: FGD5 antisense RNA 1
FGF14-IT1: hypothetical LOC283480
FITC: fluorescein isothiocyanate
FLC: free light chain
FOXN3-AS2: PRO1768
FTX: FTX transcript, XIST regulator (non-protein coding)
GACAT1: gastric cancer-associated transcript 1 (non-protein coding)
GAS5: growth arrest-specific 5 (non-protein coding)
GAS5-AS1: GAS5 antisense RNA 1
GDP: guanosine 5'-diphosphate
GEF: GDP/GTP exchange factor
GNAS-AS1: GNAS antisense RNA 1
GRM5-AS1: GRM5 antisense RNA 1
GTP: guanosine triphosphate
GWAS: genome-wide association study
H19: H19, imprinted maternally expressed transcript (non-protein coding)
HAR1A: highly accelerated region 1A (non-protein coding)
HAR1B: highly accelerated region 1B (non-protein coding)
HCG11: HLA complex group 11 (non-protein coding)
HCG18: HLA complex group 18 (non-protein coding)
HEIH: hepatocellular carcinoma upregulated EZH2-associated long non-coding RNA
HIF-1: hypoxia-inducible factor 1
HLA: human leukocyte antigen
HNRNPU-AS1: HNRNPU antisense RNA 1

HOTAIR: HOX transcript antisense RNA (non-protein coding)
HOTAIRM1: HOXA transcript antisense RNA, myeloid-specific 1
HOTTIP: HOXA distal transcript antisense RNA
HOXA11-AS1: HOXA11 antisense RNA 1 (non-protein coding)
HOXA-AS2: HOXA cluster antisense RNA 2
HOXA-AS3: HOXA cluster antisense RNA 3
HTR4-IT1: HTR4 intronic transcript 1 (non-protein coding)
HULC: hepatocellular carcinoma upregulated long non-coding RNA
IEF: isoelectric focusing
IFN: interferon
Ig: immunoglobulin
IGF2-AS: insulin-like growth factor 2 antisense
IL: interleukin
ILV: intraluminal vesicles
IMSGC: International Multiple Sclerosis Genetics Consortium
iPSc: induced pluripotent stem cells
IPW: imprinted in Prader-Willi syndrome (non-protein coding)
IQCF5-AS1: IQCF5 antisense RNA 1
JPX: JPX transcript, XIST activator (non-protein coding)
KCNIP4-IT1: KCNIP4 intronic transcript 1 (non-protein coding)
KCNQ1OT1: KCNQ1 overlapping transcript 1 (non-protein coding)
KIR: killer-cell immunoglobulin-like receptor
KRASP1: Kirsten rat sarcoma viral oncogene homolog pseudogene 1
L1CAM: L1 cell adhesion molecule
LINC00094: long intergenic non-protein coding RNA 94
LINC00116: long intergenic non-protein coding RNA 116
LINC00293: long intergenic non-protein coding RNA 293
LINC00324: long intergenic non-protein coding RNA 324
LINC00421: HCG2019585-like
LINC00570: long intergenic non-protein coding RNA 570
LINC00581: long intergenic non-protein coding RNA 581
LINC00599: hypothetical LOC157627
LINC00635: hypothetical LOC151658
LINC00657: long intergenic non-protein coding RNA 657
LINC00662: long intergenic non-protein coding RNA 662
LINC00667: hypothetical LOC339290
LINC00853: long intergenic non-protein coding RNA 853

LINC-ROR: long intergenic non-protein coding RNA, regulator of reprogramming

lncRNA: long non coding RNA

LOC100287846: patched 1 pseudogene

LOC101927156: uncharacterised LOC101927156

LOC653160: uncharacterised LOC653160

LRRC37BP1: leucine rich repeat containing 37B pseudogene 1

LRRC75A-AS1: Non-protein coding RNA 188

LUCAT1: lung cancer-associated transcript 1 (non-protein coding)

MAG: myelin-associated glycoprotein

MALAT1: metastasis-associated lung adenocarcinoma transcript 1 (non-protein coding)

MBP: myelin basic protein

MCM3AP-AS1: MCM3AP antisense RNA 1 (non-protein coding)

MEG3: maternally expressed 3 (non-protein coding)

MEG9: maternally expressed 9 (non-protein coding)

MHC: major histocompatibility complex

MIAT: myocardial infarction-associated transcript (non-protein coding)

miRNA: micro RNA

MOG: myelin oligodendrocyte glycoprotein

MRI: magnetic resonance imaging

MRPL23-AS1: MRPL23 antisense RNA 1

MS: multiple sclerosis

MSGB: multiple sclerosis genetic burden

MVB: multivesicular bodies

MZF1-AS1: uncharacterised LOC100131691

NAA: n-acetylaspartate

NAMA: non-protein coding RNA, associated with MAP kinase pathway and growth arrest

NAV2-AS5: NAV2 antisense RNA 5

NCBP2-AS2: hypothetical LOC152217

NEAT1: nuclear paraspeckle assembly transcript 1 (non-protein coding)

NFAT: nuclear factor of activated T-cells

NfH: neurofilament high protein

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NfL: neurofilament light protein

NMO: neuromyelitis optica

NRON: non-protein coding RNA, repressor of NFAT
NUTM2A-AS1: NUTM2A antisense RNA 1
OCB: oligoclonal bands
OIP5-AS1: OIP5 antisense RNA 1
OTX2-AS1: OTX2 antisense RNA 1 (head-to-head)
PANDAR: promoter of CDKN1A antisense DNA damage activated RNA
PBMC: peripheral blood mononuclear cell
PBS: phosphate-buffered saline buffer
PCAT1: prostate cancer-associated transcript 1 (non-protein coding)
PCGEM1: prostate-specific transcript 1 (non-protein coding)
PCR: polymerase chain reaction
PDXDC2P: pyridoxal-dependent decarboxylase domain containing 2, pseudogene
PLP: proteolipid protein
PP-MS: primary progressive form
PRC: polycomb repressor complex
PRINS: psoriasis-associated non-protein coding RNA induced by stress
PSMA3-AS1: hypothetical LOC379025
PTCSC1: papillary thyroid carcinoma susceptibility candidate 1 (non-protein coding)
PTCSC3: papillary thyroid carcinoma susceptibility candidate 3 (non-protein coding)
PTENP1: phosphatase and tensin homolog pseudogene 1
PTENP1-AS: PTENP1 antisense RNA
RBM5-AS1: RBM5 antisense RNA 1
RISC: RNA-induced silencing complex
RMST: rhabdomyosarcoma 2-associated transcript (non-protein coding)
RN7SK: RNA, 7SK small nuclear
RNApol-II: RNA polymerase II
RNP: ribonucleoprotein complexes
RNS: reactive nitrogen species
ROS: reactive oxygen species
RP11-399K21.11: uncharacterized LOC101929189
RPLP0: ribosomal protein, large, P0
RPS6KA2-AS1: RPS6KA2 antisense RNA 1
RQ: relative quantity
RR-MS: relapsing remitting form

RT-PCR: real time polymerase chain reaction
SD: Standard deviation
SDCBP2-AS1: SDCBP2 antisense RNA 1
SENP3-EIF4A1: SENP3-EIF4A1 readthrough (NMD candidate)
SIK3-IT1: SIK3 intronic transcript 1 (non-protein coding)
SIX3-AS1: SIX3 antisense RNA 1
SLC7A11-AS1: SLC7A11 antisense RNA 1
SNAP: NSF-attachment protein
SNARE: SNAP-attachment protein receptor
SNHG11: small nucleolar RNA host gene 11 (non-protein coding)
SNHG16: small nucleolar RNA host gene 16 (non-protein coding)
SNHG20: long intergenic non-protein coding RNA 338
SNHG5: small nucleolar RNA host gene 5 (non-protein coding)
SNHG7: small nucleolar RNA host gene 7 (non-protein coding)
SNORA73A: small nucleolar RNA, H/ACA box 73A
SNP: single nucleotide polymorphism
SOX2-OT: SOX2 overlapping transcript (non-protein coding)
SP-MS: secondary progressive form
SPRY4-IT1: SPRY4 intronic transcript 1 (non-protein coding)
ST7-AS1: ST7 antisense RNA 1
ST7-AS2: ST7 antisense RNA 2
TEM: transmission electron microscope
TERC: telomerase RNA component
TERRA: lncRNA-associated with telomeres
TF: transcription factors
TINCR: placenta-specific 2 (non-protein coding)
TLR: toll-like receptor
TMEM161B-AS1: TMEM161B antisense RNA 1
TP73-AS1: TP73 antisense RNA 1 (non-protein coding)
TRERNA1: translation regulatory long non-coding RNA 1
TSIX: TSIX transcript, XIST antisense RNA (non-protein coding)
T-UCR: ultra-conserved transcripted regions
TUG1: taurine upregulated 1 (non-protein coding)
TUNAR: TCL1 upstream neural differentiation-associated RNA
TUSC7: tumor suppressor candidate 7 (non-protein coding)
UCA1: urothelial cancer-associated 1 (non-protein coding)
UVR: ultraviolet radiation

VEGF: vascular endothelial growth factor
WT1-AS: WT1 antisense RNA (non-protein coding)
XIST: X (inactive)-specific transcript (non-protein coding)
ZFAS1: ZNFX1 antisense RNA 1
ZNRD1-AS1: ZNRD1 antisense RNA 1 (non-protein coding)

1. INTRODUCTION

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is the most common progressive disease of the central nervous system in young adults and the most common cause of serious physical disability in adults of working age [1].

MS is pathologically characterised by focal areas of inflammation, demyelination, gliosis, and axonal damage throughout the central nervous system (CNS). MS disease presentation is very heterogeneous with variable clinical manifestations that evolve over time. About 80% of patients present with relapsing-remitting disease (RR-MS). This form of the disease is characterised by relapses followed by periods of partial or complete recovery (remissions). Other subtypes of MS include primary progressive MS (PP-MS), which shows progressively worsening disability from the onset, and secondary progressive MS (SP-MS), in which patients develop RR-MS but then begin progressing with or without relapses [2,3].

Physicians have described possible MS cases since the Middle Ages. Lidwina of Schiedam is probably the first documented case, and dates back to 1421. Her biographers claim that she suffered episodes of paralysis since she was 16, and that she became progressively worse, until her death. She even became blind. We have to wait for five centuries to read about a new MS case, when Augusto Federico d'Este decided to describe the progress of his disease in a diary.

When doctors began to scientifically analyse illnesses, MS was among the first diseases to be studied. Drawings from autopsies performed as early as 1838 clearly show what we now recognise as MS. Then, in 1868, Jean-Martin Charcot carefully examined a young woman with a tremor of a sort he had never seen before. He noticed her other neurological problems, including

slurred speech and abnormal eye movements, and compared them to other patients he had seen. When she died, he examined her brain and found the characteristic scars or "plaques" of MS. Dr. Charcot wrote a complete description of the disease and the changes in the brain that accompany it. However, he was baffled by its cause and frustrated by its resistance to all his treatments. The three signs of MS, nystagmus, intention tremor, and telegraphic speech were called Charcot's triad. Charcot also observed cognitive changes, describing his patients as having a "marked enfeeblement of the memory" and "conceptions that formed slowly".

Scientists began to understand the pathogenesis and progression of the disease more in detail in the 20th century, when sophisticated techniques improved research tools [4]. Since the description of MS by Charcot in the nineteenth century, there has been an increasingly important need to accurately diagnose MS. Today the current diagnostic criteria for MS are McDonald's criteria, published in 2001 by a team led by Prof. Ian McDonald, and revised in 2005 and 2010 [5,6].

Despite the remarkable progress made by research on the mechanisms of MS, today there is no cure for multiple sclerosis. Treatment typically focuses on speeding up recovery from attacks, slowing down progression of the disease and managing MS symptoms.

MS is one of the most socially expensive disease. The costs of the disease are ascribable to age of onset, duration of the disease and welfare costs; indeed it hurts the most productive class of the population. As Owens said in his report "in 2012 the average costs for all privately insured patients with MS were $30,000 annually" [7].

1.1.1   Epidemiology

2.5 million people worldwide have MS, in particular 630,000 in Europe and 58,000 in Italy [8].

Typically, the onset of the disease ranges from 20 to 45 years, with an incidence peak of 25-30 years, although cases of childhood or late onset are reported. MS with childhood onset is a rare condition (<5% of all MS cases) [9].

The overall incidence rate of MS was 3.6 cases per 100,000 person-years (95% CI 3.0, 4.2) in women and 2.0 (95% CI 1.5, 2.4) in men [9]. Recently the gender ratio has become higher than in the past, and is indirectly a marker of an increased incidence of MS in women. This might reflect the changes in lifestyle, i.e., smoking, attitude, stress, obesity, use of oral contraceptives, later pregnancy, that could play an important role in MS aetiology [1]. Another reason for this gender prevalence might be found in the dimorphism between the two sexes, associated with genetic and hormonal factors, as well as in the intrinsic biological differences of the immune and nervous system [10].

MS is more common in high income countries, and it presents a heterogeneous prevalence in the world; precisely, in Europe and North America we find the highest incidence with 108 cases/100,000 and 140 cases/100,000, respectively, while the lowest incidence is in Sub-Saharan Africa and in East Asia (~2.0 cases/100,000) [1] [Figure 1].
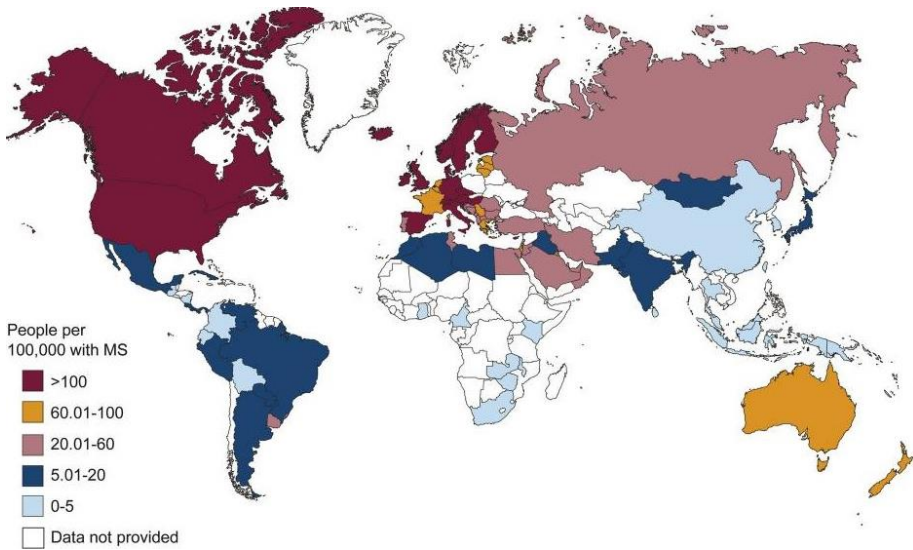
Figure 1. Global prevalence of MS in 2013 [11]

In 1975, some evidence led Kurtzke to define three different zones of global prevalence rating: high (30–80/100,000), medium (5–25/100,000) and low zones (<5/100,000). Northern United States, Canada, New Zealand, Australia, most of Northern Europe and Israel generally belong to the first group. Southern Europe, southern United States and northern Australia are the medium zones, while Asia, South America and most of Africa are included in the low zones [12]. Hence the concept of the "geographical gradient north-south". According to this idea, some genetic and environmental factors have a gradient frequency that corresponds to a latitude variation. In particular, environment, infections, smoking, exposure to sunlight and vitamin D levels were associated with MS risk. Regarding genetics, it was noticed that in high risk areas, some racial groups maintain a low prevalence of disease (Japanese and other Asians living in Great Britain, African Americans, Africans in South Africa). In addition, recent

studies have shown the correlation between the risk of MS and the place where childhood years were spent, and have underscored the impact of migrations [8].

Despite this evidence, the "gradient concept" has been currently questioned, since several exceptions have emerged [1]. For example, the idea that Italy represents a medium risk area, compared to Northern Europe, has been reviewed by epidemiological studies conducted over recent years, which have shown a prevalence of 30 to 70 cases/100,000 inhabitants, placing Italy among high risk countries [3,13]. Moreover, in our country the disease presents no reduction as the latitude diminishes from North to South. Indeed, MS has a high incidence in Sicily and Sardinia probably because of the founder effect and different genetic ancestry (in particular for Sardinia).

Given the above, further population studies are required also because the knowledge of epidemiology and natural history of MS might help to highlight the pathogenesis of the disease and lead to an effective therapy. As Simpson et al. wrote: "While classic epidemiological methods are ongoing, novel avenues for research include gene-environment interaction studies, the world of '-omic' research, and the utilization of mobile and social media tools to both access and track study populations, which means that the epidemiological discoveries of the past century may be but a glimpse of our understanding in the next few decades" [14].

1.1.2   Aetiology and pathogenesis of MS

Strong evidence underpins the fact that MS is a disease caused by different factors. Not only genetics, but also lifestyle and environmental factors predispose an individual to develop clinical MS and might act in the subclinical phase, before the onset.

Genetic factors

Epidemiological analyses have revealed that MS cluster in families, determined by genetic factors. The familial recurrence rate is about 20% and the reduction in risk changes from 3% to 1%, if we consider first degree relatives, in comparison with second and third degree relatives [2] [Figure 2].
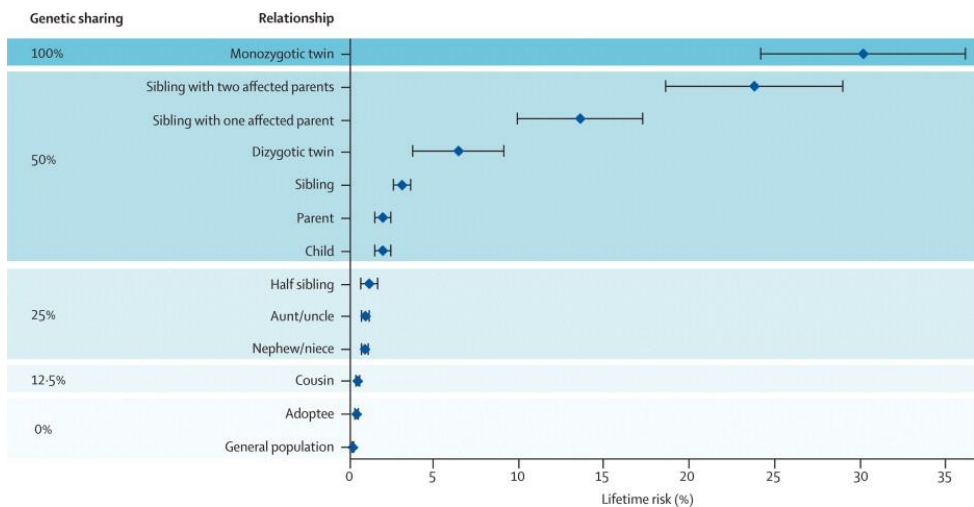
Figure 2. Risk of MS recurrence in families [2]

Analysing families with MS, it is rare to find more than 3 or 4 cases, and extended families with many affected subjects are uncommon [15].

Over the past 10 years, an international collaborative project organised by the International Multiple Sclerosis Genetics Consortium (IMSGC) – identified >100 genetic risk factors for MS. Ongoing projects increase this number to approximately 200 common risk variants and are expected to reach the limit of common genetic risk variants that are realistically detectable at this time. It was demonstrated that these variants have

enriched immunological function, and overlap with other autoimmune but not neurological disorders [16].

Genetic susceptibility to the disease is probably multifactorial, linked to many genes, and does not follow a Mendelian inheritance. Since the 1970s, the association has been known between MS and genes encoding leucocyte antigen (HLA) contained within the MHC, but it is difficult to identify the variant that drives these correlations. Indeed, this gene region is characterised by extreme polymorphism and extensive linkage disequilibrium.

In particular, HLA class II and I genes are the most relevant as modifier of MS risk [Table 1]. The *HLA-DRB1*15:01* lead to the haplotype that is mainly associated with the development of the disease. Instead, it has been confirmed that the *HLA-A*02:01* allele has a protective effect [17].

	OR	RAF	−log(p)
HLA-DRB1*15:01	3·10	13·3%	320·0
HLA-A*02:01	1·37†	25·9%	22·0
HLA-DRB1*03:01–DQB1*02:01	1·26	14·6%	9·4
HLA-DRB1*13:03–DQB1*03:01	2·40	0·9%	10·9
rs9277535_G‡	1·28	24·9%	26·4

The statistical significance for each allele is conditional on the alleles above it in the table. RAF refers to allele frequency in the UK population. −log(p) refers to the negative log of the p value in the combined analysis reported by the International Multiple Sclerosis Genetics Consortium[27] and the Wellcome Trust Case Control Consortium 2.[27] OR=odds ratio. RAF=risk allele frequency. †This allele exerts a protective effect so the OR on the risk scale would be 0·73. ‡This single-nucleotide polymorphism is in linkage disequilibrium with *HLA-DPB1*03:01*.

Table 1. Multiple sclerosis risk alleles in MHC [19]

Moutsianas et al. 2015 not only confirmed the important role of HLA class II risk alleles (*HLA-DRB1*15:01, HLA-DRB1*13:03, HLA-DRB1*03:01, HLA-DRB1*08:01 and HLA-DQB1*03:02*) and of class I protective alleles (*HLA-A*02:01,HLA-B*44:02, HLA-B*38:01* and *HLA-B*55:01*) in MS aetiology, but also described interactions involving pairs of class II alleles, such as *HLA-DQA1*01:01–HLA-DRB1*15:01* and *HLA-DQB1*03:01–HLA-DQB1*03:02* [18].

As mentioned, genome-wide association studies (GWAS) have identified ~100 non-HLA single nucleotide polymorphisms (SNPs) that are mildly associated with MS susceptibility. Independent evidence has underscored how SNP rs6897932 from the IL7R gene plays an important role in the disease risk, and new variants in specific genes involved in MS inflammatory pathways were later identified. In most cases, the role of these genes is to regulate lymphocyte function, particularly in the activation and proliferation phase of the T-cell population. They can also be involved in the cytokine cascade, such as CXCR5, IL2RA, IL7R, IL7, IL12RB1, IL22RA2, IL12A, IL12B, IRF8, TNFRSF1A, TNFRSF14, and TNFSF14. They can act as regulatory molecules, such as CD37, CD40, CD58, CD80, CD86, CLECL1, and receptor molecules implicated in signal transduction processes [17,19]. Recent studies have attributed a role in MS aetiology to the mutation with loss of function of the enzyme CYP27B1, which is responsible for the conversion of 25-hydroxyvitamin D into 1,25-dihydroxyvitamin D. This confirms the role of vitamin D in determining the disease [20].

The association between KIR (killer-immunoglobulin-like receptors) and MS have recently been studied. They are highly polymorphic receptors expressed by natural killer cells and regulate cell killing and the cytokine response. Since many HLA class I molecules act as ligands for KIR, probably some associations observed between KIR and MS susceptibility actually result from their function. They can also be expressed by CD4+ and their alterations may affect the production of specific antibodies, explaining their role in the pathogenesis of MS. In particular, MS susceptibility increases if KIR are absent, while the presence of different types of KIR may be a

protective factor. Hence the possible role of NKs as players in the pathogenesis of MS [21].

Since the majority of associations related to variants are common in the general population, we can talk about the presence of "cumulative genetic risk" in MS, defined by MS genetic burden (MSGB). Gourraud et al. 2012 defined the MSGB as "*a score based on an algorithm that incorporates each risk variant for a given individual and weighs each SNP according to its reported effect size. The MSGB score quantitatively represents the known MS genetic risk for each individual.*" The MSGB score gives an opportunity to analyse cases in the context of whole populations as well as the possibility of personalised care [22].

Epigenetic factors

Extensive data on the involvement of epigenetic mechanisms in the onset of MS have been collected in the past few years. Elements supporting this hypothesis include the probable maternal transmission of the disease, environmental risk factors, such as Vitamin D deficiency, smoking and Epstein Barr virus, which can induce epigenetic modifications.

Regarding MS, twin studies revealed epigenetic differences, particularly in the DNA methylation and acetylation pattern and in the female predominant. This gender influence suggests a possible epigenetic effect on specific genes located in the X chromosome. Moreover, the mother transmits *HLA-DRB1*15* allele more than father, underling a parent-of origin effect [23].

Some evidence showed alterations in the promoter methylation status of genes, in PBMC and/or brain samples, though histone acetylation seems to be the most crucial event in MS aetiology. Moreover some data described an

increase or decrease in acetylation, methylation and citrullation of genes involved in the inflammatory response and demyelination process [24,25]. At the post-transcriptional level, micro-RNA (miRNA) control epigenetics through the RNA-induced silencing complex (RISC) and long non-coding RNA (lncRNA). miRNA-RISC modulate cellular pathways, such as apoptosis, proliferation and differentiation [26], while lncRNA regulate gene expression at the epigenetic, transcriptional, and post-transcriptional level in cellular homeostasis [27] . Table 2 summarises the major epigenetic mechanisms involved in the pathogenesis of MS.

Factor	Epigenetic regulation	Proposed mechanism of action	Factor	Epigenetic regulation	Proposed mechanism of action
MS patients			*Experimental autoimmune encephalomyelitis (EAE) studies*		
SHP-1	Increased methylation	Increased proinflammatory gene expression (Kumagai et al. 2012; Christophi et al. 2008)	T cell factor 1	Increased acetylation/ methylation	Th17 differentiation (Ma et al. 2011)
HERV-W	Decreased methylation	Increased inflammation, reduced myelination (Kremer et al. 2013; Perron and Lang 2010)	IFN-γ/IL-17A	Decreased methylation	Increased IFN-γ, IL-17 production (Guan et al. 2011)
PAD2	Decreased methylation	Increased myelin citrullination, myelin breakdown, anti-myelin immunity (Calabrese et al. 2012)	IFN-γ/IL-17A, IL-4/ Foxp3	Increased methylation, demethylation	Increased Th1, decreased Th2 response (Guan et al. 2011)
			IL-17A	Increased methylation	Decreased Th17 differentiation (Coquet et al. 2013)
PAD4	Increased histone citrullination	Reduced myelin production (Mastronardi et al. 2006)	IRF1	Decreased acetylation	Increased Th17 differentiation (Yang et al. 2013)
miR-17, miR-20a	Decreased expression	Increased Th1 and Th17 differentiation (Cox et al. 2010)	Foxp3	Increased methylation	Decreased regulatory T cell activity (Liu et al. 2010)
miR-155, miR-326, miR-34a	Increased expression	Decreased CD47, increased macrophage mediated myelin phagocytosis (Junker et al. 2009)	miR-155	Increased expression	Increased Th17 differentiation (Escobar et al. 2013)
miR-155, miR-338	Increased expression	Reduced neurosteroid production (Noorbakhsh et al. 2011)	miR-326	Increased expression	Increased Th17 differentiation (Du et al. 2009)

Table 2. Epigenetic mechanisms in MS [24]

As Fenoglio et al. 2012, wrote: "*Preliminary studies have started to analyze the possible genetic contribution of miRNA loci variability in MS, suggesting that the research on miRNAs has finally begun to be approached in a more comprehensive and definitive manner.*" [28]

In recent years, researchers have found a large number of dysregulated miRNAs in serum, plasma and PBMC from MS patients, as well as in brain lesions. At times the miRNA profile showed similarities in both active lesions and in blood, such as the upregulation of miR-326, but the alteration can also be different sometimes. This happens for miR-323, which showed an upregulation in whole blood, active brain lesions, and T-reg cells, but not in the serum of MS patients [29].

Interestingly, growing evidence demonstrates that also lncRNAs get dragged into different cell pathways as, for example, in the regulation of immune cell activity. Regarding this issue, Spurlock et al. identified specific expression patterns in lymphocyte lineages [30]. Since it is a known fact that these cells are involved in the pathogenesis of MS, lncRNAs may control important steps of their imbalanced activity [31,32], suggesting that they could be associated with progression of the disease.

Environmental factors

Next to genetic factors, environment and lifestyle can influence the pathogenic pathways of MS and lead to the clinical disease. These factors affect the immune system to trigger and/or perpetuate the disease. [Figure 3]

The importance of environmental factors is demonstrated not only by the epidemiological characteristics of the geographical distribution of MS, but also by monozygotic twin studies. These indicate, with a concordance frequency of about 30%, that the aetiology of the disease cannot be explained only considering genetics.

In addition, several epidemiological migration studies have shown that populations tend to maintain the risk of developing MS in the area of origin

when migration occurs after the 15th year of life, while on the other hand they acquire the risk of the new country of residence when migration occurs before the 15th year of life [33,34]. These data suggest, therefore, the presence of environmental factors that affect the subject during childhood or early adolescence.
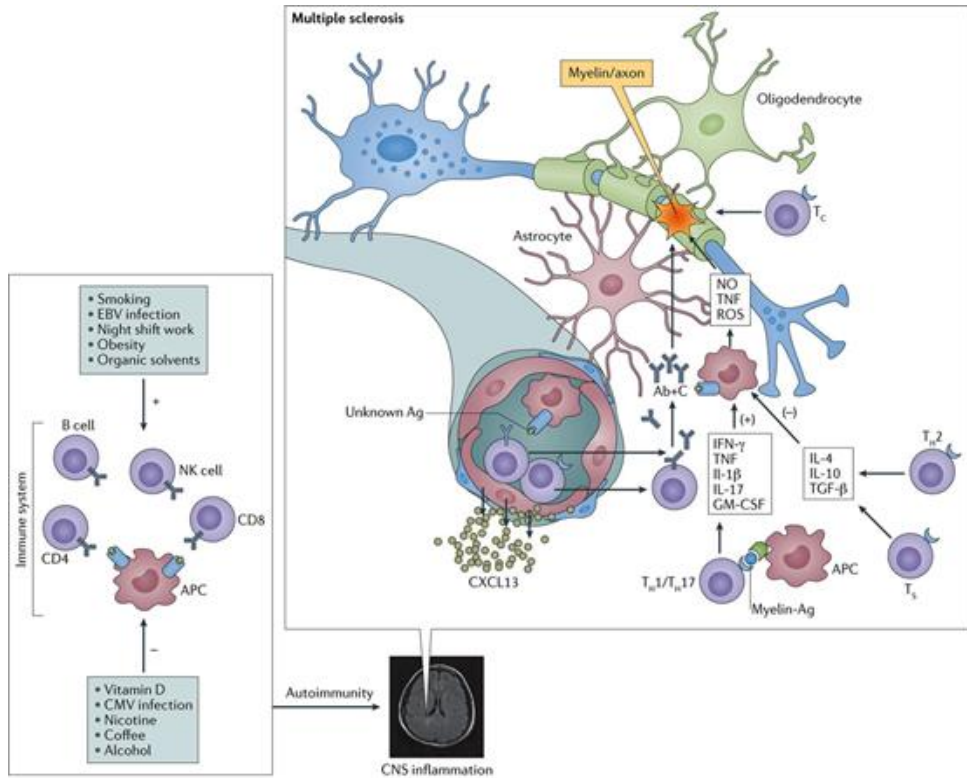
Figure 3. Environmental factors and the immune system [35]

Hence, the period of exposure to certain factors is also significant. Recent migration studies also confirm the evidence of a risk attributable to

environmental factors, without recognising a precise cut-off age for their influence [33].

Other non-genetic risk factors include smoking, Epstein-Barr virus (EBV) infections, vitamin D levels and sun exposure, obesity during adolescence and diet [Table 1] [36,37].

Factor	OR	HLA gene interaction	Combined OR (nongenetic factor + HLA allele)	Effect during adolescence	Immune system implied	Level of evidence
Smoking	~1.6	Yes	14	No	Yes	+++
EBV infection (seropositivity)	~3.6	Yes	~15	Yes	Yes	+++
Vitamin D level <50 nM	~1.4	No	NA	Probably	Yes	+++
Adolescent obesity (BMI >27 at age 20 years)	~2	Yes	~15	Yes	Yes	+++
CMV infection (seropositivity)	0.7	No	NA	Unknown	Yes	++
Night work	~1.7	No	NA	Yes	Yes	++
Low sun exposure	~2	No	NA	Probably	Yes	++
Infectious mononucleosis	~2	Yes	7	Yes	Yes	++
Passive smoking	~1.3	Yes	6	No	Yes	+
Organic solvent exposure	~1.5	Unknown	Unknown	Unknown	Unknown	+
Oral tobacco/nicotine	0.5	No	NA	Unknown	Yes	+
Alcohol	~0.6	No	NA	Unknown	Yes	+
Coffee	~0.7	No	NA	Unknown	Yes	+

Level of evidence for a role of a particular lifestyle or environmental factors in MS is not easy to define. Large prospective studies are, with few exceptions, rare in MS. CMV, cytomegalovirus; EBV, Epstein–Barr virus; HLA, human leukocyte antigen; MS, multiple sclerosis; NA, not applicable; OR, odds ratio; +++, high level of evidence: drawn from large prospective studies or if a case–control observation is supported by Mendelian randomization studies; ++, Case–control observations, if replicated and/or supported by independent methods; +, Non-replicated observations (included to enable further observations).

Table 3. Environmental risk factors for MS [35]

There is a positive correlation between smoking or passive exposure and the increased risk of MS. Interestingly, some studies underlined that smoking is also associated with the development of neutralising antibodies against some MS therapies, such as Natalizumab and IFN-β [38,39]. The hypothetical mechanism of action of smoking is described in [Figure 4].

Smoking promotes the activation of inflammation in the lungs, and leads to an altered post-translational event of inflammatory molecules. These events cause activation of resident CD4+ CNS-antigen-specific T cells through *HLA-DRB1*15:01* proteins. This hypothesis is supported by some experiments in experimental autoimmune encephalomyelitis (EAE), an MS animal model [37].
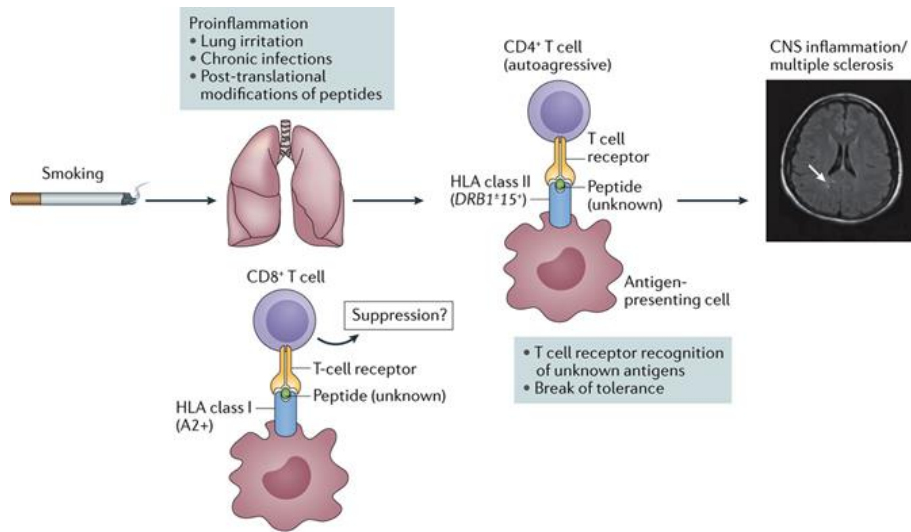
Figure 4. Mechanisms of smoking-associated processes in MS [35].

The hypothesis according to which some bacterial or viral agent can be the cause of MS is particularly important. Currently, it is unclear whether it is a general infection or a specific agent that can trigger the disease or whether tissue damage is a direct or indirect effect of the infection. Anyway, some infective agents were isolated from MS patient serum and CSF, and some of them were also found in demyelinating plaques: *Chlamydia pneumonie*, *Mycoplasma pneumonie*, EBV, HHV-6, Coronavirus [40]. Among these, some evidence suggests that the most interesting candidate is EBV. Indeed,

MS patients have higher levels of EBV nuclear antigen1 antibodies (EBNA1), and Handel et al. 2010 demonstrated that people who had EBV infection have an >2-fold increased risk of MS [41]. Furthermore, there are some interactions between *HLA DRB*15:01* and the mononucleosis agent [42]. Overall, these observations confirm a bond between EBV and MS, although its role needs to be clarified.

In recent years, several studies have focused on microbiota and found that different strains of bacteria differ in terms of capability to cause neuroinflammation. Hence the hypothesis that the type and distribution of gut bacteria could be considered as a risk factor for MS [37].

Epidemiological studies determined ultraviolet radiation (UVR) and vitamin D as protective factors against the onset of MS. This is linked with a latitude-dependent variation in the incidence and prevalence of MS. On the one hand sun exposure reduces peripheral inflammation, and on the other hand high levels of vitamin D, especially in adolescence, are protective against axonal injury. Though the physiological mechanism has not been understood, people presenting MS with high levels of vitamin D have a reduction in axonal damage and low levels of neurofilaments in biological fluids [43]. Therefore, considered together, these factors are able to mitigate a future MS risk.

Growing evidence underpins the fact that obesity, in particular during adolescence, can play a role in the pathogenesis of MS. Indeed, adolescence seems to be the crucial period in which weight affects the develop of MS. Munger underscored a strong correlation between Body Mass Index (BMI) > 27 [44], and Hedstrom 2014 too described an interaction between BMI-HLA variants [45]. Inflammatory processes are involved, since obesity promotes the production of cytokines and leptin, with a reduction in T_{reg} cells and vitamin D bioavailability. All of these events might enhance the

activation of adaptive autoreactive immune cells and can trigger neuroinflammatory activity [37].

Pathogenesis of multiple sclerosis

The exact cause of multiple sclerosis is still unknown, but to date it is clear that different factors contribute to the onset of disease processes, such as neuroinflammation, demyelination, gliosis and neurodegeneration.

Without a main risk factor, we do not know if multiple sclerosis is triggered in CNS or in peripheral regions of the body. In the most acclaimed model, a peptide molecule from a foreign antigen closely resembling part of a self-protein is presented to T cells (*molecular mimicry event*) [46], or to antigen-presenting cells (APC) *(bystander activation)* [47] that are consequently activated, and trigger an autoimmune reaction. Therefore, in case of reduced regulatory T (T_{reg}) function and/or if lymphocytes B and T show resistance to suppressive mechanisms, CNS-directed autoreactive B and T cells can be activated. The inflammation processes start with the differentiation of CD8$^+$ T cells into CD4$^+$ T helper cells (T_H1 and T_H17 cells) and with the following T, B and immune cell infiltration into the CNS. Exposure to specific environmental factors or genetic predisposition contribute to these events [Figure 5] [48].

As mentioned, autoreactive T cells escape central tolerance in the thymus. In healthy subjects, T cells with high affinity for *self*-antigen are led to apoptosis by peripheral tolerance processes. However, autoreactive lymphocytes may be released in peripheral regions due to a stochastic phenomenon. Indeed, the thymus might produce a limited number of APCs, or these cells might have a reduced capability to interact and induce T cell

apoptosis. Consistently with this theory, some studies demonstrated that these autoreactive cells overexpress β-arrestin 1, a key promoter of naïve activated CD4$^+$ T cell survival [49]. In addition, the presence of specific variants in HLA genes, linked with MS susceptibility, could help to break the tolerance.
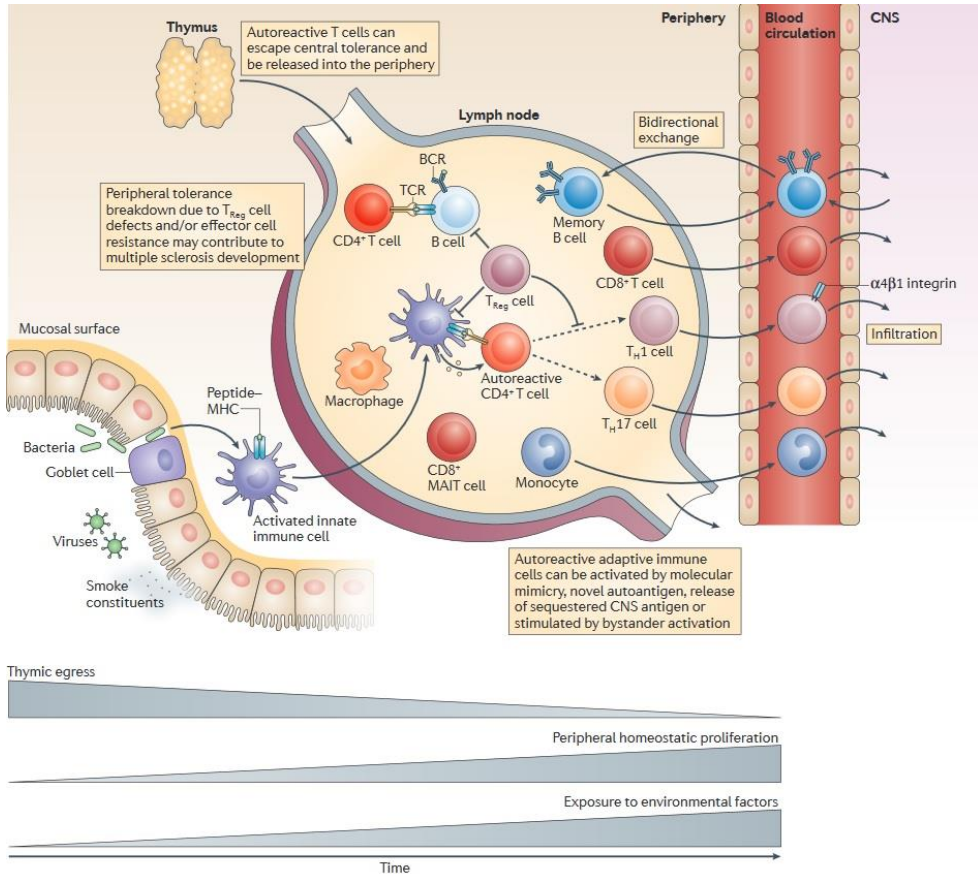
Figure 5. Immune system dysregulation outside the CNS [48]

In MS, T cells are autoreactive against the myelin component, in particular myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) , myelin associated glycoprotein (MAG) and proteolipid protein (PLP) [50].

Although these are the main protein candidates, other antigens seem to be implicated in the early disease processes. For instance, αB crystalline protein is described as preventing suppression of the inflammation [51], and neurofascin as mediator of axonal injury [52]. During the initial stage of the disease, the immune response is mainly focused on an immuno-dominant epitope and clonal lymphocytic populations mediate it. Subsequently, a phenomenon known as *epitope spreading* occurs with an increasing number of antigen molecules resulting from initial tissue damage being exposed to the extracellular environment, and becoming the target of the autoimmune response [53].

As a secondary phenomenon, there is the infiltration of autoreactive lymphocytes into the CNS. T cells migrate across the blood-brain barrier (BBB) and the CSF-barrier. Though the CNS is an immune-privileged site, its isolation is not absolute. Indeed, blood-derived innate immune cells might stay in ventricular, meningeal and perivascular spaces in order to activate CNS-resident T cells when there is an important inflammatory condition [54]. Perivascular astrocytes might contribute to change BBB permeability, producing VEGF and HIF-1 after inflammatory signal stimulation [55]. Since local regulatory processes fail in MS, T cells infiltrate and become localised in different regions, such as cortex and subcortical white matter, in the optic nerve and brainstem, or cluster around the corpus callosum and lateral ventricles. Here they form the so-called inflammation plaques, which are visible by MRI [8].

Lymphocyte migration through brain endothelium is a multi-step process, which involves a complex interaction between adhesion molecules (such as selectins and integrins), chemokines and proteases. In physiological conditions, chemokines are confined to the parenchymal side. After a

stimulus, they are exposed to vessels in order to facilitate lymphocytic extravasation. CCL19, CCL21 and CXCL12 seem to be mainly involved in MS pathogenesis [56]. After extravasation, T-lymphocytes have to cross the extracellular matrix, which is made up of type IV collagen. Here the metalloproteases (gelatinases A and B, collagenase) play a key role in helping T cells to penetrate into the white substance of the CNS.

In the CNS, infiltrated CD4^{+} T cells are re-activated by APC and recruit monocytes and naïve CD4^{+} T. Thus activated, these preferentially differentiate into T_H1 and T_H17 cells, secreting INFγ and cytokines, such as IL-17A. Following antigen recognition, the activated T CD4^{+} and CD8^{+} lymphocytes release cytokines (IL-1, TNF-α, IL-12, IL-17) and chemokines (IP-10, RANTES, MIP-1a). Moreover, they induce apoptotic signals and activation of other lymphocytic populations. Compared with T cells, B cells are also involved in disease progression, destroying myelin and fuelling of the inflammation fire.

Given the above, chronic inflammation is thus established [Figure 6].

Focal inflammatory response in the CNS results in destruction of myelin, oligodendrocyte death and Wallerian degeneration of the axons. This evolution of late plaque is the result of ineffective re-myelination by oligodendrocyte precursors, probably due to continuous inflammatory insult in the course of time. According to some evidence, the loss of oligodendrocytes might be due, at least partly, to apoptosis without mediation of immunity [57]. It has, therefore been hypothesised that the immune system acts as a trigger for a neurodegenerative process that can become spontaneous in time.

Key neurodegenerative processes in MS are the consequences of chronic inflammation. Indeed, they are associated with the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These phenomena cause mitochondrial injury and subsequent ER stress and energy deficiency. Since normal neuroaxonal functions require a lot of energy, in pathological conditions we have the loss of neuronal health with critical implications for the neuron. Therefore, neurodegeneration and demyelination seem to be mediated at least by oxidative damage, correlated with age. It remains to be established whether the initial damage is due to the release of pro-inflammatory cytokines by the cells involved in chronic inflammation, with the establishment of a specific pattern of microglia activation and the initiation of oxidative cascade or, alternatively, whether it is triggered by a hypothesised soluble demyelinating factor that is responsible for the initial damage, which is then maintained [58].

Moreover, in order to maintain ion homeostasis, different ion channels change their distribution following demyelination [Figure 7]. A series of buffering mechanisms are triggered to cope with this situation, but they do not suffice to guarantee neuron survival [57].
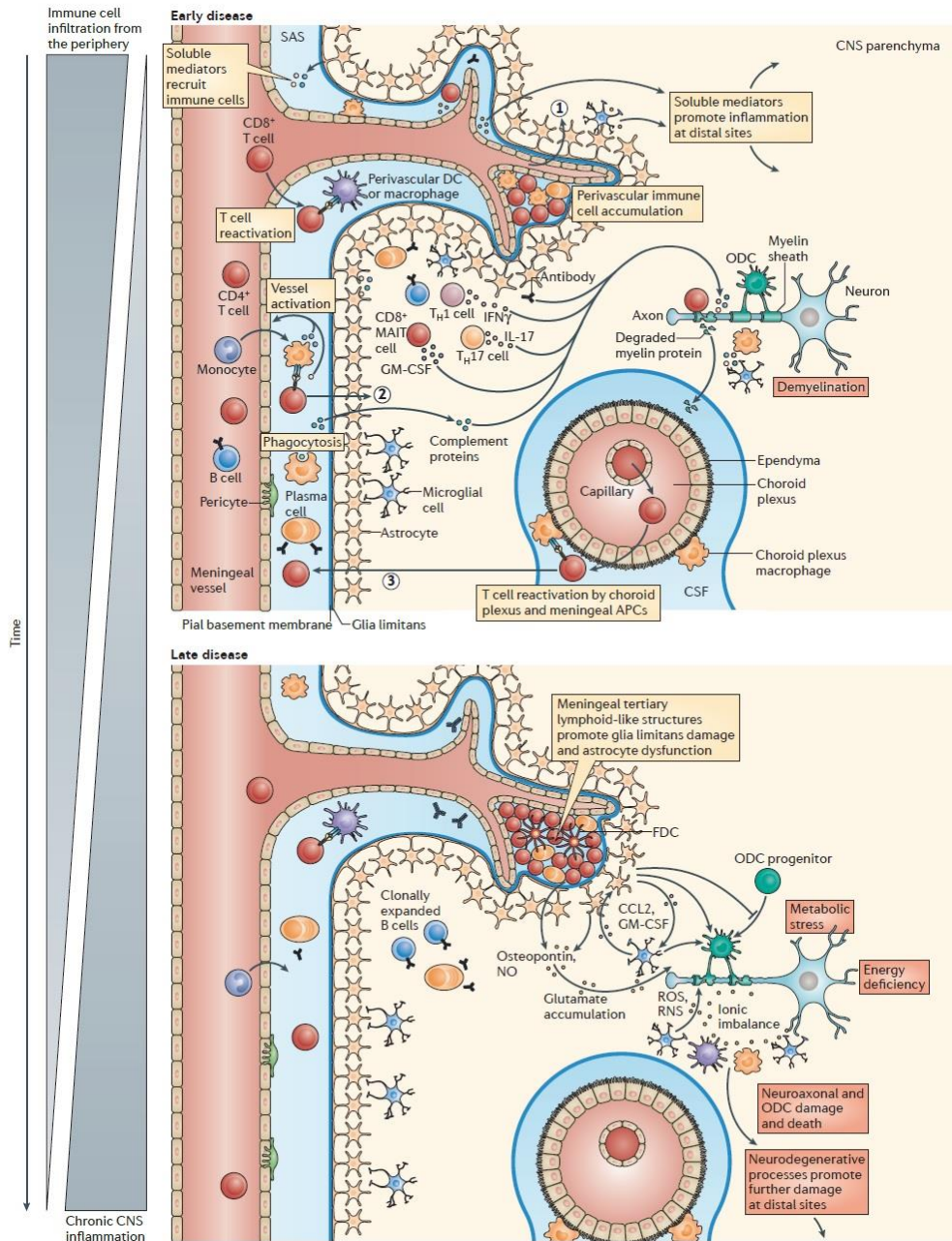
Figure 6. Immune system dysregulation in the CNS [48]
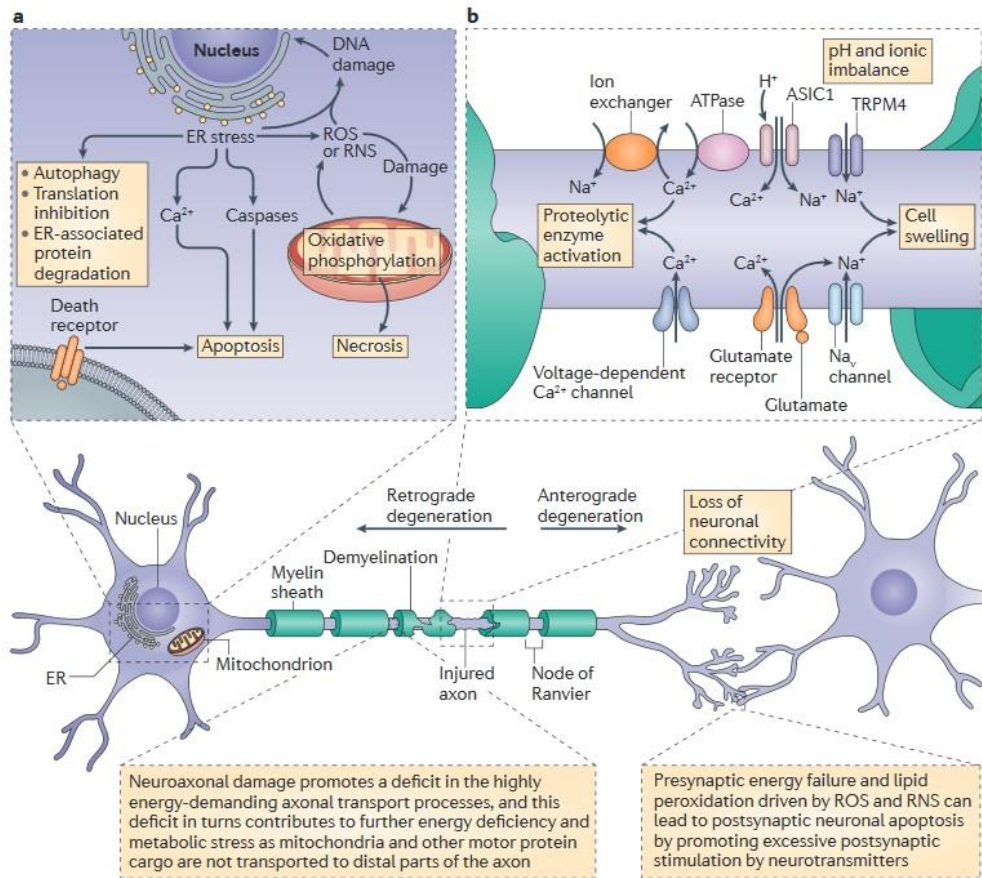
Figure 7. Key neurodegenerative processes [48]

Although neurodegeneration processes are the culmination of a cascade of inflammatory events implemented by infiltrated immune cells, they can also be sustained by cells that are already resident in the CNS. Indeed, infiltrated cells also activate CNS-resident microglia and astrocytes, and through soluble inflammatory and neurotoxic molecules they promote oligodendrocyte and neuron injury, as well as demyelinating events. In particular, astrocytes produce CCL2 and GM-CSF in response to stimulated microglia, and this leads to even further microglial recruitment. Physiologically, microglia have a neuroprotective capacity by helping to

resolve inflammation. They produce neurotrophic factors, maintain CNS homeostasis and are involved in neuroaxonal injury repair. In pathological conditions, microglia change phenotypes and their functions, and might promote tissue damage, sustaining inflammatory processes [48].

The described processes involved in MS pathogenesis are complex and, sometimes, difficult to fully understand. Though we have consistently answered many questions regarding the development of MS, important points remain to be finally clarifies in order to find an effective therapy.

1.1.3   Pathophysiology

Myelin is a fatty white substance that winds concentrically around the neural axon. Myelin function consists in isolating the nervous impulse that rapidly propagates from the cellular body along the axon, passing from one Ranvier node to the next with a saltatory conduction mode.

The main physiological effect resulting from demyelination is the prevention of efficient electrical conduction, with a reduction in action potential velocity. Depolarisation might cross the lesion but with reduced velocity, which is a feature of evoked potentials. This slow nerve conduction in the pyramidal path is probably responsible for the sense of fatigue [2]. Instead, the significant increase in sensitivity in demyelinated nerve fibres explains the rapid onset of certain clinical manifestations in MS patients and the apparent fluctuation of symptoms, in absence of relapse. An example is provided by the Uhthoff phenomenon, characterised by a decrease in visual acuity following an increase in body temperature [59]. Moreover, demyelinating axons become sensitive to temperature increase, which worsens the propagation of stimuli. A 0.5°C increase suffices to induce a block in fibre conduction with a thin or absent layer of myelin.

In the acute stages of the disease, axonal distress caused by demyelination is followed by recovery of nerve conduction (remission). During this period, the regression of oedema and peripheral inflammatory processes cause re-myelinating processes, and axons are once again able to conduct the nerve impulse and functions are restored [60]. Later, the lesion chronicises, and we can observe an evident reduction in the inflammatory component. The presence of irreversible cell damage involves constant distress of the axon. In turn, axonal damage results in regression of the symptoms and stabilisation of the neurological deficit (progressive MS), as well as a proportional degree of disability.

These phenomena, which alter conduction, are important because they are directly correlated with clinical symptoms in MS [61].

1.1.4 Clinical and diagnostic aspects

Multiple sclerosis is a heterogeneous disease characterised by different clinical manifestations, which involve the visual, motor, sensory and autonomic systems [Table 4].

	Symptoms	Signs
Cerebrum	Cognitive impairment, Hemisensory and motor, Affective, epilepsy and focal cortical deficits (rare)	Deficits in attention, reasoning and executive function (early), dementia (late) Upper motor neuron signs
Optic Nerve	Unilateral painful loss of vision	Scotoma, reduced visual acuity, color vision and relative afferent pupillary effect
Cerebellum and cerebellar pathway	Tremor Clumsiness and poor balance	Postural and action tremor, dysarthria Limb incoordination and gait ataxia
Brainstem	Diplopia, oscillopsia, vertigo, impaired swallowing, impaired speech and emotional lability, paroxisomal symptoms	Nystagmus, internuclear and other complex ophthalmoplegias Dysartria Pseudobulbar palsy
Spinal cord	Weakness, stiffness and painful spasms, bladder	Upper motor neuron signs, spasticity

	dysfunction, erectile impotence, constipation
Other	Pain, fatigue, temperature sensitivity and exercise intolerance

Table 4 Symptoms and sign of MS [2]

The progress of MS [Figure 8] is highly variable, and is classified as described below.

➢ *Clinically isolated syndrome (CIS-MS):* characterised by the appearance of a neurological episode (symptom or sign), which lasts for at least 24 hours and is due to a demyelinating process of the central nervous system. People with a CIS will not necessarily develop MS.

➢ *Relapsing-remitting MS (RR-MS)*: more common (85-90% of cases), it is characterised by episodes of neurological dysfunction without either infection or fever (relapses), followed by periods of partial or complete remission. Relapses are more frequent in the early stages of the disease. The typical symptoms observed in this form are optic neuritis (also occurring in 20% of cases), sensory deficits or cerebellar dysfunction.

➢ *Secondary progressive MS (SP-MS):* after an initial phase of RR-MS, disease progression accumulates clinical impairment, either without or with relapses. SP-MS is characterised by a persistent disability that progresses gradually over time. In this form the most commonly observed symptoms are paresis, spasticity, and gait ataxia.

➢ *Primary progressive MS (PP-MS):* typically presents at an older age. It is characterised by a worsening of neurological functions since the appearance of the first symptoms, without actual relapses or

remissions. The course is insidious with a slow increase in neurological disability.
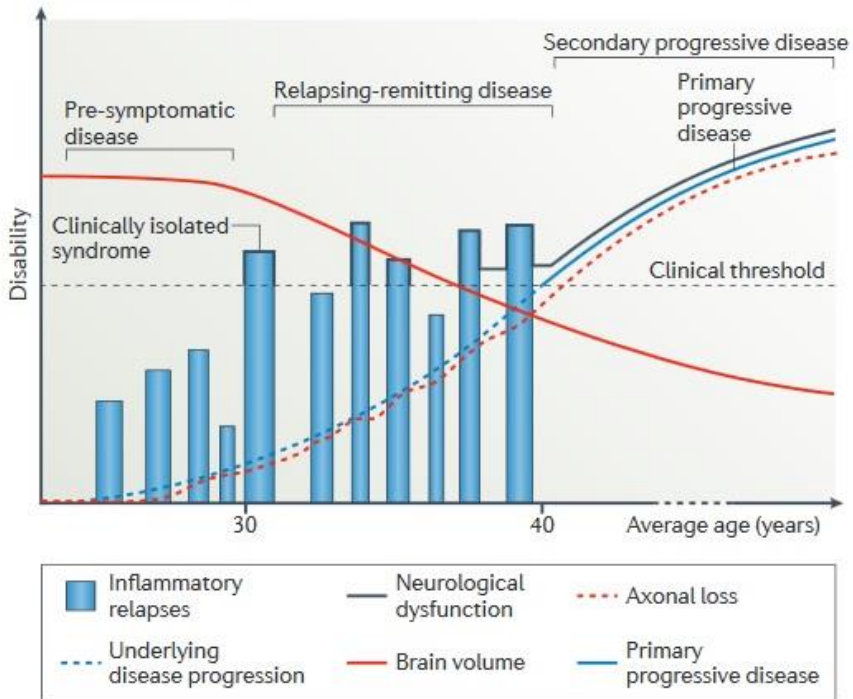
Figure 8. Progress of MS [2]

The diagnosis of MS is based on diagnostic criteria recently reviewed and published by the International Committee for Multiple Sclerosis (McDonald's Criteria) in 2010 [Figure 9] [6,62]. The diagnosis is worded as dissemination of lesions in space (DIS) and time (DIT). Magnetic resonance (MRI) plays a key role in defining the picture of MS, but the examination of CSF and neurophysiologic analysis can help as well. A standardised MRI protocol has been proposed by MAGNIMS for clinical practice [63].

Panel 2: McDonald 2010 diagnostic criteria for multiple sclerosis, with bullet points showing additional evidence required

At least two attacks with objective clinical evidence of at least two lesions
None

At least two attacks with objective clinical evidence of one lesion
Dissemination in space shown by:
- At least one T2 lesion in at least two of four areas of the CNS typically affected in demyelination: periventricular, juxtacortical, infratentorial, and spinal cord
- Further clinical attack at a different site

One attack with objective clinical evidence of at least two lesions
Dissemination in time shown by:
- Simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesions on a single scan or a new T2 and/or gadolinium-enhancing lesion on follow-up MRI
- Second clinical attack

One attack with objective clinical evidence of one lesion
Dissemination in space shown by:
- At least one T2 lesion in at least two of four areas of the CNS typically affected in demyelination: periventricular, juxtacortical, infratentorial, and spinal cord
- Second clinical attack at a different site
Dissemination in time shown by:
- Simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesions on a single scan or a new T2 and/or gadolinium-enhancing lesion on follow-up MRI
- Second clinical attack

1 year of disease progression (retrospectively or prospectively determined)
Presence of two of:
- At least one T2 brain lesion in at least one multiple sclerosis-characteristic region: periventricular, juxtacortical, or infratentorial
- At least two T2 spinal cord lesions
- Positive CSF (at least two oligoclonal bands not present in serum, elevated IgG index, or both)

Modified from Polman and colleagues.[1]

Figure 9 McDonald's criteria for MS

Brain MRI usually reveals multifocal T2-hyperintense white matter lesions in juxtacortical, infratentorial and periventricular regions. Instead, in T1-weighted images, lesions show hypointensity (Black holes) [Figure 10].
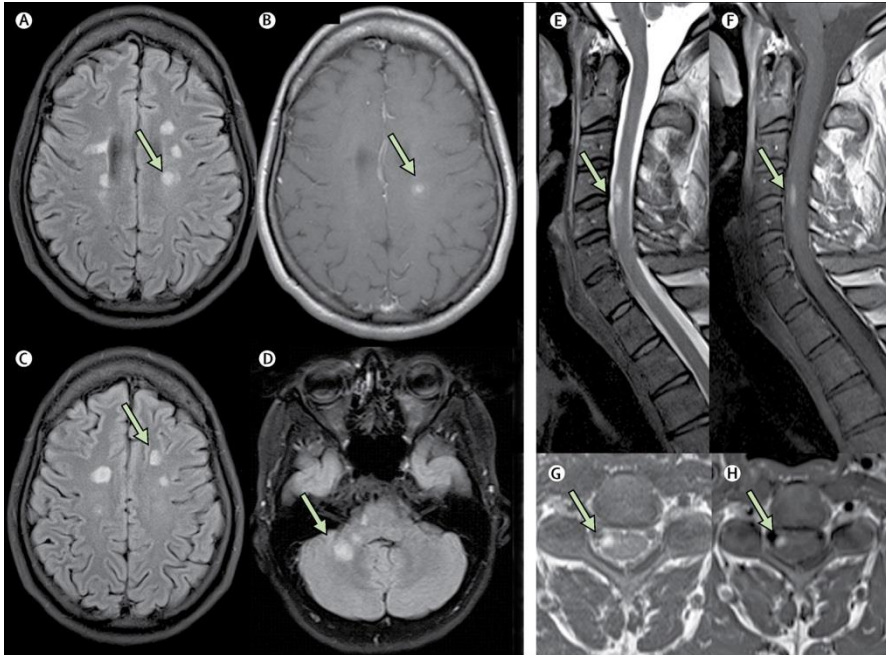
Figure 10. Typical MS lesions in the brain and spinal cord. Arrows indicate the lesions. (A) periventricular lesions. (B) periventricular lesions with contrast enhancement of one lesion. (C) juxtacortical lesions. (D) infratentorial lesions. Sagittal (E,F) and axial (G,H) scans with a cervical spinal cord lesion [64].

Sometimes, most patients need supportive evidence before an MS diagnosis, such CSF analysis. This includes white cell count (<25 cells per cm^3, predominantly lymphocytes) and protein (<1 g/L), elevated IgG index and the absence of IgG oligoclonal bands in serum (Pattern 2). Oligoclonal bands (OCBs), revealed using isoelectric focusing (IEF), are found in 90% of MS cases, but are not specific. Indeed, they are also present in other neuroinflammatory disorders. Four patterns are defined [Figure 11], namely
Pattern 1: normal polyclonal IgG distribution in CSF and in serum;
Pattern 2: OCBs in CSF and absence in serum; typical in MS;

Pattern 3: OCBs in both, CSF and serum; they indicate acute inflammatory processes in the CNS, such as encephalitis, with an important systemic component;

Pattern 4: also called "mirror pattern", presents OCBs in CSF and serum. Serum bands spread passively in the CSF compartment [65].
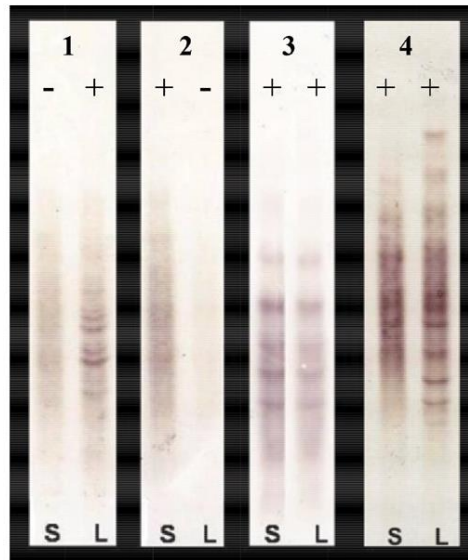
Figure 11. Oligoclonal band patterns [65]

Evoked potential in auditory, sensory or visual pathways, analysed by neurophysiological tests, allow to identify clinically silent lesions through dissemination in space, thus contributing to a diagnosis of MS. They can also be suggestive of demyelination, but with low specificity [64].

Though we have good and easily applicable criteria for MS diagnosis, they are invasive and expensive. Moreover, differential diagnosis is difficult to perform today. For these reasons, further studies are required to identify more specific and sensible biomarkers.

1.1.5   Body fluid biomarkers for multiple sclerosis

The diagnosis of multiple sclerosis, as previously described, is based on exclusion. It considers clinical evaluation and MRI analysis but, though they are precious, they have low specificity in the earliest stage. Hence, research has focused on CSF and blood as a source of dynamic, accessible and cost-effective biomarkers. Moreover, body fluid biomarkers could be useful for a differential diagnosis, given the clinical heterogeneity of demyelinating diseases [Figure 12].
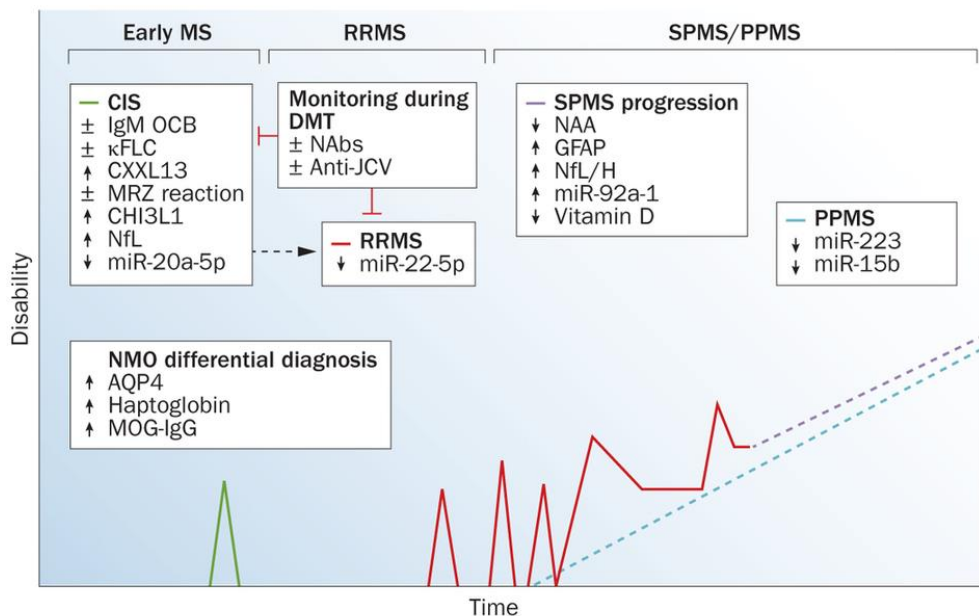
Figure 12. Biomarkers in different stages of MS [66]

Inflammatory and immunological markers

IgG-OCBs [67], IgM-OCBs [68], IgG directed against neurotropic viruses [69] and the increase in chitinase-3-like protein 1 (CHI3L1) [70] are the strongest

inflammation markers to identify CIS converters and support early MS diagnosis.

Plasma cells, besides immunoglobulin, also secrete immunoglobulin free light chains (FLCs), which can be detected in serum and CSF. There are two types of FLC, precisely kappa FLCs (k-FLC) and lambda (λ-FLC). Lymphocytes usually produce a small amount of excess FLCs over the heavy ones. These chains, which do not combine to form complete immunoglobulins, are released into blood, CSF and urine. Some evidence indicated high levels of k-FLC as a potential biomarker of CIS and RR-MS [71]. FLC have the advantage of being easily detectable by an automated nephelometric method [72].

In a systems immunology approach that compares multiple immunomodulatory treatments, Dooley et al. have demonstrated a unique B cell pathway, including B cell activation factor (BAFF) and transitional B cells, as shared across treatments [73]. These data, along with those from other recent studies of individual treatments, indicate the key role for B cells not only in the pathogenesis but also in the treatment of MS [74]. In this framework, recent studies have reported higher CSF levels of B-cell-attracting C-X-C motif chemokine 13 (CXCL13) in CIS converters than in non-converters [75]. However, CXCL13 levels are increased also in other inflammatory diseases of the CNS and lacks specificity.

Among useful markers to discriminate MS and *neuromielitis optica* (NMO), we find haptoglobin, an acute phase protein produced and secreted by the liver when serum contains anti-myelin oligodendrocytes glycoprotein IgG (MOG-IgG) and anti-aquaporin 4 IgG. In particular, the discovery of these molecules in the serum of NMO patients allowed to define this disease as pathophysiologically distinct from MS [66].

Axonal damage markers

In recent years, research on biomarkers has focused on neurodegenerative markers. In particular, in MS neurofilaments, glial fibrillary acidic protein (GFAP) and N-acetyl aspartate (NAA) play a key role as axonal damage markers.

Neurofilaments are the major components of the cytoskeleton axon. They consist of 3 different chains, namely light (NfL), intermediate and heavy (NfH). Following an axonal injury, these chains can be released in blood and CSF circulation and, in peripheral regions, and reflect neurodegenerative processes in the CNS. Indeed, high CSF and serum levels of Nf were found in patients with different neurological diseases [76,77].

In MS, elevated neurofilaments are good candidates as axonal damage biomarkers [78]. Their levels in biological fluids precede global brain atrophy in MRI and are correlated with disability [79]. Moreover, CSF NfL seem to be a promising prognostic biomarker for CIS conversion [80]. However, more extensive validation in a large cohort of patients is required because their prognostic value seems limited in individual patients [66].

GFAP is a protein of the cytoskeleton of astrocytes. Increased levels of GFAP were measured in a progressive form of MS, and were associated with disability [81,82].

NAA is the second most concentrated molecule in the brain after the amino acid glutamate. Assessed using proton magnetic resonance spectroscopy, it has a high pathological specificity for axonal density. Its levels were low in CSF from SP-MS patients, compared with RR-MS and CIS. NAA is negatively correlated with disability and positively associated with brain

atrophy [66,83]. Therefore, NNA could be a marker of neurodegenerative processes, especially in the progressive form pf MS.

Other markers

Non-coding RNA, such as microRNA (miRNA) and long non-coding RNA (lncRNA), are being intensively investigated as a new class of biomarkers. They are involved in T-cell regulation and in other inflammatory pathways.

An involvement of microRNA (miRNA) in MS pathogenesis has been extensively established [84], with particular regard to the dysregulation observed in circulating miRNA levels in CSF and serum from MS patients, compared with controls. In particular, some miRNAs appeared to be specifically dysregulated in serum or plasma of progressive patients [85–87]. This aspect confers miRNAs the potential to become a promising biomarker of disease progression or of response to therapy as with other diseases, such as cancer, where the employment of miRNAs as a new diagnostic tool is about to become an actual fact. However, the sample sizes of miRNA studies published are very small and a replicate in larger population is required.

LncRNA are abundant in the CNS, and it has been hypothesised that brain complexity requires a large number of regulatory RNAs. Under this assumption, some lncRNA involved in this context have been identified and their dysregulation has been linked to neurological disorders, such as Huntington's Disease, Alzheimer's Disease and psychiatric disorders [88–90].

To date there is little evidence of lncRNA in MS; recently, three lncRNA have been identified as deregulated in the serum of RR-MS patients, compared to controls, precisely *nuclear paraspeckle assembly transcript 1* (*NEAT1*), *taurine upregulated 1* (*TUG1*), and *7SK small nuclear* (*RN7SK RNA*) [91].

Conversely, Zhang et al. identified a subset of dysregulated lncRNA in peripheral blood mononuclear cells (PBMCs), by microarray analysis, in a population consisting of 26 MS patients [92].

Given that, this thesis investigated the role of lncRNA in MS pathogenesis by using a comprehensive methodological approach in order to achieve reliable results.

Dysregulation in lncRNA and miRNA levels detected in biological fluids could be a new source of MS biomarkers. They could be helpful for disease diagnosis, prognosis and identification of clinical subtypes, thereby aiding therapeutic decisions or the monitoring of therapeutic effects. The discovery of MS biomarkers should greatly improve the diagnosis and management of MS and, in this context, miRNAs and lncRNAs could have great value for the research of new therapeutic targets [28].

1.2 Long non-coding RNA (lncRNA)

Until recently, the main role of RNA was thought to be that of a messenger that transfers information from DNA to proteins. The discovery of different RNA subtypes has changed the scene.

More than 98% of human RNA is not translated into proteins, and 50-70% of this 98% is represented by introns. The idea that the genome exercises its function only through classical genes and proteins seems overly simplified, since a notable part of the genetic code is transcribed into RNA that is not converted into proteins. Many studies, in the past, focused on protein-encoding genes, although most of the human genome is represented by non-coding DNA, considered, in the past, as "junk" or as interfering with normal functions. Recent data have shown that this part of the genome is important and is involved both in physiological processes and in tissue homeostasis [93–95]. This idea is also supported by the point of view of evolution. Indeed, protein encoding genes have remained relatively stable, while the number of non-coding transcripts has increased considerably, in parallel with the complexity of the organism [96].

The genome has at least 10,000 transcripts with a low or no protein-coding potential, and a length of more than 200 nucleotides [97]. They are called long non-coding RNA (lncRNA). These molecules share some common features with microRNA (miRNA), as they can be spliced, capped and polyadenylated [98]. They regulate gene expression at the epigenetic, transcriptional, and post-transcriptional level in cellular homeostasis [27].

LncRNA can be classified based on their location in genome, compared to protein-coding genes [Figure 13]:

- *Intergenic (LincRNA):* localised in the genomic region in which there are no encoding genes;
- *Genic:* associated with other transcripts; they are divided into:
  - *Exonic:* lncRNA exon intersects a part of protein-coding locus on the same (*sense, S*) or on the opposite strand (*antisense, AS*);
  - *Intronic:* they reside within introns of a coding gene, but do not intersect any exons;
  - *Overlapping:* they contain a coding gene within an intron on the same strand.

However, there is a large number of lncRNAs with particular characteristics that cannot be classified using this criterion. Many lncRNAs can act as transcripts for small RNA production, so the previous classification may be ambiguous. They are, therefore, subdivided according to their functions into lncRNA-activated genes, pseudo-genes associated with telomeres (TERRA), ultra-conserved transcripted regions (T-UCR), enhancer RNA (eRNAs), circulars, and others [99,100].
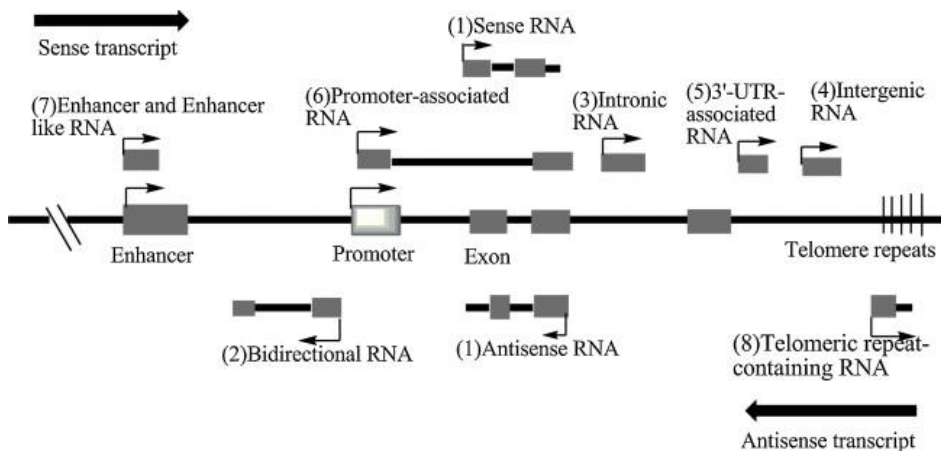
Figure 13. LncRNA classification [100]

1.2.1   Biogenesis

The lncRNA biogenesis resembles that of mRNAs and other non-coding RNA classes. They are subjected to post-transcriptional and inter/ intra-cellular transport. Most of them have a nuclear localisation, different expression and low levels of conserved sequences. These molecules are different from coding RNA because they lack a substantial open reading frame (ORF) and fail to produce protein [95].

Usually, lncRNA is transcribed by RNA polymerase II, can be polyadenylated and can be spliced with the formation of different isoforms. Only a small number of non-polyadenylated lncRNAs are expressed by promoters of polymerases III. Alternatively, some lncRNA are formed by excision during splicing and production of small nucleolar RNA [101,102]. Since lncRNA are involved in chromatin and epigenetic regulation of gene expression, they are preferentially located in the nucleus, in contrast to mRNA, which is conveyed to the cytosol for translation [94,97].

Once transcribed, they are folded into a secondary structure, which is guaranteed by their particular properties. For instance, the presence of multiple sites for hydrogen bonds enables them to adopt a thermodynamically more stable structure. They have many binding domains and are able to form double helix, pseudo-knocks, bulges or hairpin conformations. The resulting architecture consists in co-axial stacks of helices arranged in parallel or perpendicular to each other with repeated motifs. This structure gives them a high degree of stability.

Although lncRNA can acquire a tertiary structure and molecular complexes, we currently lack data about it. There is no data about whether they can form ribonucleoprotein complexes (RNPs) or whether they exist as isolated RNAs.

Some evidence suggests that they participate in a subcellular structure formation called "paraspeckles", but new structural studies are necessary to clarify lncRNA conformations in detail [103].

1.2.2 Main functions and mechanism of action

Though in the recent years much evidence has suggested the important role of lncRNA in gene expression regulation and more besides, only a small number has been well characterised. Some of these are involved in biological processes that have not been fully understood so far. The main biological functions of lncRNA include epigenetic regulation, chromatin remodelling and protein metabolism control. They can act at the transcriptional and post-transcriptional level, and are dynamically expressed during cellular differentiation and development. They are able to regulate cell cycle, genetic imprinting and stem cell reprogramming. Summarising, we can attribute lncRNA to 3 main processes, precisely chromatin modification, transcriptional and post-transcriptional regulation [104–108] [Figure 14].
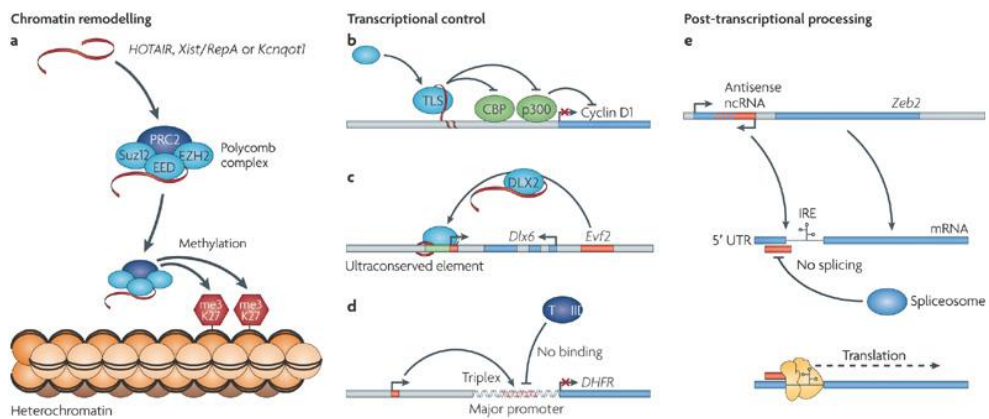
Figure 14. LncRNA functions [108]

Chromatin remodelling

LncRNA interact with chromatin-modifying complexes and histone-modifying enzymes, and may convey them to a specific genomic locus and influence chromatin status. They can be involved in global epigenetic reprogramming and during cell growth and development.

In particular, some of them bind the repressive complex Polycomb 2 (PRC2), e.g., HOTAIR or XIST (X-inactive specific transcript). Another epigenetic complex associated with lncRNA is the G9a methyltransferase, related with Air [109]. The lncRNAs might regulate the three-dimensional structure of chromosomes because they facilitate the interaction between chromosomal loci and recruiting factors involved in gene activation. This role is played, for instance, by MISTRAL and HOTTIP [110].

Recent evidence suggests the specific involvement of lncRNA, also in imprinting processes. X chromosome inactivation is mediated by XIST. XIST and Tsix anneal seem to form an RNA duplex, processed by Dicer to generate a small interfering RNA that inactivates the X chromosome [111].

Transcriptional regulation

Transcriptional regulation can be implemented at different levels and by specific mechanisms.

LncRNA can regulate neighbouring genes in cis in a sequence-independent manner by inhibiting the assembly of RNA polymerase II (RNApII) and transcription factors (TFs). Moreover, they can influence RNApII activity by interacting with the initiation complex, and influence the promoter's choice. An example can be found in the Dihydrofolate reductase (*DHFR*) locus, where a triplex in the promoter prevents TFIID binding.

Sometimes, lncRNA might prevent TFs translocation to nucleus. NRON provides an interesting example. It is an lncRNA repressor of the nuclear factor of activated T cells (NFAT), which interacts with members of the importin beta superfamily and acts as specific regulator of NFAT nuclear trafficking [112].

These RNAs might also act as co-factors or, as in the case of cyclin D1, tethered to the gene, they recruit TLS to modulate histone acetyltransferase activity of CBP and p300 and repress its transcription [111].

Post-transcriptional regulation

LncRNA play a key role also in post-transcriptional events, such as mRNA splicing, editing, transport, translation and degradation. These functions are possible because they can recognise complementary sequences and interact with mRNA.

In particular, antisense molecules can mask some sites, forming RNA duplexes. It is the case of BACE1-AS, which may prevent translational repression of BACE1 mRNA by miR-485-5p by masking the binding site for microRNA [113].

Alternatively, lncRNA can be associated to a splicing complex and regulate alternative splicing. For example, GOMAFU/MIAT can bind the splicing factor 1 (SF1) protein through its UACUAAC repeat sequences and inhibit splicing and spliceosomal complex formation [114]. Also MALAT1 has been found to regulate alternative splicing of endogenous target genes [115].

Finally, they can interact synergistically with miRNA and the silencing complex.

Mechanism of action

LncRNA expression is specific for cell types and responds to different stimuli. This suggests that they too are under transcriptional control. Hence the theory that they act as signal molecules [Figure 15 I]. Indeed, this type of lncRNA is transcribed at a specific time to integrate the answer to environmental or cellular changes. Some signal lncRNAs play an active role in transcription, while others are merely produced. Their transcription is regulatory, and guarantees rapid and performing events. In this archetype we find XIST, HOTAIR and Air.

Another mechanism of action of these molecules is that they act as a decoy. In this case, lncRNA titrate transcription factors away from chromatin. In addition, they can do the same for other protein factors, leading them into nuclear subdomains [Figure 15 II]. TERRA RNA belong to this class.

Alternatively, lncRNA can change gene expression, into cis and trans, by recruiting chromatin-modifying enzymes and, as a guide, target genes [Figure 15 III]. It is difficult to identify by the lncRNA sequence, which of them has this capability. However, it seems that COLDAIR, HOTTIP and Jpx work in this manner.
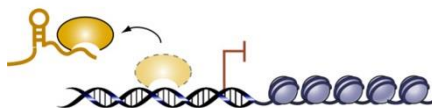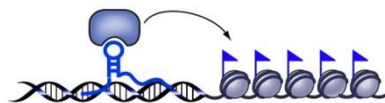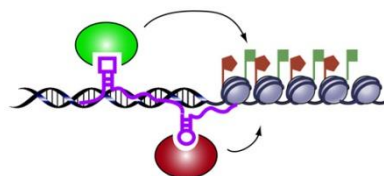
Figure 15 Mechanisms of action [101]

Lastly, they are able to bring together multiple proteins and form ribonucleoprotein complexes (RNP). On the one hand, lncRNA-RNP bring histone modifications and chromatin remodelling, and on the other hand this scaffold stabilises signalling complexes and structures in the nucleus [Figure 15 IV]. A ANRIL is an lncRNA scaffold [101].

1.2.3 Role of lncRNA in the CNS

The functions of the vast majority of known lncRNAs appear correlated with the CNS. Some evidence underscored that they are expressed in specific neuroanatomical regions, cell types or subcellular brain compartments, and are involved in important neurobiological processes. This suggests that their deregulation can contribute to the pathogenesis of neurological disorders. They play a role in brain development and neurogenesis, in neural cell differentiation, in synaptic plasticity, in stress response and in aging [104,116].

Brain development and neural cell differentiation

Some studies showed that lncRNA are critical regulators of neurogenesis. Human-induced pluripotent stem cells (iPSCs) and human embryonic stem cells (ES) present a dynamic expression of these molecules during neuronal differentiation. In particular, the 3 main players in these events are SUZ12, REST and SOX2OT. SUZ12 is a component of PRC2 and REST, and SOX2OT are the pluripotency-associated TFs [116]. These ncRNAs interact with protein components of chromatin-modifying complexes and allow to maintain ES features.

Multipotent neural stem cells (NCS) can differentiate to form neurons and glia, and lncRNA are differentially expressed in the two lineages. In particular, neurons express TUG1, MALAT1, RIAN, GTL2 etc., while glia

express GOMAFU/MIAT, SOX8OT, DLEU2, etc., as shown in Figure 16. GOMAFU is particularly interesting, as it shows dynamic regulation during differentiation of neural stem cells into oligodendrocytes [117].
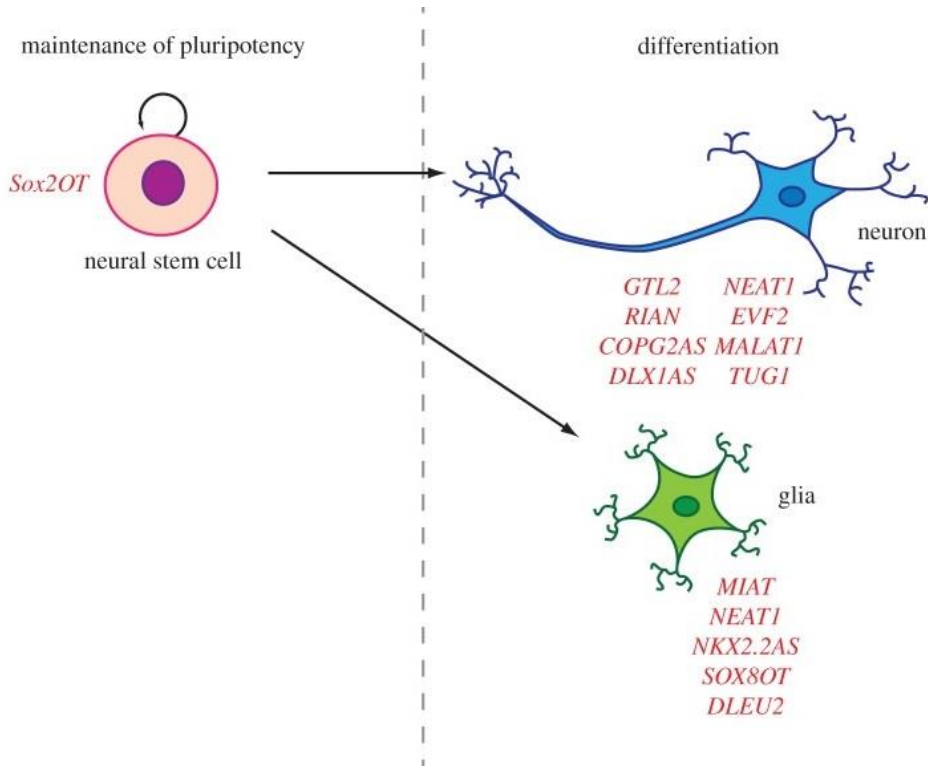
Figure 16. LncRNA in neural/glial differentiation [116]

Synaptic plasticity

Recent evidence showed that lncRNA are involved in synaptic plasticity processes, in the development of the postsynaptic dendritic compartment, and in the regulation of serine/arginine-related splicing factors that influence synapse density. MALAT-1 is the most studied in this contest. It is present especially in hippocampal neurons, and regulates synaptogenesis through

gene splicing [118]. Other long non-coding RNA are transcribed by genomic loci and are involved in these processes, i.e., loci for Calmodulin/Calcium-dependent neurogranin and kinase inhibitor II. These RNAs can be associated to plasticity-related pathologies, such as autism and intractable epilepsy. For instance, BC1/BC200 and Evf2 modulate neural plasticity and excitability [96,119]. Moreover, the analysis of some human biopsies revealed that there is an accumulation in the nucleus accumbens of heroin, cocaine and alcoholic-addicted subjects; therefore, they seem to be associated with drug dependence. Many lncRNAs are also associated with genes involved in the synaptic connection's long-term potentiation and in short-term memory consolidation [96].

Ageing

As previously described, lncRNA are implicated in development and in brain ageing processes. Indeed, downregulated transcripts in ageing have a role in such pathways. In particular, there is a 60% decrease in BC200 levels in the atrophic cortex [120]. Neuronal ageing seems correlated with the ageing of stem cells, a phenomenon that leads to a reduced capability for self-renewal, proliferation and differentiation with subsequent activation of senescence and apoptotic pathways. For this reason, lncRNA might play a key role in brain ageing. This hypothesis is also supported by their involvement in neurodegenerative diseases, typically occurring in the elderly, such as Alzheimer's disease [121].

1.2.4 Role of lncRNA in the immune system

LncRNA influences a variety of functions from innate to activation of the adaptive immune system. Development of the autoimmune system is associated with an epigenetic mechanism in response to complex environmental changes.

The development of immune cells from hematopoietic stem cells and their cell proliferation needs specific lncRNAs as regulators, and which thus participate in the differentiation [Figure 17] [122,123].
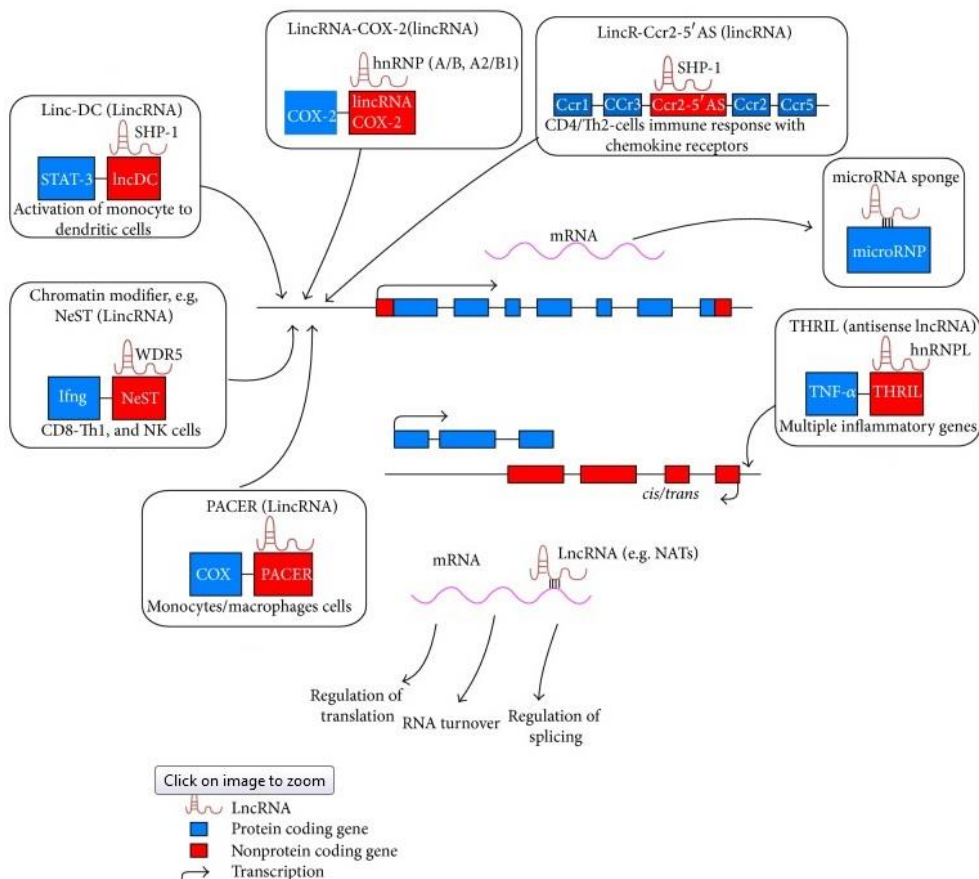
Figure 17. lncRNA in the immune system [123]

LncRNA in innate immunity

The first line of defence against pathogens is innate immunity. There is a growing list of lncRNAs involved in the process, particularly phagocytes. Bacterial or exogenous proteins can significantly change lncRNA expression through Toll-like receptor (TLR) pathways. They regulate the innate response in this manner.

In this contest, one of most important lncRNAs is lincRNA-Cox2, which acts as a regulator in macrophages. Indeed, its transcription is activated by TLR ligands and microbial pathogens, and is the last step of a complex pathway involving MyD88 and NF-kB. LincRNA-Cox2 is a repressor for several genes, such as chemokines and other inflammatory molecules, and activator for others (IL6) [124,125].

PACER, instead, seems to be solely involved in controlling COX-2 expression in epithelial and monocyte cells. It is able to bind NF-kB dimer p50/p50, preventing the bond with the Cox-2 promoter. Hence, the transcription complex can be assembled, and Cox-2 expression is favoured [126].

Instead, during HSV-1 infection, NEAT1, the main structural RNA in paraspeckles, has been linked to IL-8 expression. Moreover, it regulates HIV-1 controlling HIV-1 mRNA trafficking from nucleus to cytosol [125,127].

Cui et al. hypothesised that Lnc-IL7R could negatively regulate the expression of proinflammatory mediators, such as E-selectin, VCAM-1, IL-6 and IL-8, and the subsequent recruitment of TLR4. It acts probably through

trimethylation of H3 actin at lysine level 27, at the proximal level of inflammatory mediators [128].

Lethe and THRIL, together with the molecules described, are some examples in the large number of lncRNA implicated in innate immunity.

LncRNA in adaptive immunity

It is a known fact that lymphocytes T and B, which represent the mediators of adaptive immunity, differentially express lncRNA. In addition, also subsets (T_h1, T_h2, T_h17, T_{reg}) present a specific expression pattern. This underscores the importance of lncRNA also in immune cell differentiation.
Some important lncRNA expressed by T cells are NRON, GAS5 and LincR-Ccr2-5'AS.

NRON is an intronic lncRNA repressor of NFAT, a calmodulin/Ca^{2+} dependent TF that governs the expression of different cytokines, such as IL2. NRON, acting as a scaffold and interacting with β-importins, regulates NFAT translocation from cytoplasm to nucleus [124,129,130].
GAS5, instead, is an important repressor of T cell proliferation. Indeed, it has been linked to cell-cycle arrest in response to deprivation or other environmental conditions. GAS5 also regulate glucocorticoid receptor expression [131,132].
LincR-Ccr2-5'AS plays a role in T_h2 CD^{4+}, timocyte and mature peripheral T cell migration. So far, these data are from mice and must be confirmed in humans.

Regarding B cells, whether lncRNA have an important role in their maturation and function remains an open question. In comparison to T cells, very little knowledge of B cell function of lncRNAs is known [124,125].

1.2.5 Long non-coding and multiple sclerosis

lncRNA are good candidates as diagnostic and prognostic markers in autoimmune, inflammatory and neurodegenerative diseases as a result of their potential role in the pathogenic mechanisms. As demonstrated for miRNAs, the deregulation of some lncRNA is now recognised as a distinctive feature of some diseases. Among them, oncological and neurological diseases are the most investigated fields [89,130,133,134]. However, to date, research is only at the beginning, and there is a very little information about the role of lncRNA in multiple sclerosis.

As described above, adaptive immunity plays a key role in MS pathogenesis, and at molecular levels immune cell functions are modulated by lncRNA. Not only immune processes but also neurodegeneration is implicated in MS, suggesting the involvement of lncRNA in these processes.

In 2008 Burfoot et al. described *HLA-A*2* and *A*3* loci as protective against MS. Several lncRNA genes are located in these genomic regions, such as IL2RA locus. Among them, M21981 is upregulated in activated T cells [135,136].
FNG-AS1 (Tmevpg1), instead, is an lncRNA that is able to regulate T lymphocytes, and it is considered involved in MS because of the TMEV infection that is often used as an experimental murine model for MS [135]. Moreover, it is transcribed by a cluster of genes for cytokines, including IFNγ. Indeed, FNG-AS1 is adjacent and appears to promote its expression [137].

In 2016 Zhang et al. were the first researchers to investigate the expression profile of lncRNA in peripheral blood mononuclear cells (PBMCs). Using microarray analysis they found 2,353 upregulated lncRNAs and 389 downregulated lncRNAs in 26 MS patients, compared to healthy control subjects. Different pathogenic pathways, among immune and neurodegenerative ones, are involved in this large global de-regulation [92].

That same year, Santoro et al. identified three lncRNAs as deregulated in the serum of RR-MS patients, compared to controls precisely *nuclear paraspeckle assembly transcript 1* (*NEAT1*), *taurine upregulated 1* (*TUG1*), and *7SK small nuclear* (*RN7SK RNA*). These molecules are important players in neurodegeneration processes. However, the number of samples was small and these data need validation [91] .

The most recent paper published early in 2017 reports that, in a cohort of MS patients, the expression of *lnc-IL-7R* long non-coding RNA correlates with soluble and membrane-bound isoforms of *IL-7Ra* gene. However, there are some significant differences between the expression levels of *IL-7RB*, *IL-7RS* and *lnc-IL-7R* genes in cases and controls. Of course, we need additional studies to highlight IL7 regulation [138].

This thesis focuses on this field of research to investigate the role of cellular lncRNA in MS pathogenesis by using a comprehensive methodological approach to achieve reliable results.

1.3 Exosomes

Early studies on exosomes date back to the '80s, when Trams et al. noticed neoplastic cell line capacity to release microvesicles containing 5'-nucleotidase activity [139]. Later, electron microscopy enabled researchers to observe that, in reticulocyte cultures, multivesicular endosomes could merge with the plasma membrane, which internalised their content. By centrifugation of the supernatant, they isolated these vesicles and called them "exosomes".

Exosomes are small membrane vesicles, which can be detected in biological fluids (serum, plasma, CSF, urine, etc....) secreted by different cell types. The exosome diameter is 30-100 nm, and their density range in a sucrose gradient ranges from 1.13 to 1.19 g/mL. These vesicles present a characteristic "cup-shaped" morphology, a phospholipid bilayer containing high levels of cholesterol, sphingomyelin and ceramide with a specific membrane-micro domain, called "lipid rafts" [140,141].

The exosome membrane is characterised by the proteins involved in transport and membrane fusion, such as Rab, GTPase, Annexine and Flotillin, by the components of the ESCRT complex, including Alix, Tsg101 and HSP, integrins and tetraspanines, besides CD9, CD63, CD81. It is interesting to note that their surface can also have the markers of original cells. In particular, neural-derived exosomes present L1CAM (i.e., CD171) and NCAM [141–143].

The following paragraphs will outline biogenesis, molecular composition and main functions of exosomes.

1.3.1 Biogenesis and secretion

The biogenesis of exosomes begins with the formation of endocytic vesicles from specialised regions of the plasma membrane. This process can be clathrine-dependent, such as for the transferrin receptor, or clathrine-independent, as for GPI-anchored proteins.

Exosome formation involves the production of intraluminal vesicles (ILVs) within the multivariate bodies (MVBs). The pathways are illustrated in Figure 18.

The Endosomal Sorting Complexes Required for Transport (ESCRT) dependent biogenesis have the ESCRT complex as main player. In particular, the subunit ESCR-0 recognises ubiquitinated proteins in the cytosolic side of MVB, separates them into micro domains and then binds ESCRT-I. Later, the recruitment of ESCR-II entails RNA and protein transport within the vesicles that form. ESCR-III arrival completes the ESCRT-complex formation, following which ILV originate. Then, ubiquitin and ESCRT are recycled in the cytosol, and the free exosomes are secreted into extracellular space.

Not all exosomes form in this manner. Indeed, there is an alternative ESCRT-independent route. In this case, sphingomyelinases form the ceramide from a machine, presenting raft-based micro domains rich in sphingolipids, which triggers ILVs formation within the MVBs.

The MVBs destiny has two possibilities, namely to melt with lysosomes and degrade their content or melt with the plasma membrane and release ILVs into the extracellular environment [144].
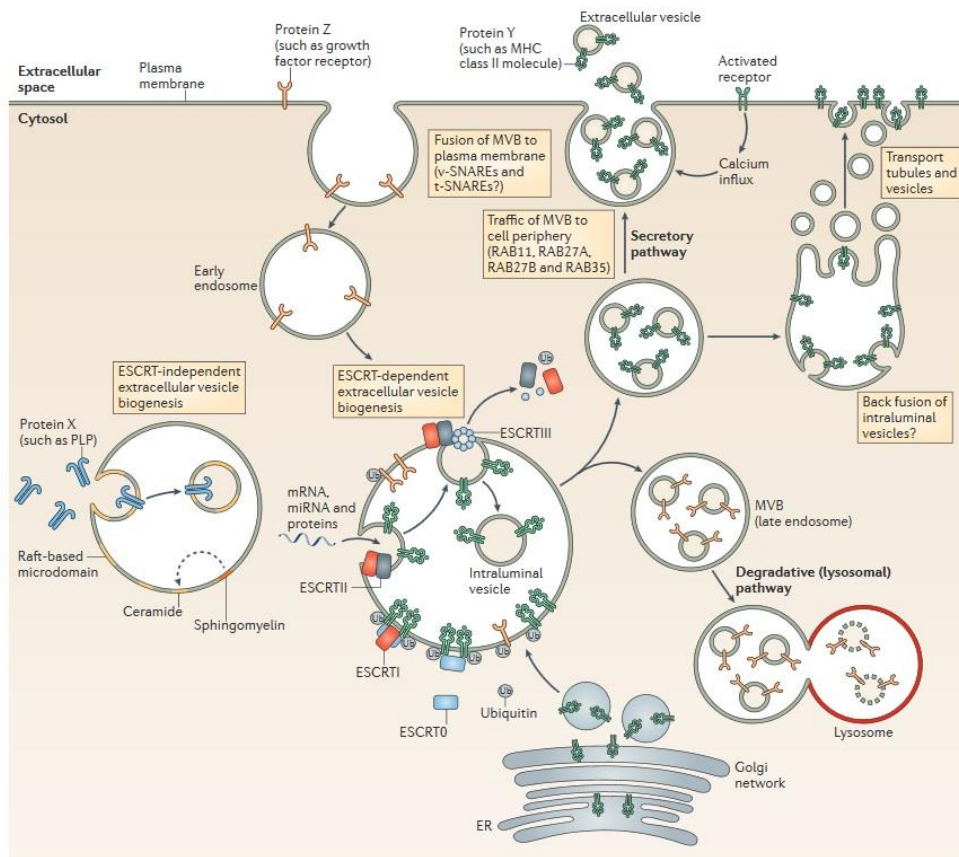
Figure 18. Exosome biogenesis [144]

Alternatively, exosomes originate by the "direct pathway". T cells and leukemic cell lines release exosomes directly from their plasma membrane. These vesicles cannot be distinguished from exosomes formed by the classic endosomal pathway because they are enriched with classical exosome

markers, such as CD63 and CD81, and have similar diameters and densities [145].

After ILVs formation within the MVBs, they are released into the extracellular environment through different steps, namely transport, binding and fusion with the plasma membrane.

Annexins and Rab family proteins mainly mediate transport and binding processes.

Annexins are proteins located in the cytosol and characterised by a phospholipid binding domain and regulated by Ca^{2+}.

Instead, Rabs are monomeric G proteins anchored to a cell membrane. Like other GTPases, they switch between two conformations, precisely an inactive form bound to guanosine diphosphate (GDP), and an active form bound to guanosine triphosphate (GTP). A GDP/GTP exchange factor (GEF) catalyses conversion from the GDP-bound to the GTP-bound form, thereby activating the Rabs. Rab effectors are heterogeneous, and different isoforms have different roles; for instance, RAB5 associates with early endosomes, RAB7 with late ones and RAB11 is implicated in recycling processes [146]. An important isoform is RAB27A. It controls exosome binding and the secretion of soluble factors during the following steps of exosome release, in stressful conditions.

Fusion events seem to be determined by soluble factors, such as soluble NSF-attachment protein (SNAP) and membrane complexes, SNAP-attachment protein receptor (SNARE), formed by VAMP-7 and VAMP-8. These are also involved in lysosomal vesicles fusion processes, but how they contribute to exosome secretion is still unknown at present [141,144,147].

1.3.2   Content

According to the exosome content database "Exocarta" (www.exocarta.org),
286 studies identified 9,769 proteins, 1,116 lipids, 4,946 mRNAs, 2,838
miRNA, and about 10 lncRNAs in exosomes derived from different cell types.
This further underscores their complexity [Figure 19].
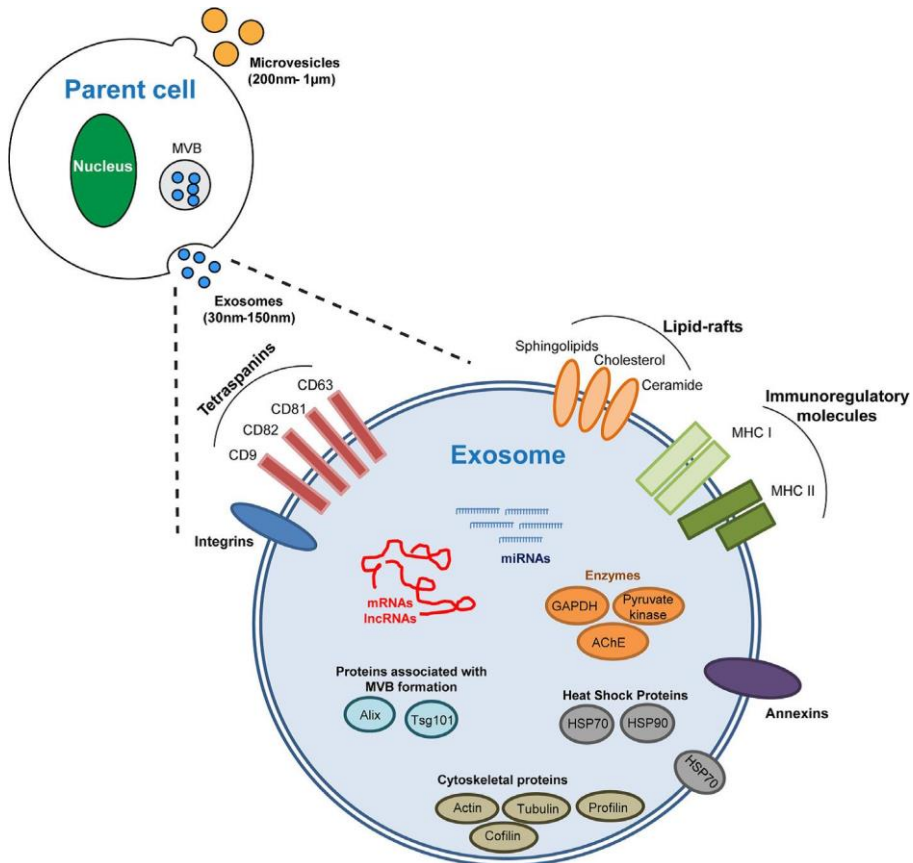
Figure 19. Exosome content [148]

Proteins

Both ubiquitous and cell-specific proteins can be transported by exosomes.

Proteins involved in exosome biogenesis, such as Rab family and annexins (I, II, V, VI), are constitutively present in them, along with some proteins that have unknown common functions. Among them, we can find cytosolic and cytoskeletal components, for instance actin and tubulin, receptors, and proteins involved in signal transduction, such as kinases as G proteins. Metabolic enzymes, pro/anti apoptotic and heat-shock proteins, which regulate the trafficking of misfolded proteins to degradation, are present as well.

Several adhesion molecules have been identified, such as intercellular adhesin molecule-1, CD146, CD9, milk-fat globules, EGF-factor VIII (MFG-E8), CD18, CD11a, CD11b, CD11c, CD166 and LFA-3 / CD58.

Tetraspanines are the most typical exosome proteins. They are a family of transmembrane proteins, which seems involved in the formation of multimeric protein networks. Since the exosome membrane is enriched with them, including CD9, CD63, CD81 and CD82, they can be considered as a subject's own exosome markers [141,143,146,149,150].

Exosomes also transport specific proteins for various parent cell types. For instance, MHC class II is present in vesicles derived from APC, CD86, and large amounts of several integrins are present, if DCs are the parent cells. In CND-derived exosomes, characterised by L1CAM (CD171) marker, we find the amyloid β, Tau and α-synuclein proteins [151–153]. This is important because it guarantees identification of the original cell type, with a possible link with pathological conditions states, and probably addresses them to a target cells.

Lipid

Limited data are available regarding lipid composition.

As with proteins, the lipid content of exosomes reflects the original parent cell. However, the lipid amount is higher in vesicles, which explains the higher rigidity of their membrane, in comparison with the plasma membrane of the cell.

Exosomes are enriched in cholesterol, diglycerides, sphingolipids, such as sphingomyelin and ceramide involved in a biogenesis pathway. Furthermore, they present phospholipids and glycerol-phospholipids, including phosphatidylcholine, phosphatidylserine, phosphatydyl-ethanolamide and phosphatydyl-inositol [154].

Nucleic Acids

RNA can be circulating in different modes, precisely cell-free, bound in protein complexes or packed in microvesicles. The presence of functional RNA in extracellular vesicles was described for the first time in mice stem cells by Ratajczak et al. in 2006 [155].

Unlike cellular mRNA, which has a length of 400-12,000 nucleotides, exosomal mRNA is long <700 nucleotides. It could be either intact or in fragments [156,157].

There are also mRNA, miRNA [158], lncRNA [159–161], t-RNA, snoRNA, snRNA [161], vault-RNA and Y-RNA [161]. Some evidence reports the absence of r-RNA, 18S and 28S, which is abundant in cells [156].

These RNA molecules play a key role in the regulation of gene expression, influencing cellular pathways and functions not only between cells of the same tissue but also between different tissues [156]. This method is efficient when there is an external stimulus and the answer must be obtained quite rapidly. There are few studies about DNA content in exosomes; therefore, this aspect needs to be explored in-depth.

1.3.3 Uptake

Exosomes interact with the target cell in different ways [Figure 20].
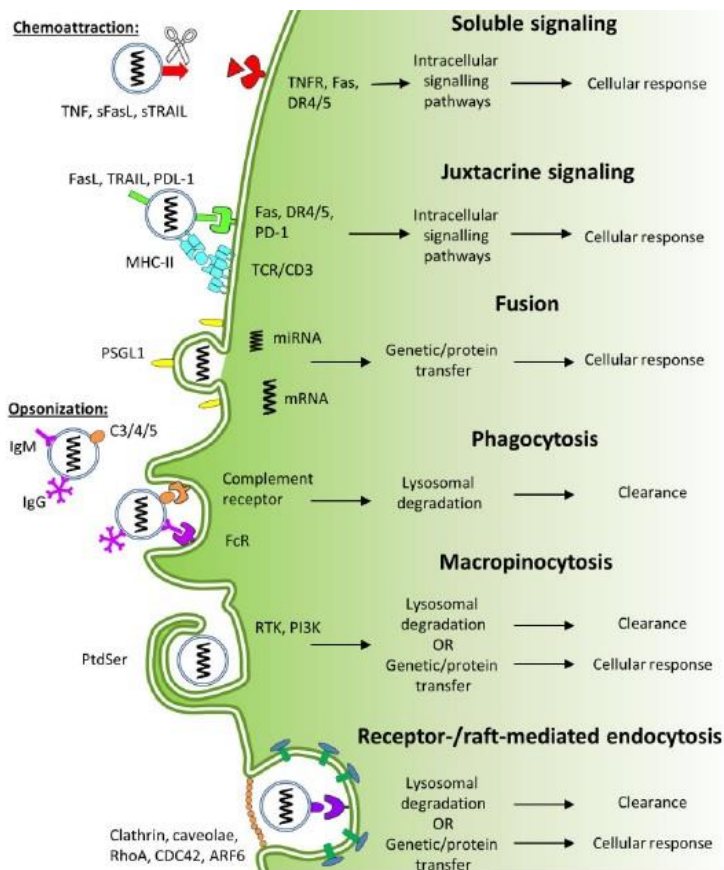
Figure 20. Exosome uptake [162] *modified*

Soluble signalling involves the proteolytic cleavage of ligands from the exosomal surface or alternative splicing, unlike juxtacrine signalling that requires the juxtaposition of ligands and receptors on the surfaces of both the exosome and the target cell. The membrane bond is mediated by FasL, TRAIL and TNF that can be cleaved by metalloproteinases to form soluble cytokines.

Exosomes could also be internalised by fusion. During this process, vesicles merge with the cell membrane, but the effective mechanism is still unknown.

Alternatively, exosomes are taken up by means of phagocytic events. It is an actin-mediated mechanism that requires opsonisation of vesicles and the presence of specific receptors, such as FcRs, on the cell surface. This process is dependent on actin, PI3K and dynamin 2.

Another uptake mode is macropinocytosis. In this case, the plasma membrane creates protrusions with actin filaments, with the subsequent formation of an invagination, which incorporates the particles. Exosome macropinocytosis is dependent on Na^+ and PI3K.

Finally, we have two endocytosis processes mediated by receptors or by raft. The first, also called clathrin-mediated endocytosis, requires clathrin and adaptor protein complexes. The second one is caveolae-mediated endocytosis regulated by distinct combinations of dynamin, flotillin and/or Rab proteins [162].

Due to the lack information about exosome uptake, further studies are required to better understand cell-to-cell communication mediated by these vesicles.

1.3.4   Biological functions in the CNS and in the immune system

As mentioned in the description of exosome biogenesis, they would be an alternative to lysosomal degradation. Indeed, proteins resistant to some proteases might be eliminated through this pathway.

However, the most important role of these vesicles is cell-to-cell communication. Obviously, in order to mediate any kind of biological function, exosomes must transfer their content into the target cell.

Recent studies suggest that they are capable of stimulating receptors located on the target cell membrane by releasing specific ligands. Moreover, it seems that they are able to transfer superficial receptors from cell to cell, and to change their cell surface distribution with the consequent influence on cellular metabolism and function [163].

As cargo molecules, they can transport enzymes, infectious agents [164], organelles, such as mitochondria [165] and growth factors, and control differentiation and proliferation pathways [141].

Exosomes are also actively synthetised during oxidative stress, irradiation and hypoxia events [166].

There is evidence of a possible role of these vesicles in neurodegenerative and immune-mediated disorders, such as multiple sclerosis [144,152,163,167,168].

The role of exosomes in the CNS

In the CNS, exosomes occur during normal development and physiology, acting as mediators of intercellular communication and playing functional

roles not only during development but also during normal neuronal regeneration.

First, in 2011 Lachenal et al. explained the role of exosomes in normal CNS physiology. They demonstrated how Ca^{2+} and glutamatergic synaptic activity influence exosome secretion from hippocampal and cortical neurons [169].

A few years later, Frühbeis et al reported an important reciprocal communication between neurons and oligodendrocytes mediated by these vesicles. Oligodendrocytes release exosomes in response to neuronal stress signals, and they are internalised along the endocytic pathway. In neurons, they release proteins, glycolytic enzymes, mRNAs and miRNAs to axons with a neuroprotective function [170]. Not only oligodendrocytes, but also other microglial cells and astrocytes communicate with exosomes that use neurons [171].

Exosomes play a crucial role also in synaptic plasticity and during myelin membrane biogenesis [172].

Regarding synaptic plasticity, exosomes allow the transport of proteins, mRNAs and miRNAs from the postsynaptic terminal to the presynaptic terminal [173]. In addition, inverse transport occurs through sinaptotagmine-4 (Syt-4), a transmembrane protein of synaptic vesicles. Korkut et al. reported that Syt-4 is present in presynaptic exosomes, and since Syt-4 is essential for retrograde signalling, this transport is an integral part of presynaptic control [174]. Moreover, MVB fusion with the plasma membrane is related to synaptic activity, supporting the role of these vesicles in synaptic plasticity [175].

It was observed that, during myelin membrane biogenesis, exosomes have a regulatory function between glial cells and axons. On the one hand they contribute to the elimination of excess membrane [170], and on the other hand they are involved in the biogenesis of the membrane itself [176].

The role of exosomes in the immune system

The immune response, both adaptive and innate, is included in the large number of biological functions of exosomes.

A highly significant role played by exosomes is induction and promotion of the adaptive immune response. Extensive data showed the role of exosomes in T and B cell activation, also related to DC.

It is widely known that antigen (Ag) presentation to T cells is mediated by APC. Recent studies showed that this happens not only directly, but also through co-stimulatory signals issued by exosomes secreted by APC. Furthermore Sprent et al. 2005 demonstrated that these vesicles express MHC class I, B7 and ICAM1, molecules that can activate CD8$^+$ T cells also in the absence of APC [177]. This feature is important in the CNS, an immunologically-privileged organ with limited MHC molecule expression.

Exosomes might also downregulate adaptive immunity, inhibiting T and B cells. They can act on myeloid cell precursors or, as in the case of tumours, increase TGF-β1 expression. In addition, a direct immunosuppressive effect is involved in the development of immune tolerance. These events have DC as main players. Indeed, mast cells secrete exosomes that are able to regulate the expression of MHC II, CD80, CD86 and CD40 in DC [178].

In innate immunity, instead, exosomes participate in chemotaxis events. For example, vesicles secreted by platelets act as cargo for chemotactic factors,

such as sphingosine-phosphate and arachidonic acid, and exert their function on NK cells, monocytes, T and B cells [179].

Exosomes are also involved in TLR-mediated processes. In the CNS, TLR7 can be stimulated by Letc and miRNA 21, transported by vesicles, and modify neuronal growth. Moreover, they control cytokine expression in PBMC through these receptors [163].

Overall, these observations suggest the existence of a specific signalling mechanism between immune cells and other target tissues mediated by microvesicles.

1.3.5 The role of exosome in multiple sclerosis

As previously described, exosomes play an important regulatory role in SNC and in immune system. Therefore, it's not surprising that recently, a large number of study has been focused on their involvement in neurodegenerative and autoimmune disease, such as MS.

As reported in the previous paragraph, myelination processes include cell-to-cell communication mediated by microvesicles. In particular, oligodendrocytes secrete exosomes, under neuronal influence, and delay myelin formation during CNS development. On the other hand, DC-derived exosomes promote oligodendrocyte growth and increase myelination, promoting repair events. This evidence underscores the possible importance of these vesicles in the regeneration process of damaged myelin sheaths, when there is neuronal stress associated with disease pathogenesis [170,171,180].

Another important mechanism in MS pathogenesis is related to the transmigration of exosomes through capability BBB immune cells. Activated

lymphocytes, monocytes, platelet and endothelial cells too might secrete vesicles, the content of which increases trafficking through BBB, sustaining proinflammatory processes.

It seems that endothelial cell-derived exosomes transfer the ICAM-1 receptor, namely integrin Mac-1, to monocytes. Moreover, those derived from T cells stimulate their expression, enhancing their transmigratory ability [163,181]. Along with these aspects, it is interesting to note that vesicles released by platelets too promote BBB trafficking. Indeed, within them researchers found high levels of P-selectin, which by interacting with PSGL1 and PECAM-1 increase the expression of integrin α4-β1 on T cells [163,182]. The facilitated transmigration of proinflammatory cells through the BBB, supported by microvesicles, favours and maintains pathogenic MS processes.

Given the above evidence, in recent times attention has focused on the possibility of considering exosomes and their cargo as potential MS biomarkers. The discovery of an association between their content and disease activity, the capability of select specific neural-derived vesicles, as well as accessibility in biologic fluids make exosomes an attractive candidate as biomarkers for various disease, in this case for MS.

For instance, Saenz-Cuesta et al, noticed a general increase in exosomes present in serum and CSF of MS patients, especially in the RR-MS form, during relapses [183]. Moreover, Verderio et al. 2012 showed how a higher amount of myeloid-derived vesicles in CSF from MS patients was associated with the inflammatory stage and correlated with MRI lesion data [184]. These are just two studies, but they might support the possible role of exosomes as promising biomarkers.

Nevertheless, very little data is published in the Literature, and more studies are required to confirm the feasibility of exosomes and their cargo as biomarkers.

2. AIM AND STUDY DESIGN

The aim of this study was to identify a specific signature of long non-coding (lnc) RNA expression in Peripheral Blood Mononuclear Cells (PBMCs) and in neural-derived exosomes from patients with multiple sclerosis (MS), compared to healthy controls, to determine the role of these transcripts in disease pathogenesis, and also to investigate their possible use as biomarkers of MS susceptibility and progression.

LncRNA represents a class of non-coding transcripts, whose functional importance has recently emerged in many diseases, including neurological disorders. LncRNAs are highly expressed in the CNS and in the immune system, where they are involved in crucial physiological processes. The discovery of an alteration in transcripts involved in some of these specific processes sheds light on partially known or entirely new aetiopathogenic mechanisms.

Interestingly, a part of exosome cargo is made up of miRNA and lncRNA, which could be considered possible disease-specific markers themselves. Within exosomes, lncRNA are well protected and transported for long distances from parental cells to other targets. Therefore, we could identify an ideal biomarker in the periphery that reflects the pathogenic status of the CNS, which could reveal disease progression and activity, as well as contribute to the choice of therapy.

Given these premises, we investigated the role of cellular and exosomal lncRNA in MS pathogenesis by using a comprehensive methodological approach in order to achieve reliable results.

To this end, we initially performed an exploratory analysis by using specific arrays that led to screen 90 lncRNAs involved in autoimmunity and in the human inflammatory response, related to disease status, in PBMC from 10 MS patients in order to identify the dysregulated ones. The additional validation step was designed to validate best hits lncRNAs in a larger population consisting of 30 cases and 25 controls, and by using specific Taqman probes for the quantitative real-time PCR assay. Lastly, a replication step was performed in an independent Belgian cohort consisting of 24 cases and 23 controls, with the droplet digital PCR system [Figure 21].

Validation Analysis
-Validation preliminary lncRNA by RT-PCR analysis using specific Taqman probes
-30 MS patients vs 25 ctrls

Replication Analysis
-Replication of best hits in a Belgian cohort using digital droplet PCR.
- 24 MS patients vs 23 ctrls

Explorative Analysis
- Microarrays to screen 90 lncRNA in PBMC
- 10 MS patients vs 6 ctrls
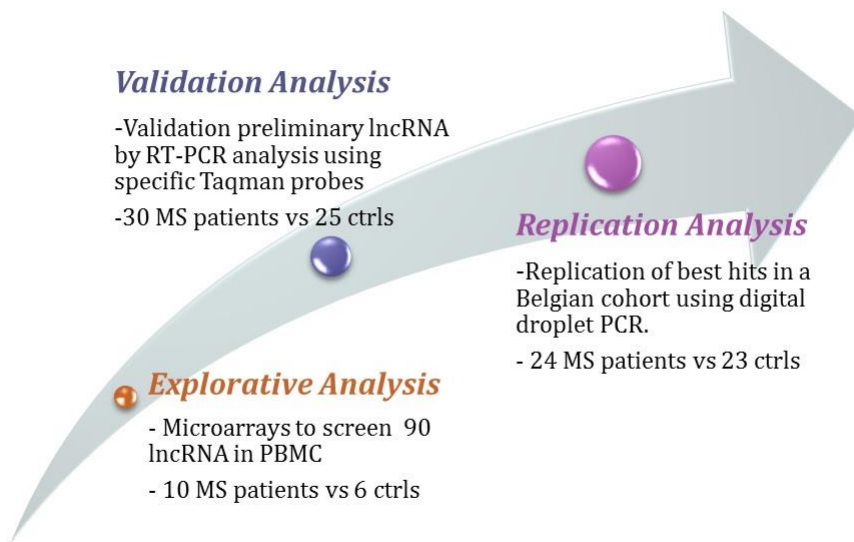
Figure 21. Study design for lncRNA from PBMC analysis

In parallel, we investigated lncRNA expression levels in neural-derived exosomes from the serum of MS patients, in comparison with healthy controls. We tuned the exosome extraction protocol, then we characterised them morphologically by using Transmission Electron Microscopy (TEM) and by citofluorimetry considering surface markers. Once neural-derived

exosomes had been isolated, we extracted lncRNAs and analysed their expression using specific arrays in RT-PCR [Figure 24].
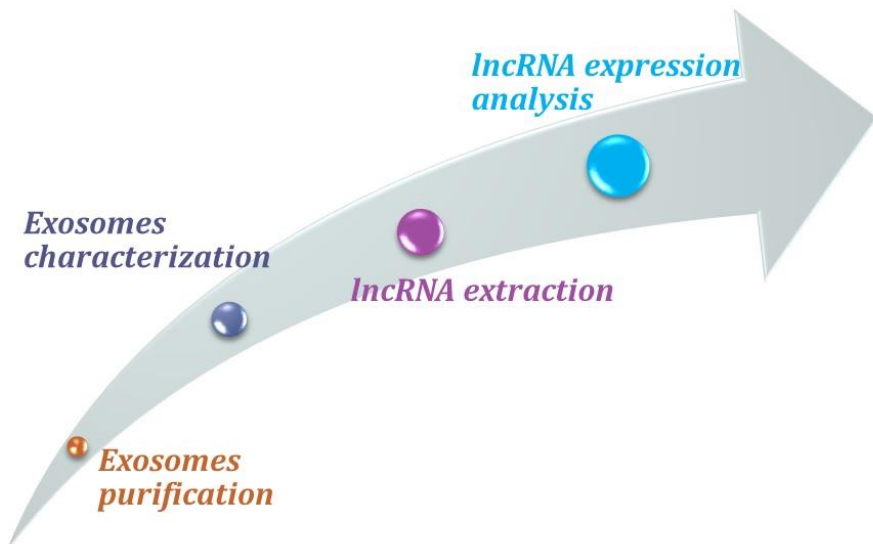
Figure 22. Study design for exosomal lncRNA analysis

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Lastly, we analysed the cellular and exosomal lncRNA profile, correlating them, when possible, with clinical aspects.

3. MATERIALS AND METHODS

3.1 Long non-coding expression profile in peripheral blood mononuclear cells from multiple sclerosis patients

3.1.1 Population and sample collection

Three cohorts of MS patients were recruited and their characteristics are summarised in the Table 5.

	Controls	MS-all	RR-MS	PP-MS
Italian discovery population				
N	6	10	5	5
Gender (M:F)	2:4	2:8	1:4	1:4
Mean age at onset, years±SD	35.4±2.9	37.5±5.8	36.8±1.7	45.5±2.0
Italian validation population				
N	25	30	22	8
Gender (M:F)	13:12	12:18	9:13	3:5
Mean age, years±SD	33.9±12.7	47.6±10.8	44.4±9.4	53.7±10.5
Mean age at onset, years±SD	NA	33.1±11.19	31.1±11.0	41.2±7.3
Mean disease duration, years±SD	NA	12.3±8.4	12.3±8.9	10.8±6.0
Median EDSS (range)	NA	2 (0-7)	1.5 (0-5)	6 (3-7)

Belgian replication population				
N	23	24	17	7
Gender (M:F)	11:12	6:18	2:15	4:3
Mean age, years±SD	51.5±19.0	51.5±21.5	49.5±22.3	51.5±21.5
Mean age at onset, years±SD	NA	20.1±24.3	36.9±10.0	42.1±7.4
Mean disease duration, years±SD	NA	23.6±14.3	23.9±14.7	23.6±14.3
Median EDSS (range)	NA	2.5 (0-6.5)	2.5 (0-4)	3 (2.5-6.5)

Table 5. Population characteristics. RR=relapsing-remitting; PP=primary progressive; OCB=oligoclonal Bands

Italian patients were enrolled at the Multiple Sclerosis Centre of the Cà Granda Foundation, Scientific Institute for Hospitalisation and Care, Ospedale Maggiore General University Hospital of Milan, while Belgian patients came from the University Hospitals of Leuven. The control group consisted of healthy volunteers matched by ethnic background and age.

The Italian exploratory population consisted of 5 subjects affected by relapse-remitting (RR) MS, 5 with the primary progressive form and 6 controls.

The Italian validation cohort included 28 RR-MS patients, 8 progressives, 2 patients with an undefined diagnosis and 25 controls.

The Belgian replication group was formed by 17 RR-MS, 7 PP-MS and 23 controls [table 5].

All patients were submitted to the standard procedure for MS and signed the informed consent. In particular, medical history, physical and neurological examination, screening laboratory tests and brain Magnetic Resonance Imaging were performed. Diagnoses were based on current consensus criteria [62].

14 mL of blood were sampled in BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube for each case and control. When the sample reached room temperature, it was centrifuged at 1,500 G for 15 minutes. After centrifugation, mononuclear cells and platelets appeared in a whitish layer just under the plasma layer [Figure 23]. The plasma was removed, the cell layer was collected in a tube and the cells were washed twice with PBS 1% and centrifuged at 680 G for 8 minutes.
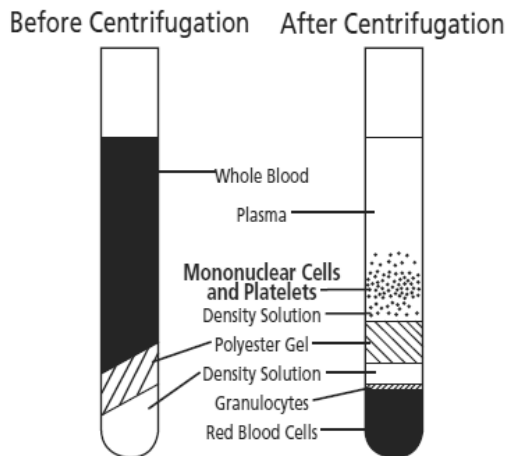
Figure 23 BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube.

Then, we aspirated as much supernatant as possible without disturbing the cell pellet, and resuspended cell pellet in 1 mL of Trizol reagent.

The samples were stored at -80°C.

3.1.2 RNA isolation and purification from peripheral blood mononuclear cells (PBMC).

We used the following protocol to extract RNA from PBMC:

a) transfer 1 mL of sample to a new Eppendorf; add 0.2 mL of chloroform per 1 mL of Trizol reagent;

b) vortex samples vigorously for 15 seconds and incubate them in ice for 5 minutes;

c) centrifuge at 15,000 G for 25 minutes at 4°C; following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase;

d) transfer the aqueous phase into a new Eppendorf and precipitate the RNA by mixing with isopropanol; use the same amount of the aqueous phase;

e) place 2 µL of glycogen in the Eppendorf cup and incubate overnight at -30°C;

f) the next day, vortex the sample and centrifuge it at 15,000 G for 15 minutes at 4°C;

g) remove the supernatant and add 500 µL of ethanol;

h) vortex the sample and centrifuge it at 15,000 G for 15 minutes at 4°C;

i) remove the supernatant and air dry the RNA pellet for 20 minutes;

j) add 22 µL of RNAse-free water and quantify the amount of RNA and purity by using the NanodropTM Lite Spectrophotometer (Thermo Fisher Scientific); RNA purity was measured by optical density and only samples with an OD 260/280 ratio ranging from 1.8 to 2 and an OD 260/230 of 1.8 or greater were used.

3.1.3 Retrotrascription in cDNA, pre-amplification and genic expression analysis.

Exploratory analysis.

LncRNAs were retro-transcribed using a cDNA Synthesis First Strand kit (Qiagen) for use with SYBR green technology arrays, following the procedure described below:

a) briefly (10-15 seconds) spin down all reagents;
b) prepare the genomic DNA elimination mix for each RNA sample, according to Table 6.

Component	Amount
RNA	25 ng – 5 µg
Buffer GE	2 µl
Nuclease-free water	variable
Total volume	**10 µL**

Table 6. DNA elimination mix

c) incubate the genomic DNA elimination mix at 42°C for 5 minutes, then immediately place on ice for at least 1 minute;
d) prepare the reverse-transcription mix according to Table 7:

Component	Amount
5x Buffer BC3	4 µL
Control P2	1 µL
R3 Reverse Transcriptase mix	2 µL
Nuclease-free water	3 µL
Total volume	**10 µL**

Table 7. Reverse-transcription mix

e) add 10 µL of the reverse-transcription mix to each tube containing 10 µL of the DNA elimination mix; blend gently by pipetting up and down;

f) incubate at 37°C for 60 minutes; then, immediately stop the reaction by incubating at 95°C for 5 minutes;

g) add 91 µL of nuclease-free water. Store at -20°C.

cDNA was pre-amplified using the commercial kit RT^2 lncRNA PreAMP cDNA synthesis (Qiagen) in association with a specific primer mix RT^2 lncRNA PreAMP Primer Mix for Human lncFinder (Qiagen):

a) thaw the RT^2 PreAMP PCR Mastermix and the RT^2 lncRNA PreAMP Primer Mix at room temperature;

b) prepare the pre-amplification mix according to Table 8;

Component	Amount
RT^2 PreAMP PCR Mastermix	12.5 µL
RT^2 lncRNA PreAMP Primer Mix	7.5 µL
Total volume	20 µL

Table 8. Pre-amplification mix

c) pipet 5 µL of cDNA into a PCR tube and add 20 µL of pre amplification mix;

d) mix gently by pipetting up and down; spin briefly to remove any air bubbles and collect all the liquid at the bottom of the tube;

e) place the tubes in the real-time cycler and start the programme according to the following cycling conditions:

1 cycle 95°C for 10 min.

12 cycles 95°C for 15 sec.

       60°C for 2 min.

Hold at 4°C.

lncRNAs expression levels were evaluated by human LncProfiler qPCR array (SBI), a panel of long non-coding RNAs related by pathway or disease, in SYBR green Technology using StepOnePlus Real time PCR System (Applied BioSystems) [Figure 24].

	1	2	3	4	5	6	7	8	9	10	11	12
A	21A	7SK	7SL	Air	AK023948	Alpha 280	Alpha 250	ANRIL	anti-NOS2A	antiPeg11	BACE1AS	BC200
B	CAR Intergenic	DHFR upstream	Dlo3os	DISC2	DLG2AS	E2F4 antisense	EgoA	EGO B	Emx2os	Evf1 and EVF2	GAS5-family	Gomafu
C	H19	H19 antisense	H19 upstream	HAR1A	HAR1B	HOTAIR	HOTAIRM1	HOTTIP	Hoxa11as	HOXA3as	HOXA6as	HULC
D	IGF2AS	IPW	Jpx	Kcnq1ot1	KRASP1	L1PA16	p21	RoR	SFMBT2	VLDLR	LOC285194	LUST
E	Malat1	mascRNA	MEG3	MEG9	MER11C	ncR-uPAR	NDM29	NEAT1	Nespas	NRON	NTT	p53 mRNA
F	PCGEM1	PR antisense	PRINS	PSF inhibiting	PTENP1	RNCR3	SAF	SCA8	snaR	SNHG1	SNHG3	SNHG4
G	SNHG5	SNHG6	Sox2ot	SRA	ST7OT	TEA ncRNAs	Tmevpg1	TncRNA	Tsix	TUG1	UCA1	UM9-5
H	WT1-AS	Xist	Y RNA-1	Zeb2NAT	Zfas1	Zfhx2as	18S rRNA	RNU43	GAPDH	LAMIN A/C	Human U6	No assay control

Figure 24. Human LncProfiler qPCR array (SBI)

a) dilute 10 µL of cDNA in 90 µL of water;

b) prepare the RT-PCR mix according to Table 9;

Component	Amount
2x RT^2 SYBRgreen PCR Mastermix	1275 µL
Nuclease-free water	1175 µL
cDNA	100 µL
Total volume	25 µL/well

Table 9. RT-PCR mix

c) in a plate place 25 µL of RT-PCR mix in each well and start the programme according to the following cycling conditions:

1 cycle 95°C for 10 min.

40 cycles 95°C for 15 sec.

       60°C for 30 sec.

Hold at 4°C.

Validation analysis

lncRNAs were retro-transcribed using SuperScript III Reverse Transcriptase (LifeTechnologies).

a) Prepare the mix according to Table 10;

Component	Amount
RNA	5 ng tot max
Oligo(dT) 50µMol	1 µL
Annealing buffer	1 µL
Total volume	variable

Table 10. SuperScript III Reverse Transcriptase mix.

b) incubate the mix at 56°C for 5 minutes, then immediately place on ice for at least 1 minute;

c) add 10µL of 2X First-Strand Reaction Mix and 2 µL of SuperScript III/ RNAase OUT Enzime Mix to each sample;

h) incubate at 50°C for 50 minutes. Then immediately stop the reaction by incubating at 85°C for 5 minutes. Store at -20°C.

Customised plates with TaqMan probes were drawn for best hits and lncRNA expression levels were measured in RT-PCR with the StepOnePlus Real time PCR System (Applied BioSystems). Two housekeeping genes, 18S and GAPDH, provided normalisation [Table 11].

lncRNA	Assay ID	lncRNA	Assay ID
HOTAIR	Hs003296680_s1	TUG1	Hs00215501_m1
HULC	Hs01909631_s1	XIST	Hs02758991_g1
ANRIL	Hs04259476_m1	AIR	Hs04332496_m1
GOMAFU	Hs00402814_m1	Sox2ot	Hs00415716_m1
H19	Hs00262142_g1	BACE1-AS	Hs04232267_s1
MALAT1	Hs00273907_s1	**Housekeeping gene**	**Assay ID**
MEG9	Hs01593046_s1	GAPDH	Hs02758991_g1
Nespas	Hs00294858_m1	18S	Hs19999999_m1
NRON	Hs04274940_s1		

Table 11. lncRNA Taqman probes

a) Prepare the RT-PCR mix according to Table 12;

Component	Amount
Taqman Universal PCR Mastermix	5 µL
Probe	0.5 µL
cDNA	1 µL
Nuclease free water	3.5 µL
Total volume	10 µL/well

Table 12. RT-PCR mix

b) In a plate, place 10 µL of RT-PCR mix in each well and start the programme according to the following cycling conditions:

1 cycle 95°C for 10 min.

40 cycles 95°C for 15 sec.

60°C for 30 sec. Hold at 4°C.

Replication analysis.

lncRNA were retro-transcribed using Multiscribe Reverse Transcriptase (Thermo Fisher Scientific).

a) Prepare the mix according to Table 13.

Component	Amount
10X RT Buffer	2 µL
25X dNTPs Mix(100 mM)	0.8 µL
10X RT random primers	2 µL
Multiscribe reverse transcriptase	1 µL
RNase inhibitor	1 µL
Nuclease-free Water	3.2 µL
RNA	10 µL
Total volume	20 µL

Table 13. Multiscribe Reverse Transcriptase mix.

b) Place in the thermal cycler at these conditions:

1 cycle 25°C for 10 min.

1 cycle 37°C for 120 min.

1 cycle 85°C for 5 sec.

Hold at 4°C.

lncRNA expression levels were measured by droplet digital PCR (QX200 ddPCR BioRad), using Taqman Probes [Table 14].

lncRNA	Assay ID	Housekeeping gene	Assay ID
NRON	Hs04274940_s1	GAPDH	Hs02758991_g1
TUG1	Hs00215501_m1		
GOMAFU	Hs00402814_m1		

Table 14. Taqman probes for replication analysis.

a) Prepare the ddPCR mix according to Table 15.

Component	Amount
ddPCR SuperMix	11.5 µL
Taqman probes	1.15 µL
Nuclease-free water	7.85 µL
Total volume	20.5 µL

Table 15. ddPCR mix

b) In a plate, in each well, place 20.50 µL of ddPCR mix and 2.5 µL of cDNA.

c) Spin briefly to remove any air bubbles.

d) After loading a 20 µL ddPCR reaction, load 70 µL of droplet generating oil in the bottom wells of the DG8 cartridge [Figure 25].
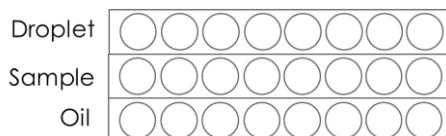
Figure 25. DG8 cartridge

e) Attach a gasket across the top of the DG8 cartridge and place it in the QX200 droplet generator, which produces about 20,000 droplets per sample.

f) Droplets should be transferred to a 96-well plate by pipetting gently.

g) After heat sealing, place the PCR plate in a thermal cycler for PCR using the following protocol:

1 cycle: 95°C for 10 min.

1 cycle: 95°C for 30 min.

70 cycles: 56°C for 1 min (annealing)

            98°C for 10 min.

Hold at 12°C.

h) Following PCR amplification of the target in the droplets, place the ddPCR plate in a QX200 droplet reader. The droplet reader and the QuantaSoft software count the PCR-positive and the PCR-negative droplets to provide absolute quantification of the target.

3.1.4   Statistical analysis.

To analyse data from human LncProfiler qPCR arrays (SBI), we used a software based on the ΔΔCt method and provided for the normalisation of five housekeeping genes. The P-values of expression data obtained from cases and controls were calculated using Student's t test values of $2^{(-\Delta Ct)}$.

For validation analysis-related quantification (RQ) of lncRNA, expression levels of cases were compared with that of controls using GraphPad Prism 6 scientific software and R.

Regarding ddPCR data, Poisson statistical analysis of the numbers of positive and negative droplets yielding absolute quantitation of the target sequence was performed by QuantaSoft software (BioRad). R was used to compare cases and controls and disease forms.

3.2 Long non-coding expression profile in exosomes isolated from serum of multiple sclerosis patients

3.2.1   Population and sample collation

6 mL of blood were sampled in BD Vacutainer® Blood CollectionTube (without anticoagulant) for each case and control. When the sample reached room temperature, it was centrifuged at 1,500 G for 10 minutes. 500 µL of serum were aliquoted in cryovials and stored at -80°C.

An MS patient cohort was recruited and the characteristics are summarised in Table 16.

	Controls	MS-all	RR-MS	PP-MS
N	10	17	10	7
Gender (M:F)	5:5	4:13	3:7	1:6
Mean age, years±SD	46±14.66	40±15.87	36±9.80	54±14.65
Mean age at onset, years±SD	NA	52±6.06	33±11.08	50±15.25
Mean disease duration, years±SD	NA	4±3.78	3±4.26	5±3.02
Median EDSS (range)	NA	2 (1-4.5)	2 (1-3.5)	2 (2-4.5)

Table 16. Population characteristics for exosomal analysis. RR=relapsing-remitting; PP=primary progressive; OCB=oligoclonal Bands

3.2.2 Exosome purification and characterisation

Exosome isolation

The Exo-Flow™ Exosome Purification kit (SIB) was used to isolate exosomes from serum. We followed the manufacturer's protocol:

1. Place 500 µL of serum in a new Eppendorf and centrifuge for 25 min. at 3,000 G;
2. transfer the supernatant into a new Eppendorf and add 125 µL of ExoQuick precipitation reagent;
3. mix by inversion and place at 4°C for 1 hour and 30 minutes;
4. centrifuge at 1,500G for 30 min. at 4°C;
5. remove the supernatant and repeat centrifugation for 5 minutes;
6. remove all supernatant and resuspend the exosome pellet in 500 µL of PBS 1%;

7. Store at -80°C.

Exo-Flow FACS magnetic bead preparation

1. Briefly vortex bead slurry and then pipette 40 μL of it into each Eppendorf.
2. Place the samples on a magnetic stand for 5 minutes.
3. Carefully remove the supernatant. Make sure not to disturb the magnetic beads.
4. Remove samples from the magnetic stand and add 500 μL of Bead Wash buffer. Invert a few times.
5. Place samples on a magnetic stand and repeat steps 2-4 for 2 washes.
6. Remove all liquid so only beads are on the side of the tube.

Binding capture antibody to beads

7. Remove tubes from the magnetic stand and add 10 μL of biotinylated capture antibody (Anti-CD171 (L1CAM), Abcam ab24345 1:5; Anti-CD81, Abcam ab109201, Anti-TSG101, Abcam ab125011; AntiCD63, Abcam ab125011) per sample. Mix by pipetting up and down.
8. Place tubes in ice for 2 hours. Flick the tube every 30 minutes to gently mix.
9. Add 200 μL Bead Wash buffer and flick to mix.
10. Place samples on the magnetic stand for 5 minutes.
11. Carefully remove the supernatant. Make sure not to disturb the magnetic beads.
12. Remove samples from the magnetic stand and add 500 μL of Bead Wash buffer. Invert a few times.
13. Place samples on the magnetic stand and repeat steps 10-12 for 3 washes.

14. Suspend capture antibody beads in 400 μL of Bead Wash buffer.

Exosome capture

15. Add 100 μL of concentrated, isolated exosomes to each bead sample for a total volume of 500 μL.
16. Incubate overnight on a rotating rack at 4°C for capture.
17. Place samples on a magnetic stand for 5 minutes.
18. Carefully remove the supernatant. Make sure not to disturb the magnetic beads.
19. Remove samples from the magnetic stand and add 500 μL of Bead Wash buffer. Invert a few times.
20. Place samples on a magnetic stand and repeat steps 3-5 for 2 washes.

Exosome staining

21. Add 240 μL of Exosome Stain Buffer and 10 μL of Exo-FITC exosome stain to reach a final volume of 250 μL per sample.
22. Place tubes on ice for 2 hours. Flick the tube every 30 minutes to gently mix.
23. Place samples on the magnetic stand for 5 minutes.
24. Carefully remove the supernatant. Make sure not to disturb the magnetic beads.
25. Remove samples from the magnetic stand and add 500 μL of Bead Wash buffer. Invert a few times.
26. Place samples on the magnetic stand and repeat steps 23-25 for 3 washes.
27. Resuspend samples in 300 μL of Bead Wash Buffer for flow cytometry.

Exosome elution

28. Place the stained exosomes/bead complexes on the magnetic stand for 5 minutes.
29. Remove samples from the magnetic stand and remove buffer.
30. Add 300 μL Exosome Elution Buffer. Invert a few times.
31. Incubate on a rotating rack at 25°C for 40 minutes.
32. Place samples on the magnetic stand for 5 minutes.
33. Carefully remove the supernatant containing your eluted exosomes and transfer to a fresh tube.

Fluorescence Activated cell sorting (FACS) analysis

The antibody-bead complexes were sorted by FACS. We selected the exosomes of neural origin by using the antibody anti-CD171, also known as L1CAM (L1 cell adhesion molecule), diluted 1:5 in 1% PBS (initial concentration 500 ng/μL).

Transmission electron microscopy for exosome characterisation

We performed negative staining to improve contrast and easily differentiate edges and features of the sample:

1) place 10 μL of sample on the grid covered by Forward (200 mesh);
2) leave at room temperature for 10 minutes;
3) dry the grids, placing them laterally on the filter paper;
4) place the grids on a drop of saturated uranium acetate (dissolved in water) for 5-10 minutes;
5) analyse the grid using TEM Leo912ab 80 kw.

Western Blot

We performed Western Blot analysis according to standard protocols. Briefly, exosomes were dissolved in PBS with LDS Sample Buffer (Life Technologies®) and separated using 4-12% Bolt® Bis-Tris Precast Gels (Life Technologies®) with MOPS SDS running buffer (Thermo-Fisher Scientific®). Then samples were electro-transferred to PVDF membranes (Thermo-Fisher Scientific®) for 2 hours at 60V at room temperature, and the membranes were immunoblotted with primary antibodies overnight and then incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce®,Thermo-Fisher Scientific) for 1 hour. Immuno-positive bands were detected by enhanced chemiluminescence (Pierce®, Thermo-Fisher Scientific) according to the manufacturer's instructions. The primary antibodies anti-L1CAM, anti-TSG101 and anti-VPS35 came from Abcam®, anti-CD9 was purchased from Santa Cruz Biotecnology®.

3.2.3   Long non-coding RNA isolation from exosomes

LncRNA extraction

lncRNA were isolated from exosomes using Total Exosome RNA and protein isolation (Ambion).

1) Transfer 300 µL of isolated exosomes to a new Eppendorf (if the volume is less than 300 µL, bring to volume with 1% PBS).
2) Under the hood, add 300 µL of Denaturing solution 2x and leave on ice for 5 minutes.
3) Add 600 mL of chloroform.
4) Vortex and centrifuge for 30 minutes at maximum speed at 4°C.

5) Carefully remove the aqueous (upper) phase without disturbing the lower phase or the interphase, and transfer it to a fresh tube. Note the volume obtained.

6) Add 1.25 volumes of 100% ethanol to the aqueous phase, and mix thoroughly.

7) Pipet 700 µL of the lysate/ethanol mixture onto the Filter Cartridge and centrifuge at 10,000 rpm for 2 minutes.

8) Discard the flow-through, and repeat until all the lysate/ethanol mixture has passed through the filter.

9) Pipet 700 µL of Wash Solution 1 on the filter.

10) Centrifuge for 2 minutes at 10,000 rpm.

11) Place the collection tube filter in a new Eppendorf and add 500 µL of Wash Solution 2/3 directly on the filter.

12) Centrifuge for 2 minutes at 10,000 rpm.

13) Add 500 µL of Wash Solution 2/3 and repeat centrifugation.

14) Remove the eluted liquid and repeat centrifugation.

15) Place the collection tube filter in a new Eppendorf and add 30 µL of Elution Solution (preheated at 90°C) directly on the filter.

16) Centrifuge for 2 minutes at 10,000 rpm.

17) Store at -80°C.

Qualitative and quantitative analysis of lncRNA.

LncRNA were analysed using Agilent 2100 Bioanalyzer and RNA 6000 Nano (Agilent) kit.

Using automated electrophoresis, the Agilent 2100 Bioanalyzer system provides sizing, quantitation, and purity assessments for RNA.

Depending on the amount of RNA, it uses different chips, precisely the NANO chip, the PICO chip or, for RNA≤200nt the Small chip.

For NANO and PICO chip, RNA quality is indicated by the RNA integrity number (RIN), a tool designed to estimate the integrity of total RNA samples. Using this tool, sample integrity is no longer determined by the ratio of the ribosomal bands alone, but by the entire electrophoretic plot of the RNA sample, including the presence or absence of degradation products. Interpretation of an electropherogram is thus facilitated, comparison of samples is enabled and repeatability of experiments is ensured.

The absolute value of RIN ranges between 0 and 10, where 0 represents completely degraded RNA, while 10 is the best quality RNA.

The protocol is described below:

1) bring the RNA 6000 Nano (Agilent) kit to room temperature;
2) spin the RNA ladder and place it in a new Eppendorf;
3) denature the RNA ladder at 70°C for 2 minutes and place it immediately on ice;
4) place the RNA ladder in the RNase-free Eppendorf and store at -70°C;
5) denature 5 µL of RNA sample at 70°C for 2 minutes and immediately place it on ice;
6) prepare the gel:
    - pipette 550 µL of RNA gel Matrix on the filter;
    - centrifuge at 1,500 G for 10 minutes at room temperature;
    - place 65 µL of filtered gel in a new Eppendorf;
    - store at 4°C for at least 4 weeks;
7) vortex the RNA dye concentrate for 10 sec. and spin;
8) pipette 1 µL on the filtered gel and vortex;
9) centrifuge for 10 min. at 13,000 G at room temperature;
10) Allow the gel-dye mix to balance at room temperature for 30 minutes before use, and protect the gel- dye mix from light during this time;

11) take a new RNA Nano chip out of its sealed bag;

12) place the chip on the chip priming station;

13) pipette 9.0 µL of the gel-dye mix at the bottom of the well indicated with a "G" and dispense the gel-dye mix;

14) set the timer to 30 seconds, ensuring that the plunger is positioned at 1 mL, and then close the chip priming station; the lock of the latch will click when the Priming Station is correctly closed;

15) press the plunger of the syringe down until it is held by the clip;

16) wait for exactly 30 seconds and then release the plunger with the clip release mechanism;

17) visually check that the plunger moves back at least to the 0.3 mL mark;

18) wait for 5 seconds, then slowly pull back the plunger to the 1 mL position;

19) open the chip priming station;

20) pipette 9.0 µL of the gel-dye mix in each of the marked wells;

21) load the marker: pipette 5 µL of the RNA 6000 Nano marker (diluted 1:2) into the well marked with the ladder symbol and each of the 12 sample wells [Figure 26A];

22) load the ladder and the samples:
    - pipette 1 µL of the RNA ladder into the well marked with the ladder symbol [Fig 26B];
    - pipette 1 µL of RNase-free sample in the first well;
    - pipette 1 µL of each sample into each of the 12 sample wells [Fig 26C];

23) vortex using the IKA vortexer for 1 minute;

24) run the Agilent 2100 Bioanalyzer [Figure 26].
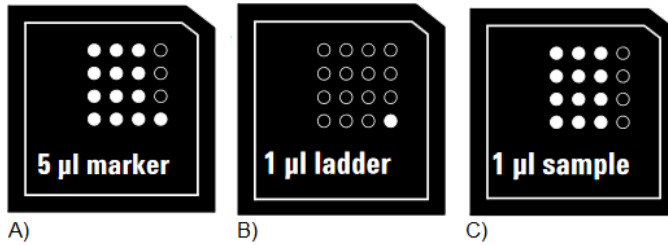
Figure 26. Loading on chip.

Retrotranscription, pre-amplification and lncRNA expression analysis by RT-PCR

In order to obtain cDNA from lncRNA, RT^2 preAMP cDNA synthesis kit (Qiagen):

a) prepare the genomic DNA elimination mix for each RNA sample, according to Table 17;

Component	Amount
RNA	8 µL
Buffer GE	2 µL
Total volume	10 µL

Table 17. DNA elimination mix

b) incubate the genomic DNA elimination mix at 42°C for 5 minutes, then immediately place on ice for at least 1 minute;

c) prepare the reverse-transcription mix according to Table 18;

Component	Amount
5x Buffer BC3	4 µL
Control P2	1 µL
cDNA Synthesis Enzyme mix	1 µL
RNase Inhibitor	1 µL
Nuclease-free water	3 µL
Total volume	10 µL

Table 18. Reverse-transcription mix

d) incubate the reverse-transcription mix at 37°C for 60 minutes, then at 95°C for 5 minutes. Store at -20°C.

cDNA was pre-amplified using the commercial kit RT^2 lncRNA PreAMP cDNA synthesis (Qiagen) as previously described on page 78.

After step e) add 2 µL of Side Reaction Reducer and incubate at 37°C for 15 minutes, and then at 95°C for 5 minutes. Add 84 µL Nuclease-free water and store at -20°C. Gene expression levels of lncRNA derived from neural exosomes were evaluated using RT^2 lncRNA PCR arrays LAHS-004ZC

	1	2	3	4	5	6	7	8	9	10	11	12
A	AIRN	ATXN8OS	BANCR	BCYRN1	BDNF-AS	BOK-AS1	CCAT1	CCAT2	CDKN28-AS1	DANCR	DIO3OS	DISC2
B	DLX6-AS1	EGOT	EMX2OS	FAS-AS1	FENDRR	FTX	GACAT1	GAS5	GNAS-AS1	H19	HAR1A	HAR1B
C	HEIH	HOTAIR	HOTAIRM1	HOTTIP	HOXA11-AS	HOXA-AS2	HOXA-AS3	HULC	IGF2-AS	IPW	JPX	KCNIP4-IT1
D	KCNQ1OT1	KRASP1	FALEC	LINC00570	LINC00581	LINC00599	LINC00853	LINC-ROR	LUCAT1	MALAT1	MEG3	MEG9
E	MIAT	MRPL23-AS1	NAMA	NEAT1	NRON	OIP5-AS1	OTX2-AS1	PANDAR	PCAT1	PCGEM1	PRINS	PTCSC1
F	PTCSC3	PTENP1	PTENP1-AS	RBM5-AS1	RMST	RPS6KA2-AS1	SIX3-AS1	SNHG16	SOX2-OT	SPRY4-IT1	ST7-AS1	ST7-AS2
G	TERC	TINCR	TMEM161B-AS1	TRERNA1	TSIX	TUG1	TUNAR	TUSC7	UCA1	WT1-AS	XIST	ZFAS1
H	ACTB	B2H	RPLP0	RN7SK	SNORA73A	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 27 . Human LncFinder RT^2 LncRNA array (QIAGEN)

(Qiagen) [Figure 27] and Human Inflammatory response and autoimmunity RT^2 lncRNA PCR arrays LAHS-004Z [Figure 28] .

	1	2	3	4	5	6	7	8	9	10	11	12
A	ASML1-AS1	ABCA11P	AC000120.7	AC007228.9	AC016629.8	AC068196.1	AC104820.2	CEP83-AS1	CROCCP2	CTC-444N24.11	CTC-487M23.5	CTD.3185P2.1
B	DLEU2	EPB41L4A-AS	ERICH1-AS1	FAM211A-AS1	FGD5-AS1	FGF14-IT1	FLJ31306	FOXN3-AS2	GAS	GAS5-AS	GRM5-AS1	HCG11
C	HCG18	HNRNPU-AS1	HOTAIR	HTR4-IT1	IQCF5-AS	JPX	LINC00094	LINC00116	LINC00293	LINC00324	LINC00338	LINC00421
D	LINC00635	LINC00657	LINC00662	LINC00667	LL22NC03.N27C7.1	LOC653160	LRRC37BP1	MALAT1	MCM3AP-AS1	MEG3	NAV2-AS5	NCBP2-AS2
E	NEAT1	NUTM2A-AS1	IP5-AS1	PDXDC2P	RMST	RP11-1134I14.8	RP11-282O18.3	RP11-29G8.3	RP11-325K4.3	RP11-363E7.4	RP11-363G2.4	RP11-367N14.3
F	RP11-38P22.2	RP11-399K21.11	RP11-473I1.10	RP11-473M20.16	RP11-498C9.15	RP11-549J18.1	RP11-819C21.1	RP11-84C13.1	RP11-96D1.10	RP1-239B22.5	RP6-24A23.7	SDCBP2-AS1
G	SENP3-EIF4A1	SIK3-IT1	SLC7A11-AS1	SNHG11	SNHG16	SNHG5	SNHG7	TP73-AS1	TUG1	XIST	ZFAS1	ZNRD1-AS1
H	ACTB	B2M	RPLP0	RN7SK	SNORA73A	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 28. Human Inflammatory response and autoimmunity RT^2 LncRNA array (QIAGEN).

These arrays contain 5 housekeeping genes (ACTB, B2H, RPLP0, RN7SK, SNORA73A), a control for genomic DNA contamination (HGDC), 3 wells for retrotranscription quality (RTC) and 3 positive controls (PPC).

a) Prepare the RT-PCR mix according to Table 19.

Component	Amount
2x RT^2 SYBRgreen PCR Mastermix	1275 µL
Nuclease free water	1173 µL
cDNA	102 µL
Total volume	25 µL/well

Table 19. RT-PCR mix

b) In a plate, place 25 µL of RT-PCR mix in each well and start the programme according to the following cycle conditions:

1 cycle 50°C for 2 min.

1 cycle 95°C for 10 min.

40 cycles 95°C for 15 sec.

60°C for 1 min.

Hold at 4°C.

Melting curve:

95°C for 15 sec.

60°C for 1 min.

95°C for 30 sec.

60°C for 15 sec.

4. RESULTS

4.1 Long non-coding expression profile in peripheral blood mononuclear cells from multiple sclerosis patients.

4.1.1 Exploratory analysis

LncRNAs expression profiling performed by lncProfiler array showed a generalised dysregulation in MS patients, compared to controls [Figure 29 A]. Some of this dysregulation presented different trends in RR-MS and PP-MS [Figure 29 B-C]. In particular, a strong difference in 12 lncRNAs in MS patients, in comparison with controls, was observed.

A)
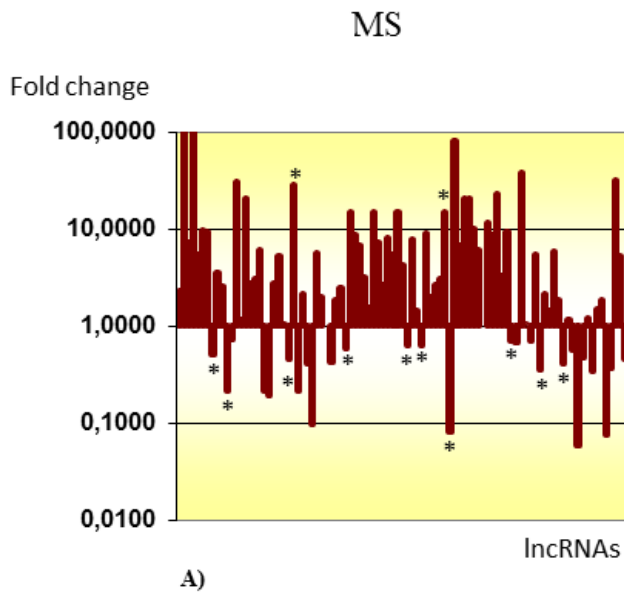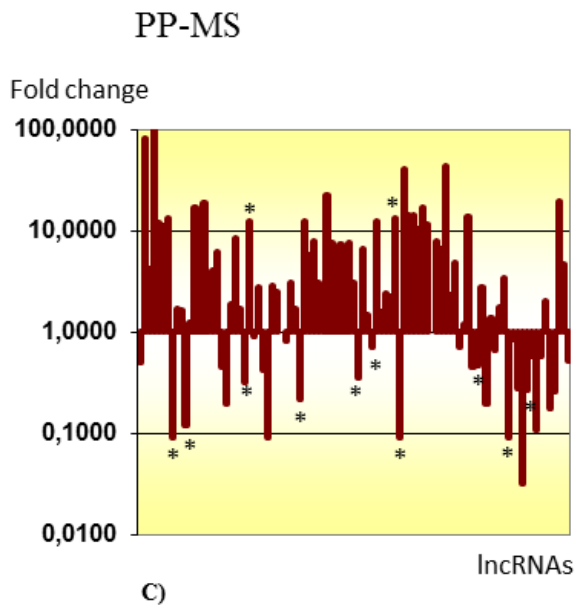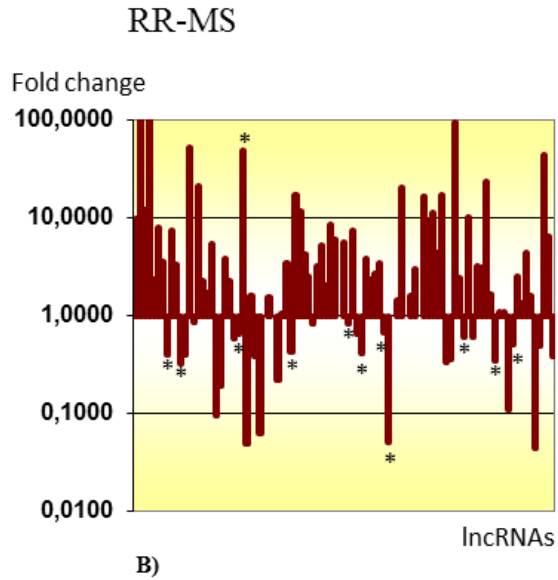
Figure 29. LncRNA expression profile in A) total MS patients (n=10) vs controls (n=6), in B) RR-MS (n=5) vs controls and in C) PP-MS (n=5) and controls (n=5). Statistically significant lncRNAs dysregulation is marked with *.

4.1.2 Validation analysis

Results were subsequently validated in an independent cohort [Table 5] and 10 out of 12 lncRNAs were downregulated in MS, compared to controls [Figure 30; exact p-values in Appendix 1]. ANRIL [Figure 30 A], TUG1 [Figure 29 K], XIST [Figure 30 L] (p<0.0001) and SOX2OT [Figure 30 I] (p<0.001) were strongly downregulated in RR-MS, while GOMAFU [Figure 30 D], HULC [Figure 30 E] (p<0.0001) and BACE-1AS [Figure 30 B] (p<0.001) showed a robust downregulation both in RR and Progressive MS, in comparison with controls.

E) HULC

F) MALAT-1

G) MEG9

H) NESPAS

I) SOX2OT

J) NRON
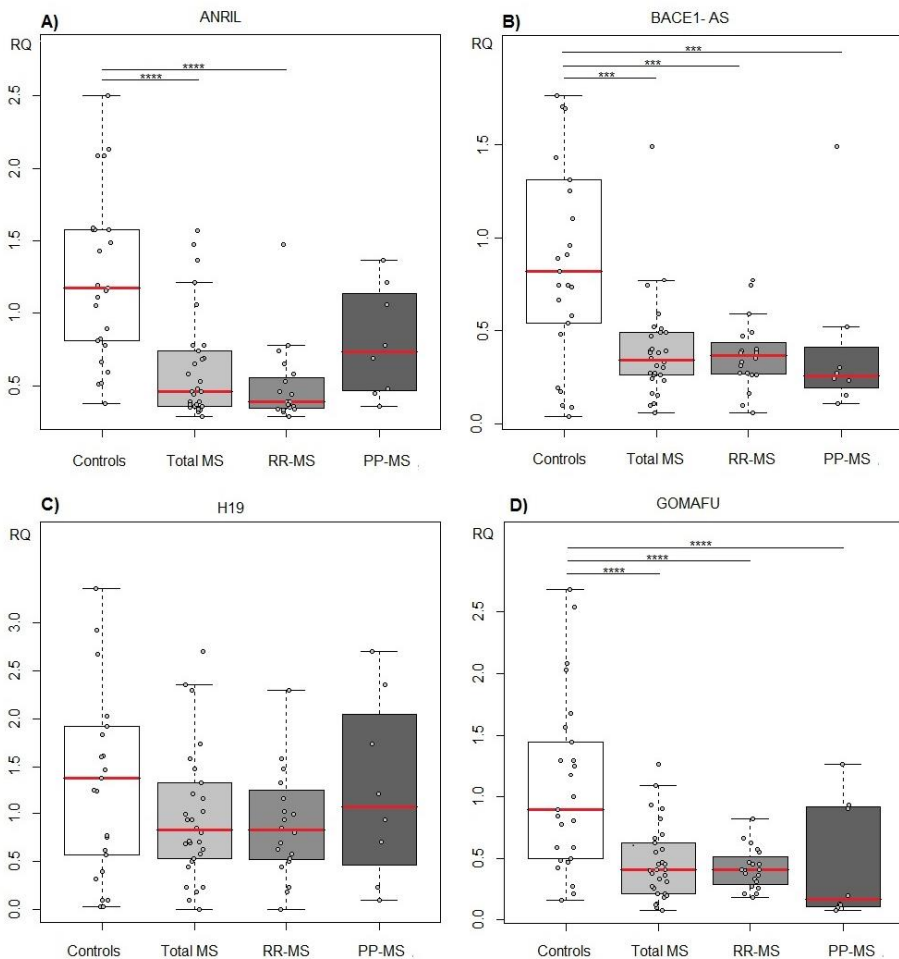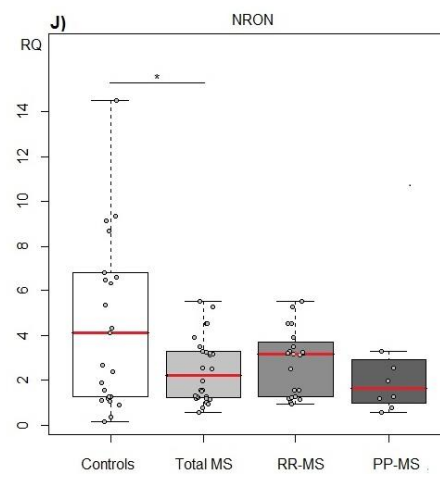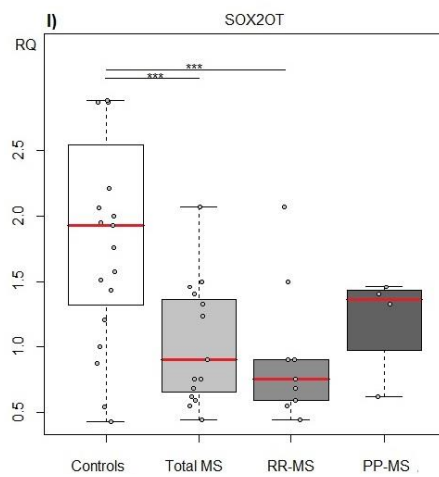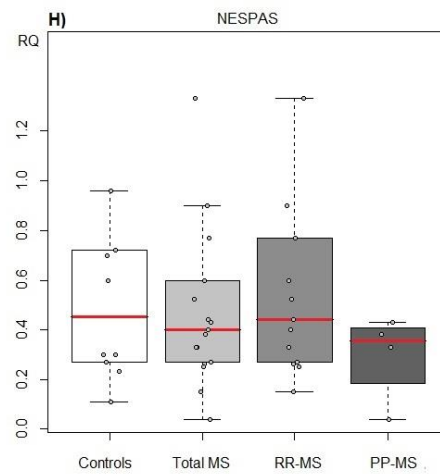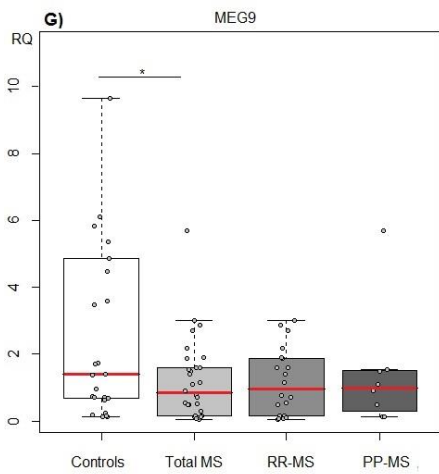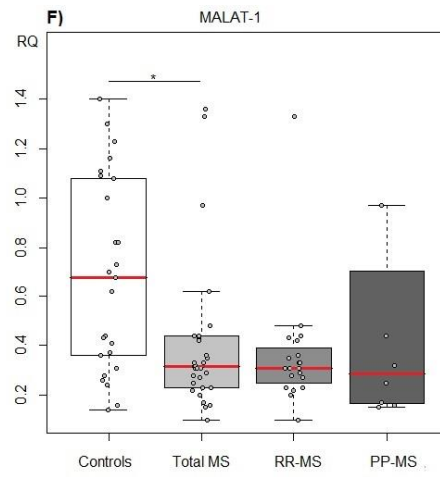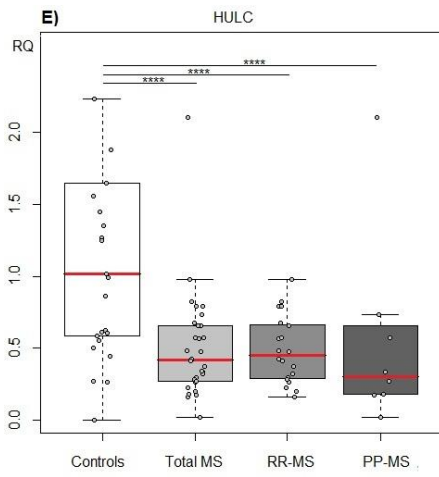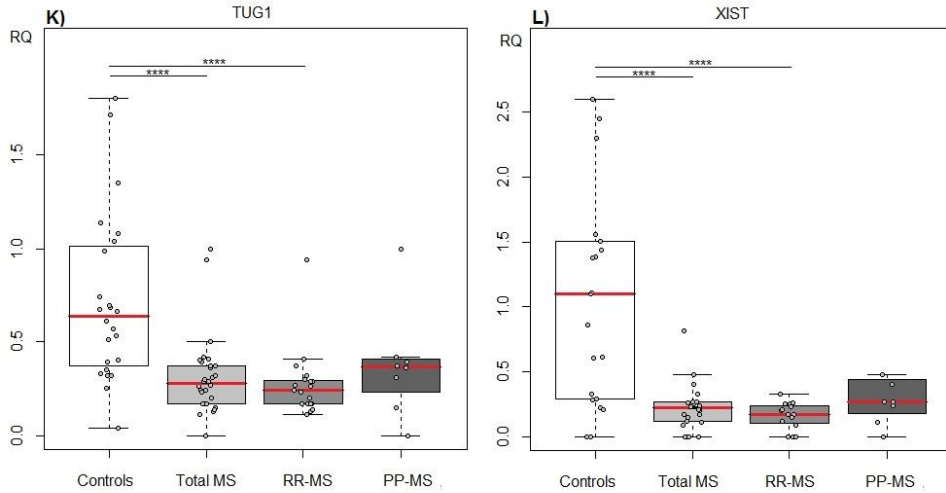
Figure 30. Validation Analysis. LncRNA expression levels obtained by RT-PCR, in the Italian cohort, are relative and expressed as relative quantification (RQ). In the last 2 bars, MS patients are divided by disease form (RR-MS=relapsing remitting; PP-MS=primary progressive). GAPDH and 18S were used as normaliser genes. The median value of each group is indicated in red.
Normality Test: Shapiro-Wilk's test. *p<0.05; **p=0.01, ***p<0.001; ****p<0.0001 calculated by non-parametric Kruskall-Wallis test. *Post-hoc test*: Dunn's test.

ANRIL (p=0.024 r=0.976) and TUG1 (p=0.007 r=0.993) correlated with disability expressed by EDSS [Figure 31], whereas NRON with disease duration (R=0.949; p=0.05) [Figure 32].
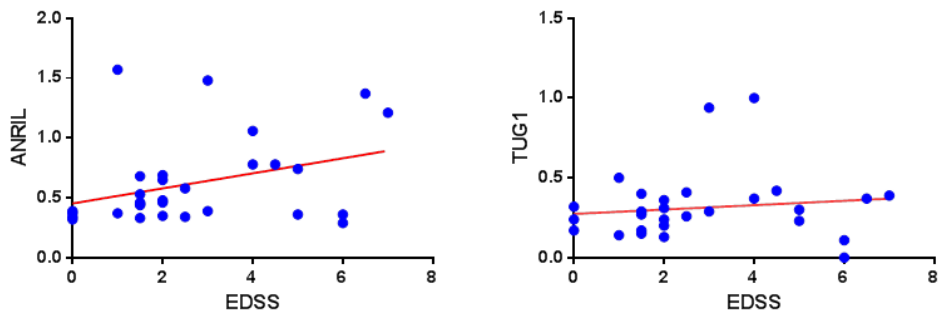
Figure 31. Correlation between lncRNAs expression levels and Expanded Disability Status Scale (EDSS) in the validation cohort. ANRIL (p=0.024 R=0.976) and TUG1 (p=0.007 R=0.933) expression levels correlated with EDSS. Correlation was calculated by Spearman's test.
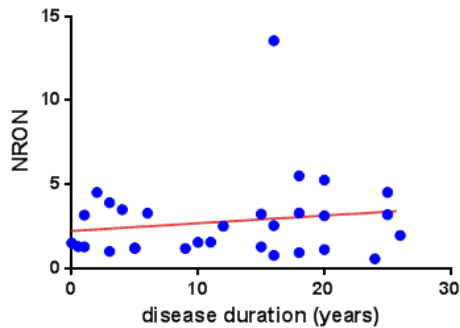
Figure 32. Correlation between NRON expression levels and disease duration (years) in the validation cohort. p=0.05 R=0.949. Correlation was calculated by Spearman's test.

4.1.3 Replication analysis

After validation, we replicated NRON, TUG1 and GOMAFU in an independent Belgian cohort [Table 5], using another method, the ddPCR. We chose these lncRNAs because of their biologic implications with the disease. We confirmed that NRON and TUG1 [Figure 33, A and B, respectively] had lower levels in MS patients, compared with controls (p<0.05 and p<0.0001 respectively). In particular, TUG1 was dysregulated both in RR-MS and PP-

MS forms [Figure 33 B]. Conversely, the replication study failed for GOMAFU [Figure 33 C].
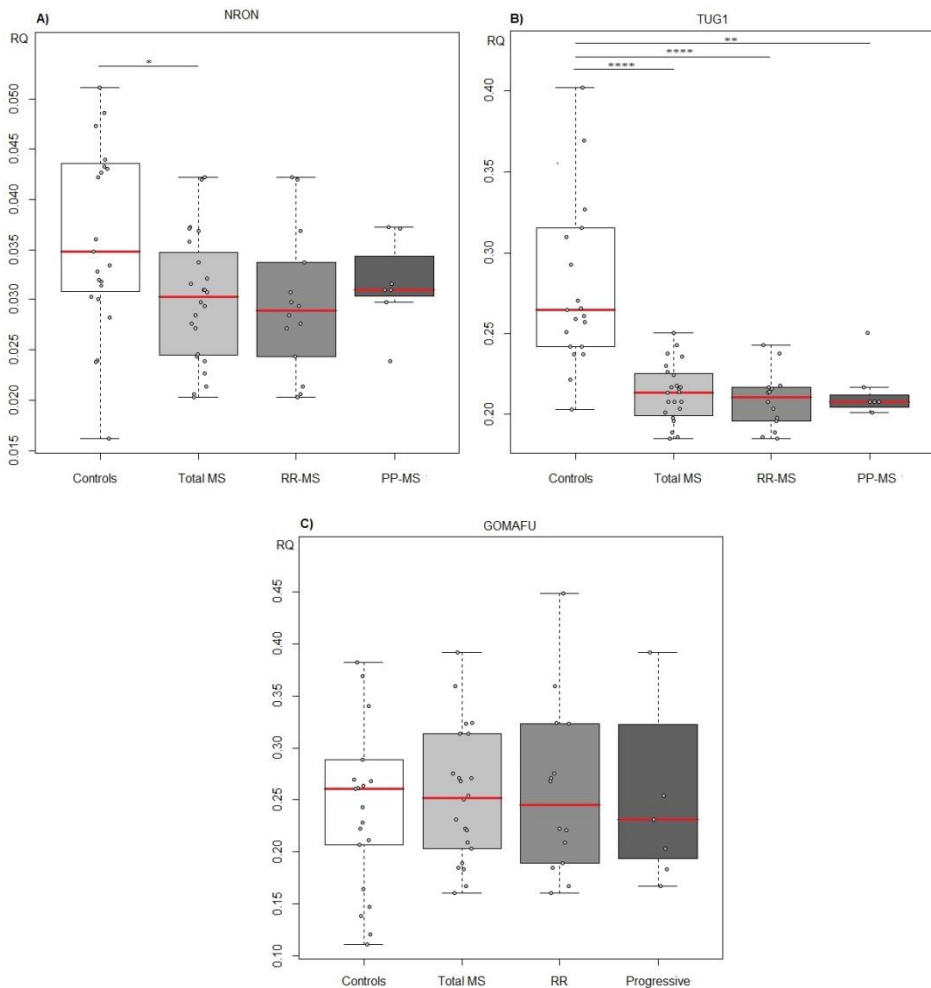
Figure 33. Replication analysis. LncRNA expression levels obtained by ddPCR, in the Belgian cohort, are relative and expressed as relative quantification (RQ). In the last 2 bars MS patients are divided by disease form (RR-MS=relapsing remitting; PP-MS=primary progressive). GAPDH and POLR2A were used as normaliser genes. The median value of each group is indicated in red.
Normality Test: Shapiro-Wilk's test. *p<0.05; **p<0.01, ****p<0.0001 calculated by non-parametric Kruskall-Wallis test. *Post-hoc test*: Dunn's test.

Correlations between the EDSS score and lncRNA expression levels failed to reach the significance threshold, although a trend toward a positive correlation between the NRON level and disease duration was found (p=0.048 r=0.471) [Figure 34].
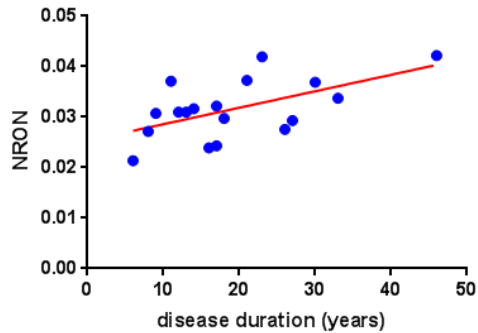
Figure 34. Correlation between NRON expression levels and disease duration (years) in the replication cohort. p=0.048 R=0.471. Correlation was calculated with Spearman's test.

4.2 Long non-coding expression profile in neural-derived exosomes from multiple sclerosis patients.

4.2.1   Exosome purification and characterisation

Neural-derived exosomes were isolated from serum of MS patients and healthy controls, and then submitted to microscopy, biochemistry and cytofluorimetric analysis.

Microscopy analysis

First, the presence of exosomes was evaluated, using the TEM. The global exosomes, marked by CD81, and specifically the neural-derived ones marked by L1CAM were present in our samples, and had the size and morphology typical of these vesicles, i.e., cup shape and spherical. [Figure 35].

A)   **B)**
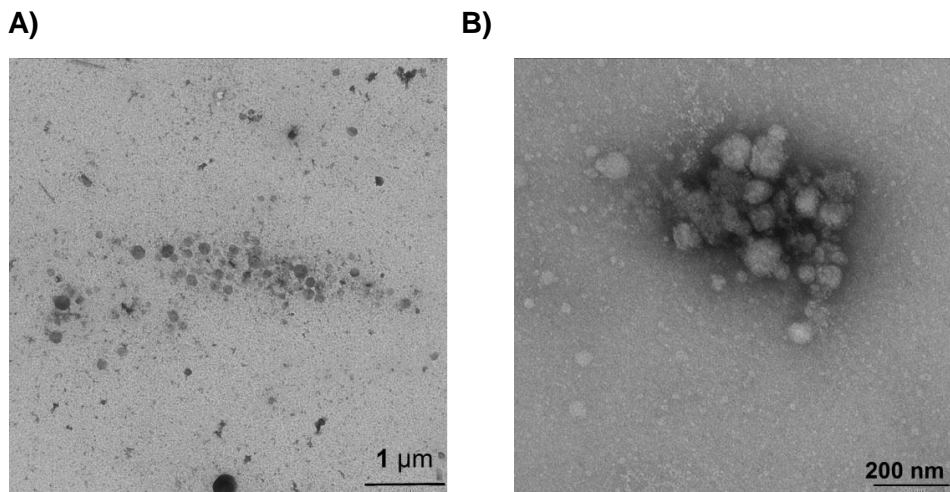
Figure 35. Exosomes extracted from serum. A) CD81 positive global exosomes. B) L1CAM positive neural-derived exosomes

Second, we evaluated whether there are any differences in the morphology and/or integrity of exosomes extracted from frozen serum and those

extracted from fresh serum. As we can notice in figure 36, integrity did not change. Both fresh and frozen serum exosomes presented the intact phospholipid membrane, suggesting that the freezing process at -80°C does not affect their integrity.

A)

B)
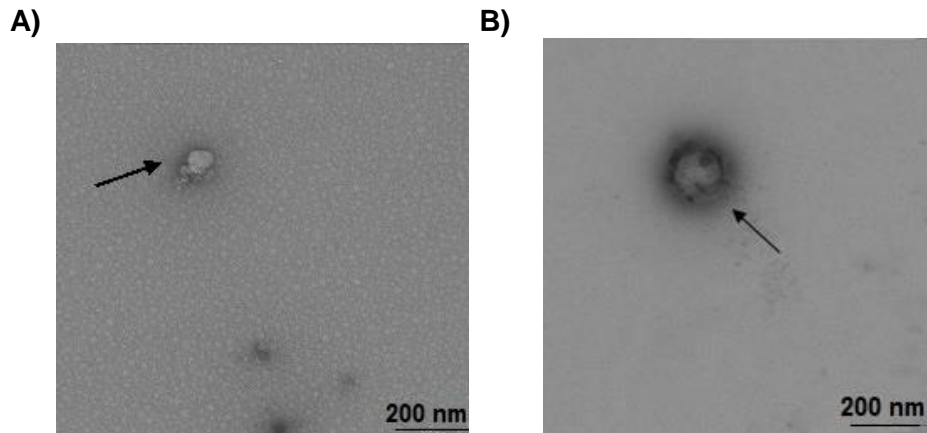
Figure 36. Integrity of exosomes isolated from frozen A) and fresh serum B). The exosomal vesicles are indicated by arrows.

Freezing seems to stress the exosome membrane shape, which appears more jagged in frozen than in fresh vesicles [Figure 37]. However, this aspect does not affect exosomal integrity.

A)
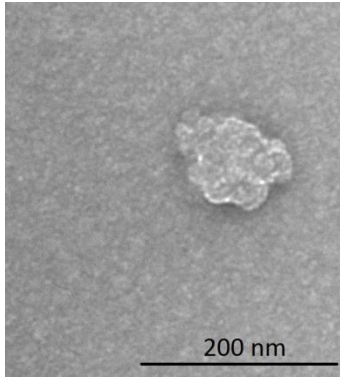
B)
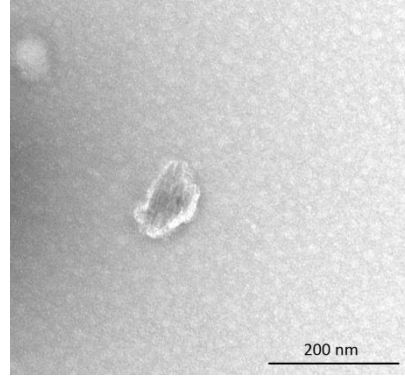
Figure 37. Morphology of exosomes isolated from frozen A) and fresh serum B).

Western blot analysis

Western Blot analysis was performed to characterise and be sure that isolated vesicles were exosomes.

CD9, TSG101, retromer (VPS35) protein and L1CAM expression was evaluated using specific antibodies in neural-erived exosome samples. All proteins analysed were detected [Figure 38].
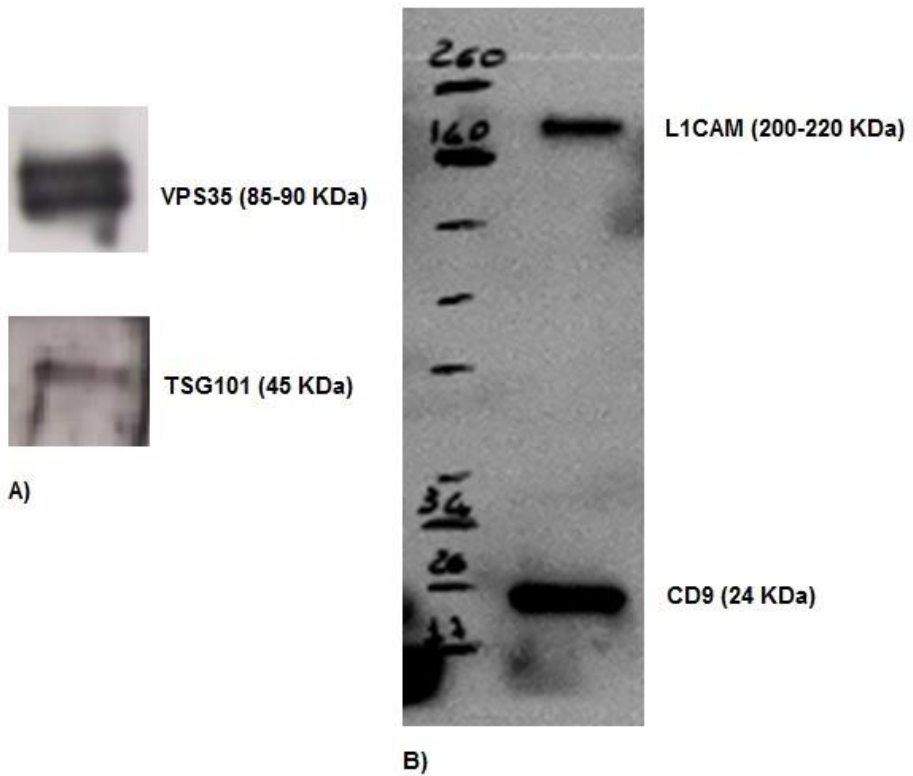
Figure 38. Western Blot analysis. A) VPS35 and TSG101. B) L1CAM (neural marker) and CD9 (general exosome marker)

Fluorescence-activated cell-sorting (FACS) analysis

A complex bead-antibody that is able to recognise the L1CAM (or CD171), a specific membrane marker of neural-derived exosomes, was used. The exosomes were stained with fluorescein isothiocyanate (FITC), a fluorophore, which binds post-translational modifications that are physiologically present on the exosome surface. Once the bead-exosome bond was formed, a FACS analysis, using the cytofluorimeter, was performed [Figure 39].
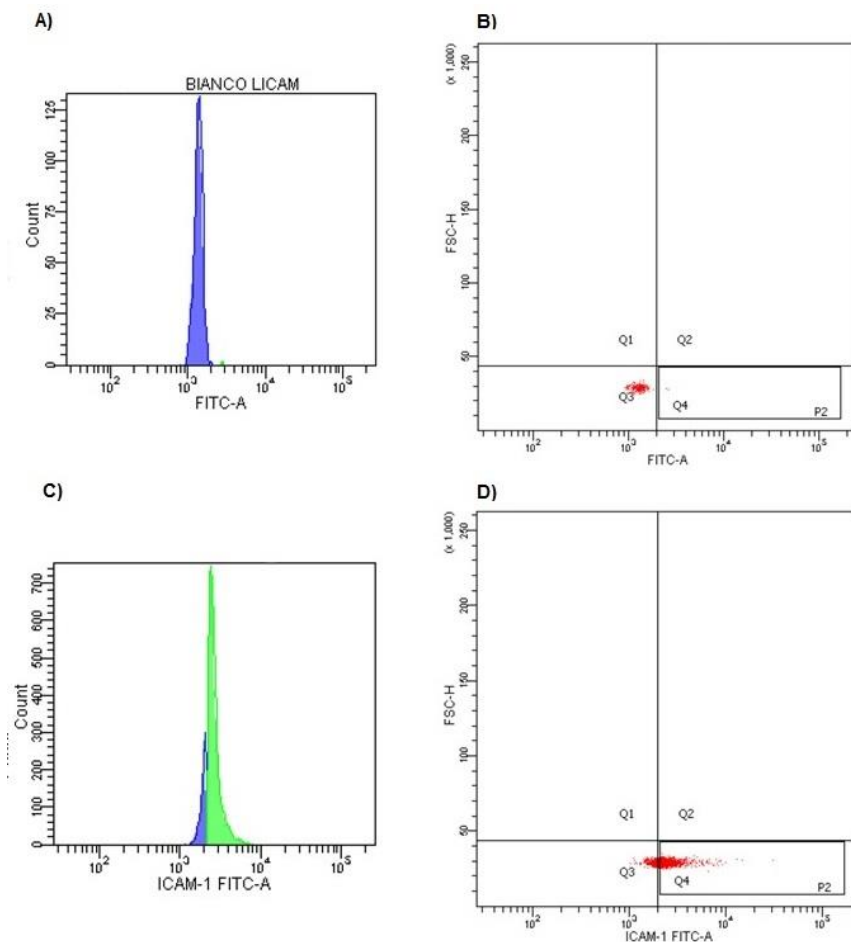
Figure 39. FACS analysis. A; B) Negative control. C; D) Neural derived exosomes from serum of an MS patient.

The negative control was obtained by conjugation of the bead-antibody complex without exosomes. Its distribution diagram presents the energy absorbed by the bead-antibody conjugates (blue peak), in the absence of fluorescent emission FITC (green peak) [Figure 39 A]. Therefore, in the dot plot, the bead-antibody system is located in quadrant Q3 due to the absence of FITC fluorescence positivity [Figure 39 B]. Regarding the sample with exosomes, presenting the L1CAM marker and labelled in FITC, the distribution diagram shows an emission peak (green peak) [Figure 39 C], and in the respective dot plot we can notice a signal shift in quadrant Q4, where fluorescence is positive [Figure 39 D].

4.2.2 Exosomal RNA analysis by Bioanalyzer (Agilent)

RNA analysis, in terms of amount, purity and non-coding RNA enrichment, was performed using Bioanalyzer (Agilent) [Figure 40; Appendix 1], and the samples were similar (mean±SD: 13.67±3.50 ng/µL).
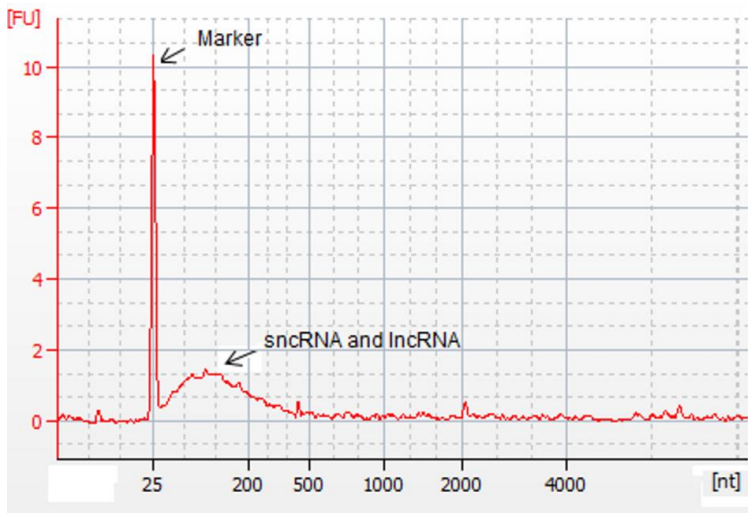
Figure 40. Exosomal RNA analysis

4.2.3 Long non-coding expression profiles

Human LncFinder RT^2 lncRNA array

LncRNA expression profile performed by the Human LncFinder RT^2 array showed generalised upregulation of lncRNA in 17 MS patients, compared to 10 controls [Table 16, Figure 41]. Indeed, 49 lncRNA were upregulated, while 9 were downregulated [Table 21].
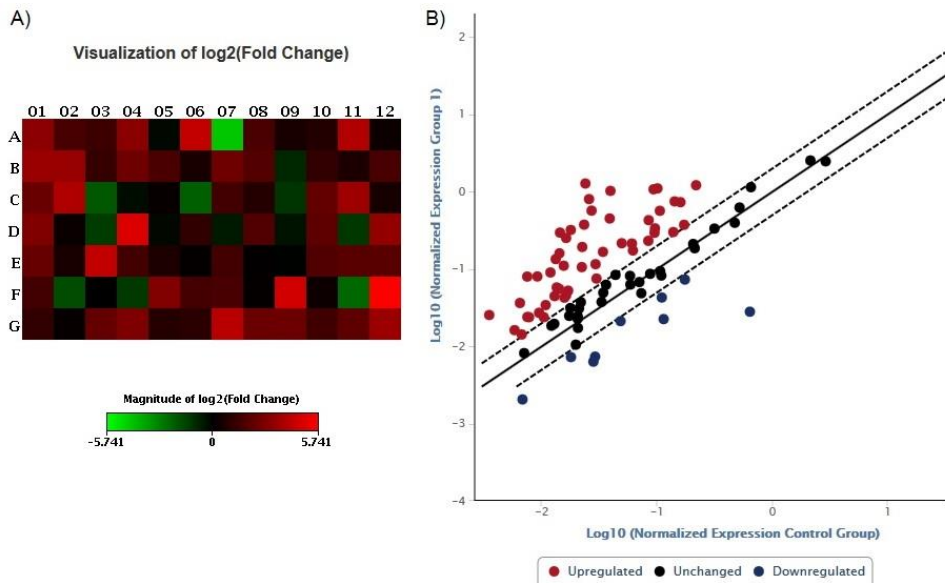
Figure 41. LncRNA expression profile MS vs CTRLS. A) Cluster diagram of fold change values for the 84 investigated lncRNAs. The data obtained are relative and expressed as fold change (fold difference), which is normalised lncRNA gene expression in the multiple sclerosis (MS) group divided by normalised lncRNA gene expression in the control group. Fold change values in MS patients versus controls. Each square represents a single lncRNA. Green squares represent lower than median level of lncRNA expression; black squares represent median level of lncRNA expression and red squares represent higher than median level of lncRNA expression. B) The scatter plot compares the normalised expression of every gene on the array between cases and controls by plotting them against one another to quickly visualise large gene expression changes. The central line indicates unchanged gene expression.

	Fold Change		Fold Change
AIRN	5,3068	LINC00853	2,5117
ATXN8OS	2,1745	LINC-ROR	2,6185
BANCR	1,2839	LUCAT1	1,678
BCYRN1	5,185	MALAT1	3,5342
BDNF-AS	1,0803	MEG3	0,3145
BOK-AS1	71,3861	MEG9	4,2323
CCAT1	0,029	MIAT	4,6327
CCAT2	1,1483	MRPL23-AS1	2,0796
CDKN2B-AS1	0,5053	NAMA	13,2398
DANCR	1,0148	NEAT1	3,2396
DIO3OS	10,1154	NRON	1,9417
DISC2	0,8611	OIP5-AS1	0,9611
DLX6-AS1	17,1683	OTX2-AS1	2,3515
EGOT	3,9779	PANDAR	0,8223
EMX2OS	0,8704	PCAT1	0,9351
FAS-AS1	4,7581	PCGEM1	3,9904
FENDRR	2,0674	PRINS	2,1218
FTX	1,0268	PTCSC1	2,1646
GACAT1	5,6064	PTCSC3	1,2158
GAS5	2,0971	PTENP1	0,2454
GNAS-AS1	0,6296	PTENP1-AS	1,4238
H19	4,7603	RBM5-AS1	0,7594
HAR1A	1,3627	RMST	11,6429
HAR1B	2,3427	RPS6KA2-AS1	1,4139
HEIH	1,3031	SIX3-AS1	2,1714
HOTAIR	4,4783	SNHG16	1,023
HOTAIRM1	0,2611	SOX2-OT	21,3974
HOTTIP	1,1933	SPRY4-IT1	0,6499
HOXA11-AS	1,9499	ST7-AS1	0,1593
HOXA-AS2	0,5652	ST7-AS2	18,6688
HOXA-AS3	4,4473	TERC	3,5735
HULC	0,7393	TINCR	0,9667
IGF2-AS	1,4677	TMEM161B-AS1	2,7706
IPW	6,6666	TRERNA1	5,8405
JPX	7,7961	TSIX	3,3364
KCNIP4-IT1	0,9111	TUG1	4,6459
KCNQ1OT1	5,1331	TUNAR	29,3266
KRASP1	1,6577	TUSC7	3,967
FALEC	0,9365	UCA1	7,7836
LINC00570	5,0492	WT1-AS	3,7676
LINC00581	1,3383	XIST	6,4054
LINC00599	2,9659	ZFAS1	6,1643

Table 20. Fold regulation of lncRNA. Upregulated lncRNA are indicated in red, while the downregulated lncRNA are blue. The comparison is cases vs controls.

Results from array analysis led to the identification of the 6 most significantly deregulated lncRNA (expressed as fold increase/decrease over controls): Antisense of IGF2R non-protein coding RNA (AIRN) (5.30-fold increase over controls, p=0.04); FAS antisense RNA 1 (FAS-AS1) (4.76-fold increase over controls, p=0.02); Hox transcript antisense RNA (HOTAIR) (4.47-fold increase over controls, p=0.03); Non-protein coding RNA, associated with MAP kinase pathway and growth arrest (NAMA) (13.24-fold increase over controls, p=0.01); Translation regulatory long non-coding RNA 1 (TRERNA1) (5.84-fold increase over controls, p=0.01) and HOXA cluster antisense RNA 2 (HOXA-AS2) (0.56-fold increase over controls, p=0.04).

Considering the data divided by disease form, we can notice different profiles between RR-MS and PP-MS, compared with controls [Figure 42].
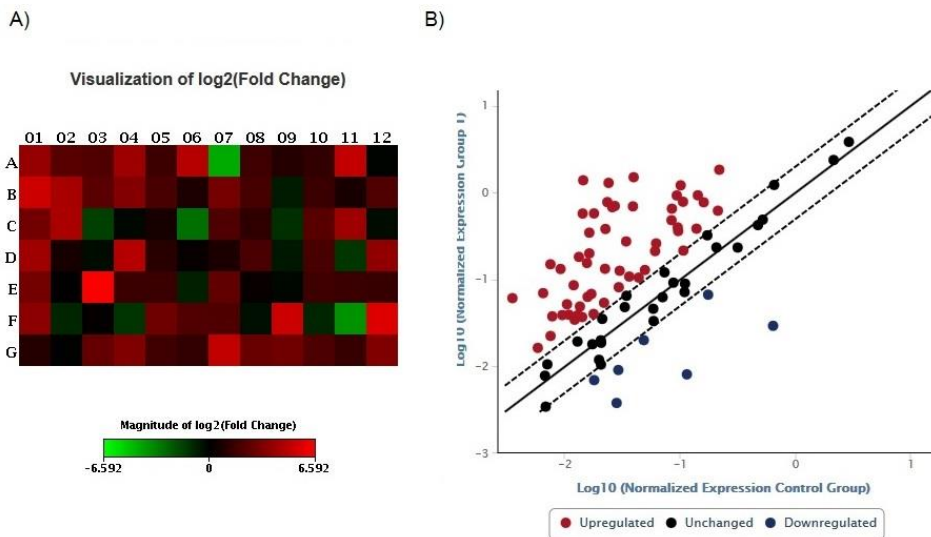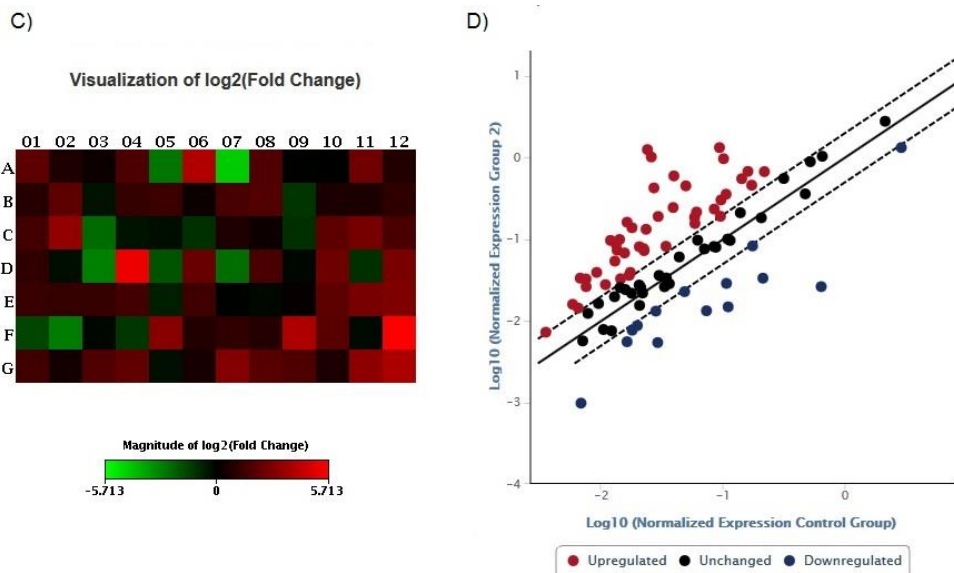
Figure 42. LncRNA expression profile. A) RR-MS vs CTRLS. C) PP-MS vs CTRLS A) and C) Cluster diagram of fold change values for the 84 investigated lncRNAs. The data obtained are relative and expressed as fold change (fold difference), which is the normalised lncRNA gene expression in the multiple sclerosis (MS) group divided by the normalised lncRNA gene expression in the control group. Fold change values in MS patients versus controls. Each square represents a single lncRNA. Green squares represent lower than median level of lncRNA expression; black squares represent median level of lncRNA expression and red squares represent higher than median level of lncRNA expression. A) RR-MS vs CTRLS; C) PP-MS vs CTRLS. B) and D) The scatter plot compares the normalised expression of every gene on the array between cases and controls by plotting them against one another to quickly visualise large gene expression changes. The central line indicates unchanged gene expression.

RR-MS patients showed a general upregulation of lncRNA, compared to controls [Figure 42 A) and B); Table 22]. In particular, 5 lncRNA are significantly deregulated, precisely AIRN (10.77-fold increase over controls, p=0.04); DLX6 antisense RNA 1 (DLX6-AS1) (46.95-fold increase over controls, p=0.01); FAS-AS1 (11.37-fold increase over controls, p=0.001); HOTAIR (9.31-fold increase over controls; p=0.02); and TRERNA1 (6.61-fold increase over controls, p=0.003).

PP-MS patients showed a different profile [Figure 42 C) and D); Table 23], where only SOX-2OT have a significant upregulation (8.95-fold increase over controls, p=0.02).

	Fold Change		Fold Change
AIRN	10,7651	LINC00853	4,2471
ATXN8OS	5,2368	LINC-ROR	3,3804
BANCR	4,2671	LUCAT1	0,9881
BCYRN1	20,1295	MALAT1	2,7999
BDNF-AS	2,7386	MEG3	0,4702
BOK-AS1	48,0805	MEG9	10,8322
CCAT1	0,0436	MIAT	8,9593
CCAT2	1,7744	MRPL23-AS1	1,2347
CDKN2B-AS1	1,3755	NAMA	53,1906
DANCR	2,6572	NEAT1	4,4288
DIO3OS	21,3012	NRON	5,3503
DISC2	0,7246	OIP5-AS1	1,0152
DLX6-AS1	46,9541	OTX2-AS1	8,6787
EGOT	12,9723	PANDAR	0,917
EMX2OS	3,235	PCAT1	0,6667
FAS-AS1	11,37	PCGEM1	2,6641
FENDRR	3,8904	PRINS	1,5821
FTX	2,0486	PTCSC1	1,9525
GACAT1	5,9534	PTCSC3	9,7282
GAS5	2,82	PTENP1	0,5375
GNAS-AS1	0,5297	PTENP1-AS	1,0671
H19	3,8354	RBM5-AS1	0,4567
HAR1A	1,0573	RMST	8,5755
HAR1B	2,7987	RPS6KA2-AS1	3,3255
HEIH	2,3203	SIX3-AS1	4,9221
HOTAIR	9,3173	SNHG16	0,7406
HOTAIRM1	0,3138	SOX2-OT	22,5515
HOTTIP	1,8277	SPRY4-IT1	0,2077
HOXA11-AS	1,9227	ST7-AS1	0,0573
HOXA-AS2	0,2533	ST7-AS2	12,7308
HOXA-AS3	5,3095	TERC	2,3263
HULC	1,7355	TINCR	0,8888
IGF2-AS	0,9722	TMEM161B-AS1	6,6464
IPW	5,7147	TRERNA1	6,6155
JPX	12,0409	TSIX	4,0493
KCNIP4-IT1	0,638	TUG1	5,3668
KCNQ1OT1	17,0463	TUNAR	38,6241
KRASP1	1,6006	TUSC7	5,3226
FALEC	1,2164	UCA1	7,3817
LINC00570	10,7635	WT1-AS	4,7688
LINC00581	3,2692	XIST	3,4567
LINC00599	2,1851	ZFAS1	5,4442

Table 21. Fold regulation of lncRNA in RR-MS patients compared with controls. Upregulated lncRNA are indicated in red, while the downregulated lncRNA are blue.

	Fold Change		Fold Change
AIRN	3,1964	LINC00853	0,4661
ATXN8OS	1,7115	LINC-ROR	2,8359
BANCR	1,2163	LUCAT1	1,3821
BCYRN1	3,8263	MALAT1	4,1873
BDNF-AS	0,1474	MEG3	0,5807
BOK-AS1	29,1424	MEG9	5,1786
CCAT1	0,0389	MIAT	2,6756
CCAT2	2,0739	MRPL23-AS1	2,6801
CDKN2B-AS1	0,7081	NAMA	1,2604
DANCR	1,079	NEAT1	4,3129
DIO3OS	3,6269	NRON	1,1726
DISC2	1,3188	OIP5-AS1	4,7397
DLX6-AS1	2,0876	OTX2-AS1	1,4662
EGOT	2,8172	PANDAR	0,7163
EMX2OS	0,487	PCAT1	0,8072
FAS-AS1	2,337	PCGEM1	3,698
FENDRR	2,7356	PRINS	3,6759
FTX	1,4825	PTCSC1	5,2281
GACAT1	2,1912	PTCSC3	0,2703
GAS5	2,8588	PTENP1	0,1528
GNAS-AS1	0,389	PTENP1-AS	0,9262
H19	2,1069	RBM5-AS1	0,5039
HAR1A	1,075	RMST	9,6436
HAR1B	1,3461	RPS6KA2-AS1	1,3878
HEIH	0,8318	SIX3-AS1	2,6025
HOTAIR	4,3623	SNHG16	1,7563
HOTAIRM1	0,1865	SOX2-OT	8,9563
HOTTIP	1,5035	SPRY4-IT1	1,5976
HOXA11-AS	1,0451	ST7-AS1	0,6827
HOXA-AS2	0,8763	ST7-AS2	12,3155
HOXA-AS3	1,9817	TERC	3,3092
HULC	0,9396	TINCR	1,2213
IGF2-AS	1,1038	TMEM161B-AS1	3,8222
IPW	5,0004	TRERNA1	2,8968
JPX	4,246	TSIX	1,0657
KCNIP4-IT1	2,5651	TUG1	3,0932
KCNQ1OT1	2,0226	TUNAR	9,2619
KRASP1	0,8597	TUSC7	3,1577
FALEC	0,2012	UCA1	3,354
LINC00570	16,1472	WT1-AS	1,8258
LINC00581	0,4354	XIST	12,0989
LINC00599	9,2861	ZFAS1	7,7799

Table 22. Fold regulation of lncRNA in PP-MS patients compared with controls.
Upregulated lncRNA are indicated in red, while the downregulated lncRNA are in blue.

Moreover, we performed a direct comparison between the subgroups RR-MS and PP-MS [Figure 43; Table 23]. We found FAS-AS1 (4.87-fold increase over controls, p=0.015) and MRPL23 antisense RNA 1 (MRPL23-AS1) (2.68-fold increase over controls, p=0.038) upregulated in RR-MS, in comparison with PP-MS.

Figure 43. LncRNA expression profile RR-MS vs PP-MS. A) Cluster diagram of fold change values for the 84 investigated lncRNAs. The data obtained are relative and expressed as fold change (fold difference), which is the normalised lncRNA gene expression in the relapsing-remitting multiple sclerosis (RR-MS) group divided by the normalised lncRNA gene expression in the progressive MS (PP-MS). Each square represents a single lncRNA. Green squares represent lower than median level of lncRNA expression; black squares represent median level of lncRNA expression and red squares represent higher than median level of lncRNA expression. B) The scatter plot compares the normalised expression of every gene on the array between cases and controls by plotting them against one another to quickly visualise large gene expression changes. The central line indicates unchanged gene expression.

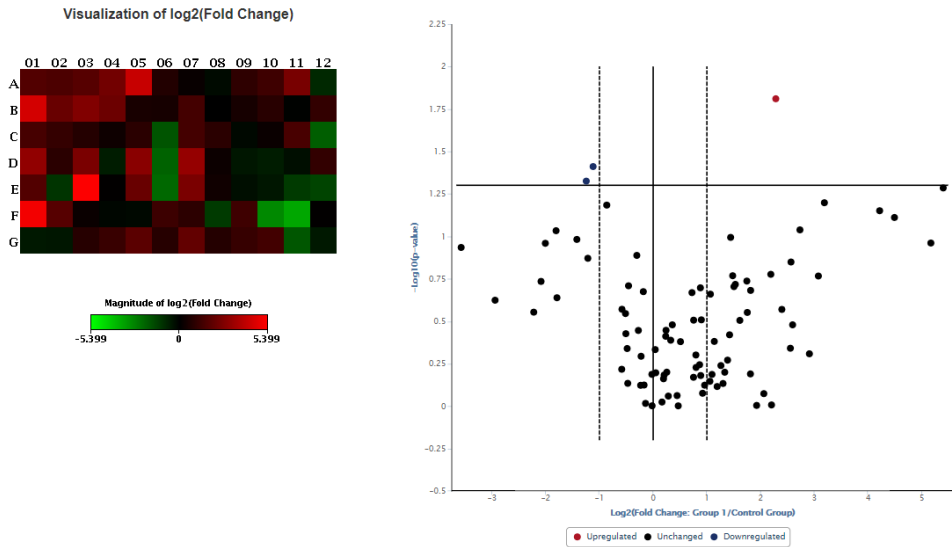|	Fold Change		Fold Change
AIRN	3,3679	LINC00853	9,111
ATXN8OS	3,0597	LINC-ROR	1,192
BANCR	3,5083	LUCAT1	0,7149
BCYRN1	5,2608	MALAT1	0,6687
BDNF-AS	18,5805	MEG3	0,8097
BOK-AS1	1,6498	MEG9	2,0917
CCAT1	1,1205	MIAT	3,3485
CCAT2	0,8556	MRPL23-AS1	0,4607
CDKN2B-AS1	1,9425	NAMA	42,2
DANCR	2,4626	NEAT1	1,0269
DIO3OS	5,8732	NRON	4,5628
DISC2	0,5495	OIP5-AS1	0,2142
DLX6-AS1	22,4917	OTX2-AS1	5,9193
EGOT	4,6046	PANDAR	1,2803
EMX2OS	6,6432	PCAT1	0,826
FAS-AS1	4,8652	PCGEM1	0,7204
FENDRR	1,4221	PRINS	0,4304
FTX	1,3819	PTCSC1	0,3735
GACAT1	2,717	PTCSC3	35,9878
GAS5	0,9864	PTENP1	3,5172
GNAS-AS1	1,3617	PTENP1-AS	1,1521
H19	1,8204	RBM5-AS1	0,9062
HAR1A	0,9836	RMST	0,8892
HAR1B	2,0792	RPS6KA2-AS1	2,3962
HEIH	2,7896	SIX3-AS1	1,8913
HOTAIR	2,1359	SNHG16	0,4217
HOTAIRM1	1,683	SOX2-OT	2,518
HOTTIP	1,2157	SPRY4-IT1	0,13
HOXA11-AS	1,8397	ST7-AS1	0,084
HOXA-AS2	0,289	ST7-AS2	1,0337
HOXA-AS3	2,6793	TERC	0,703
HULC	1,847	TINCR	0,7277
IGF2-AS	0,8808	TMEM161B-AS1	1,7389
IPW	1,1428	TRERNA1	2,2837
JPX	2,8358	TSIX	3,7995
KCNIP4-IT1	0,2487	TUG1	1,735
KCNQ1OT1	8,428	TUNAR	4,1702
KRASP1	1,8619	TUSC7	1,6856
FALEC	6,0448	UCA1	2,2008
LINC00570	0,6666	WT1-AS	2,6119

Table 23. Fold regulation of lncRNA in RR-MS patients compared with PP-MS.
Upregulated lncRNA are indicated in red, while the downregulated lncRNA are in blue.

Human Inflammatory response and autoimmunity array

The LncRNA expression profile performed by the Human RT^2 lncRNA Inflammatory Response & Autoimmunity array showed a general deregulation of lncRNA in MS patients, compared to controls [Figure 44]. We found 26 upregulated lncRNAs and 18 downregulated ones [Table 24].

Figure 44. LncRNA expression profile MS vs CTRLS. A) Cluster diagram of fold change values for the 84 investigated lncRNAs. The data obtained are relative and expressed as fold change (fold difference), which is the normalised lncRNA gene expression in the multiple sclerosis (MS) group divided by the normalised lncRNA gene expression in the control group. Fold change values in MS patients versus controls. Each square represents a single lncRNA. Green squares represent lower than median level of lncRNA expression; black squares represent median level of lncRNA expression and red squares represent higher than median level of lncRNA expression. B) The scatter plot compares the normalised expression of every gene on the array between cases and controls by plotting them against one another to quickly visualise large gene expression changes. The central line indicates unchanged gene expression.
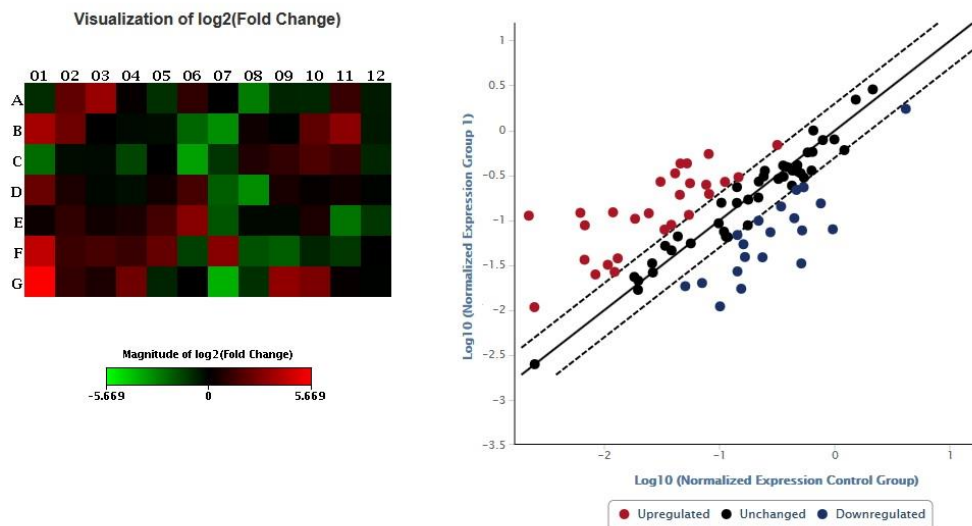
	Fold Change		Fold Change
A2ML1-AS1	0,5018	LRRC37BP1	0,2382
ABCA11P	4,3576	MALAT1	0,1126
AC000120.7	10,2979	MCM3AP-AS1	1,4428
AC007228.9	1,0808	MEG3	1,1072
MZF1-AS1	0,471	NAV2-AS5	1,2773
AC068196.1	2,073	NCBP2-AS2	0,9445
LOC101927156	0,9937	NEAT1	1,2102
CEP83-AS1	0,1486	NUTM2A-AS1	2,121
CROCCP2	0,5738	OIP5-AS1	1,2777
CTC-444N24.11	0,5541	PDXDC2P	1,513
CTC-487M23.5	2,4051	RMST	2,8835
CTD-3185P2.1	0,6641	LOC100287846	8,118
DLEU2	12,9914	RP11-282O18.3	0,2675
EPB41L4A-AS1	5,3959	RP11-29G8.3	0,8711
ERICH1-AS1	1,0055	RP11-325K4.3	0,8929
LRRC75A-AS1	0,8592	RP11-363E7.4	1,5281
FGD5-AS1	0,8261	RP11-363G2.4	0,1645
FGF14-IT1	0,2047	RP11-367N14.3	0,4359
PSMA3-AS1	0,1093	RP11-38P22.2	19,5085
FOXN3-AS2	1,2358	RP11-399K21.11	2,4206
GAS5	0,9814	RP11-473I1.10	2,9837
GAS5-AS1	4,2531	RP11-473M20.16	2,3388
GRM5-AS1	8,7239	RP11-498C9.15	4,6608
HCG11	0,6855	RP11-549J18.1	0,3677
HCG18	0,1893	RP11-819C21.1	8,2466
HNRNPU-AS1	0,8358	RP11-84C13.1	0,2847
HOTAIR	0,8552	RP11-96D1.10	0,2358
HTR4-IT1	0,3363	RP1-239B22.5	0,5813
IQCF5-AS1	0,985	RP6-24A23.7	0,4186
JPX	0,083	SDCBP2-AS1	1,0119
LINC00094	0,4552	SENP3-EIF4A1	50,8655
LINC00116	1,6559	SIK3-IT1	2,1685
LINC00293	2,1804	SLC7A11-AS1	1,5442
LINC00324	3,2526	SNHG11	5,6271
SNHG20	2,3655	SNHG16	0,5705
LINC00421	0,5554	SNHG5	0,9969
LINC00635	4,9197	SNHG7	0,0648
LINC00657	1,5379	TP73-AS1	0,4822
LINC00662	0,9028	TUG1	9,3893
LINC00667	0,8033	XIST	6,806
LL22NC03-N27C7.	1,2884	ZFAS1	1,1431
LOC653160	2,9827	ZNRD1-AS1	0,9591

Table 24. Fold regulation of lncRNA in MS patients compared with controls.
Upregulated lncRNA are indicated in red, while the downregulated lncRNA are in blue.

In particular, MZF-AS1 (0.47-fold decrease over controls, p=0.03), CEP83 antisense RNA 1 (CEP83-AS1) (0.15-fold decrease over controls, p=0.02), RP11-282O18.3 (0.27-fold decrease over controls, p=0.02), RP11-84C13.1 (0.28-fold decrease over controls, p=0.04), Small nucleolar RNA host gene 7 (SNHG7) (0.064-fold decrease over controls, p=0.04) and TP73 antisense RNA 1 (TP73-AS1) (0.48-fold decrease over controls, p=0.04) were significantly downregulated, while RP11-38P22.2 (19.5-fold increase over controls, p=0.04) was upregulated.

Considering the disease subgroups, RR-MS patients showed a significant downregulation in RP11-363G2.4 (0.07-fold decrease over controls, p=0.008) and in TP73-AS1 (0.76-fold decrease over controls, p=0.02), while RP11-38P22.2 levels are upregulated (22.32-fold increase over controls, p=0.04) [Figure 45 A) B); Table 25]. We found a general downregulation in lncRNA expression analysed in PP-MS, in particular FGF14-IT1 (0.08-fold decrease over controls, p=0.007) and RP11-282O18.3 (0.14-fold decrease over controls, p=0.04) were significantly altered [Figure 45 C) D); Table 26].

C)

Visualization of log2(Fold Change)

Magnitude of log2(Fold Change)

−6.394   0   6.394

D)

Log10 (Normalized Expression Group 2)

Log10 (Normalized Expression Control Group)

● Upregulated   ● Unchanged   ● Downregulated
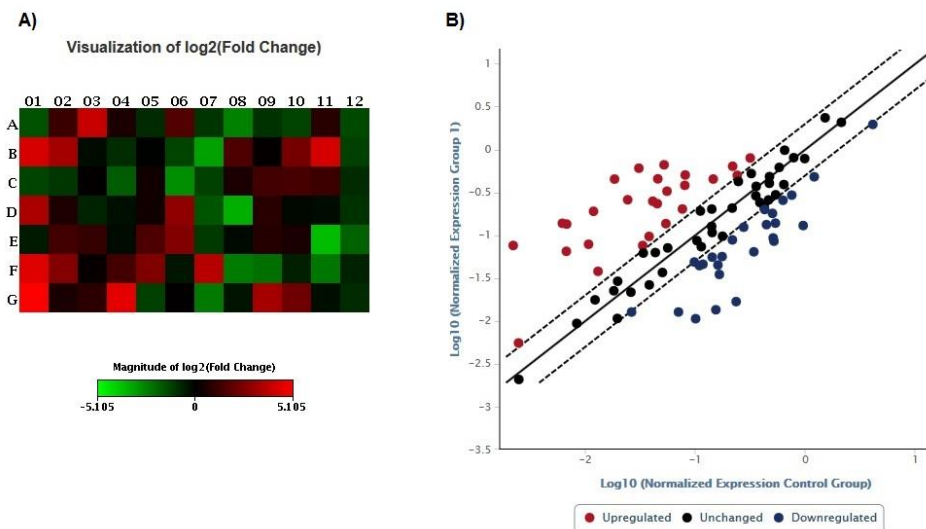
Figure 45. LncRNA expression profile. A) RR-MS vs CTRLS. C) PP-MS vs CTRLS A) and C) Cluster diagram of fold change values for the 84 investigated lncRNAs. The data obtained are relative and expressed as fold change (fold difference), which is the normalised lncRNA gene expression in the multiple sclerosis (MS) group divided by the normalised lncRNA gene expression in the control group. Fold change values in MS patients versus controls. Each square represents a single lncRNA. Green squares represent lower than median level of lncRNA expression; black squares represent median level of lncRNA expression and red squares represent higher than median level of lncRNA expression. A) RR-MS vs CTRLS; C) PP-MS vs CTRLS. B) and D) The scatter plot compares the normalised expression of every gene on the array between cases and controls by plotting them against one another to quickly

visualise large gene expression changes. The central line indicates unchanged gene expression.

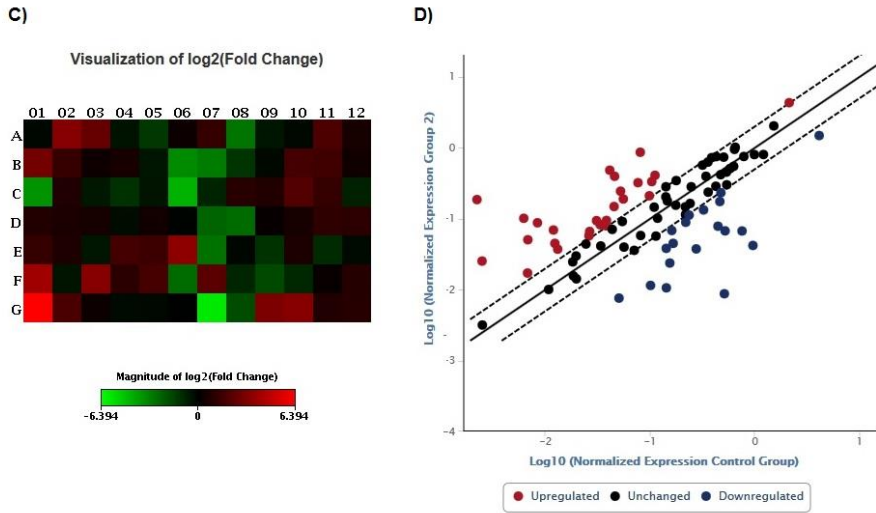|	Fold Change		Fold Change
A2ML1-AS1	0,3225	LRRC37BP1	0,3003
ABCA11P	2,2431	MALAT1	0,088
AC000120.7	16,0241	MCM3AP-AS1	1,724
AC007228.9	1,4789	MEG3	0,9007
MZF1-AS1	0,5586	NAV2-AS5	0,8355
AC068196.1	3,12	NCBP2-AS2	0,4986
LOC101927156	0,4837	NEAT1	0,6926
CEP83-AS1	0,1646	NUTM2A-AS1	2,5284
CROCCP2	0,5032	OIP5-AS1	2,08
CTC-444N24.11	0,3915	PDXDC2P	0,8395
CTC-487M23.5	1,7261	RMST	2,9103
CTD-3185P2.1	0,3585	LOC100287846	6,0858
DLEU2	19,984	RP11-282O18.3	0,4519
EPB41L4A-AS1	9,6772	RP11-29G8.3	0,8587
ERICH1-AS1	0,8369	RP11-325K4.3	1,6274
LRRC75A-AS1	0,5489	RP11-363E7.4	1,453
FGD5-AS1	0,9599	RP11-363G2.4	0,0717
FGF14-IT1	0,3904	RP11-367N14.3	0,2596
PSMA3-AS1	0,1058	RP11-38P22.2	22,3299
FOXN3-AS2	2,9225	RP11-399K21.11	6,2372
GAS5	1,0673	RP11-473I1.10	1,1224
GAS5-AS1	5,1926	RP11-473M20.16	2,5543
GRM5-AS1	19,7105	RP11-498C9.15	5,9175
HCG11	0,4071	RP11-549J18.1	0,7329
HCG18	0,391	RP11-819C21.1	12,76
HNRNPU-AS1	0,4688	RP11-84C13.1	0,1811
HOTAIR	1,0432	RP11-96D1.10	0,2109
HTR4-IT1	0,2804	RP1-239B22.5	0,6504
IQCF5-AS1	1,271	RP6-24A23.7	0,1887
JPX	0,136	SDCBP2-AS1	0,6254
LINC00094	0,4062	SENP3-EIF4A1	34,4077
LINC00116	1,4303	SIK3-IT1	1,4412
LINC00293	2,5259	SLC7A11-AS1	1,8454
LINC00324	2,6537	SNHG11	24,4853
SNHG20	2,2866	SNHG16	0,4093
LINC00421	0,5523	SNHG5	1,025
LINC00635	10,6959	SNHG7	0,1824
LINC00657	1,5138	TP73-AS1	0,7585
LINC00662	0,6118	TUG1	9,9741
LINC00667	0,7952	XIST	4,7576
LL22NC03-N27C7.	1,2394	ZFAS1	0,8137
LOC653160	7,3337	ZNRD1-AS1	0,5522

Table 25. Fold regulation of lncRNA in RR-MS patients compared with controls. Upregulated lncRNA are indicated in red, while downregulated lncRNA are in blue.

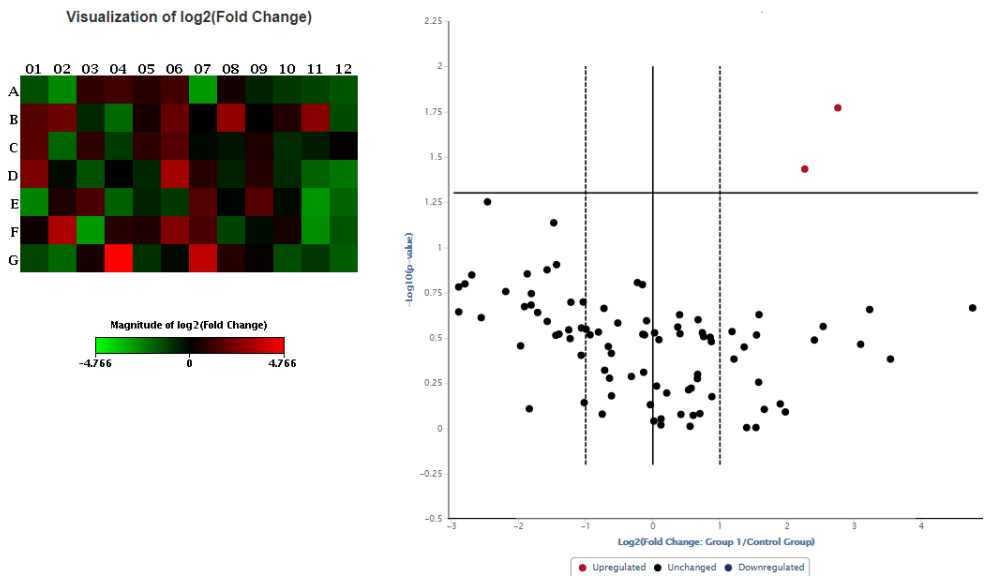|	Fold Change		Fold Change
A2ML1-AS1	0,8858	LRRC37BP1	0,1769
ABCA11P	10,2345	MALAT1	0,1546
AC000120.7	5,8325	MCM3AP-AS1	1,1476
AC007228.9	0,7221	MEG3	1,4436
MZF1-AS1	0,3783	NAV2-AS5	2,2047
AC068196.1	1,2255	NCBP2-AS2	2,1474
LOC101927156	2,5076	NEAT1	2,4803
CEP83-AS1	0,1302	NUTM2A-AS1	1,6921
CROCCP2	0,6792	OIP5-AS1	0,6828
CTC-444N24.11	0,8663	PDXDC2P	3,2267
CTC-487M23.5	3,6841	RMST	2,8494
CTD-3185P2.1	1,4669	LOC100287846	11,7581
DLEU2	7,4678	RP11-282O18.3	0,1363
EPB41L4A-AS1	2,5463	RP11-29G8.3	0,8873
ERICH1-AS1	1,273	RP11-325K4.3	0,4127
LRRC75A-AS1	1,5285	RP11-363E7.4	1,6305
FGD5-AS1	0,681	RP11-363G2.4	0,478
FGF14-IT1	0,0893	RP11-367N14.3	0,8486
PSMA3-AS1	0,1141	RP11-38P22.2	16,3983
FOXN3-AS2	0,4086	RP11-399K21.11	0,7168
GAS5	0,8811	RP11-473I1.10	10,4876
GAS5-AS1	3,2905	RP11-473M20.16	2,0882
GRM5-AS1	3,059	RP11-498C9.15	3,429
HCG11	1,3396	RP11-549J18.1	0,1515
HCG18	0,0745	RP11-819C21.1	4,7048
HNRNPU-AS1	1,7577	RP11-84C13.1	0,5094
HOTAIR	0,6624	RP11-96D1.10	0,2722
HTR4-IT1	0,4248	RP1-239B22.5	0,5031
IQCF5-AS1	0,7097	RP6-24A23.7	1,1658
JPX	0,044	SDCBP2-AS1	1,8785
LINC00094	0,5269	SENP3-EIF4A1	84,0804
LINC00116	1,9989	SIK3-IT1	3,6668
LINC00293	1,8047	SLC7A11-AS1	1,228
LINC00324	4,2254	SNHG11	0,8496
SNHG20	2,471	SNHG16	0,8745
LINC00421	0,5596	SNHG5	0,9619
LINC00635	1,8126	SNHG7	0,0172
LINC00657	1,5695	TP73-AS1	0,2693
LINC00662	1,4888	TUG1	8,6874
LINC00667	0,8137	XIST	10,785
LL22NC03-N27C7.	1,3541	ZFAS1	1,7696
LOC653160	0,9381	ZNRD1-AS1	1,9506

Table 26. Fold regulation of lncRNA in PP-MS patients, compared with controls.
Upregulated lncRNA are indicated in red, while the downregulated lncRNA are in blue.

Comparison of the exosomal lncRNA profile between RR-MS and PP-MS groups did not reveal any significant differences [Figure 46, Table 27].

Figure 46. LncRNA expression profile RR-MS vs PP-MS. A) Cluster diagram of fold change values for the 84 investigated lncRNAs. The data obtained are relative and expressed as fold change (fold difference), which is the normalised lncRNA gene expression in the relapsing-remitting multiple sclerosis (RR-MS) group divided by the normalised lncRNA gene expression in the progressive MS (PP-MS). Each square represents a single lncRNA. Green squares represent lower than median level of lncRNA expression; black squares represent median level of lncRNA expression and red squares represent higher than median level of lncRNA expression. B) The scatter plot compares the normalised expression of every gene on the array between cases and controls by plotting them against one another to quickly visualise large gene expression changes. The central line indicates unchanged gene expression.

	Fold Change		Fold Change
AIRN	0,3671	LINC00853	1,6892
ATXN8OS	0,1699	LINC-ROR	0,6525
BANCR	1,8398	LUCAT1	1,5952
BCYRN1	2,3113	MALAT1	0,593
BDNF-AS	1,6665	MEG3	0,2844
BOK-AS1	2,2655	MEG9	0,2188
CCAT1	0,1347	MIAT	0,1812
CCAT2	1,2927	MRPL23-AS1	1,4693
CDKN2B-AS1	0,6514	NAMA	2,6343
DANCR	0,487	NEAT1	0,2851
DIO3OS	0,4289	NRON	0,6393
DISC2	0,3359	OIP5-AS1	0,4783
DLX6-AS1	2,9011	OTX2-AS1	2,9177
EGOT	3,9304	PANDAR	0,9373
EMX2OS	0,6078	PCAT1	2,9814
FAS-AS1	0,2551	PCGEM1	0,9096
FENDRR	1,3237	PRINS	0,1436
FTX	3,7292	PTCSC1	0,2793
GACAT1	1,018	PTCSC3	1,1545
GAS5	6,7614	PTENP1	9,3867
GNAS-AS1	1,0389	PTENP1-AS	0,1346
H19	1,5176	RBM5-AS1	1,6257
HAR1A	5,8091	RMST	1,4851
HAR1B	0,3796	RPS6KA2-AS1	5,3039
HEIH	3,1612	SIX3-AS1	2,5678
HOTAIR	0,2734	SNHG16	0,4256
HOTAIRM1	1,8296	SOX2-OT	0,8526
HOTTIP	0,477	SPRY4-IT1	1,3365
HOXA11-AS	1,8084	ST7-AS1	0,1541
HOXA-AS2	2,9941	ST7-AS2	0,3354
HOXA-AS3	0,9179	TERC	0,4194
HULC	0,8013	TINCR	0,2656
IGF2-AS	1,4467	TMEM161B-AS1	1,3182
IPW	0,5702	TRERNA1	27,2082
JPX	0,6963	TSIX	0,5229
KCNIP4-IT1	1,0649	TUG1	0,9018
KCNQ1OT1	4,8038	TUNAR	11,6426
KRASP1	0,8984	TUSC7	1,5879
FALEC	0,3592	UCA1	1,0869
LINC00570	0,9747	WT1-AS	0,3697

Table 27. Fold regulation of lncRNA in RR-MS patients, compared with PP-MS.
Upregulated lncRNA are indicated in red, while downregulated lncRNA are in blue.

5. DISCUSSION

Emerging evidence has revealed that lncRNAs play a pivotal role in the regulation of immunological functions and autoimmunity [135]. In order to highlight the role of these molecules in MS, we conducted an in-depth investigation of the cellular and exosomal lncRNA expression profile in different cohorts of MS patients using different methods.

In order to reduce any potential confounding factors, blood withdrawal was performed early in the morning between 8.00 am and 10.00 am, and all patients were not under immunomodulating or anti-inflammatory therapies at the time of sampling.

The results from cellular lncRNAs showed a general dysregulation of their expression levels in MS patients, compared to controls, in the exploratory analysis cohort, both considering the overall MS population, compared to controls, and when comparing lncRNA levels stratified according to the different MS forms. The further validation step performed in independent Italian MS and control populations confirmed the dysregulation observed in the initial exploratory step.

Among the validated lncRNAs, we further focused on NRON, TUG1 and GOMAFU for the subsequent replication step in the Belgian cohort. These lncRNAs were chosen because of their possible implication in the pathogenesis of MS due to their involvement in inflammatory and neurodegenerative processes. NRON and TUG1 were replicated in the Belgian cohort, whereas GOMAFU did not pass the replication step, despite a trend toward downregulation of its expression level in patients.

NRON is an lncRNA repressor of the nuclear factor of activated T cells (NFAT), which interacts with members of the importin-beta superfamily and acts as specific regulator of NFAT nuclear trafficking [112]. NFATs regulate the transcriptional induction of genes encoding for immune modulators/activators, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFNγ), CD5, CD25, CD28, CD40, interleukin- (IL-) 2, IL-3, IL-4, IL-5, IL-13, IL-8 [185]. In T lymphocytes, NFAT proteins govern gene expression that regulates their development, activation, differentiation, as well as the induction and maintenance of T-cell tolerance. Furthermore, data from murine and cell line models highlight the role of NFAT/Ca^{2+} calmodulin pathway in CNS. In particular, it participates in signalling cascades that are pivotal for Schwann cell myelination [185]. Dietz et al. also demonstrated that NFAT1 and NFAT2 deficit attenuate EAE in animal models, underscoring the importance of this pathway in the pathogenesis of MS [186].

NRON expression level in PBMC of MS patients was found to be lower in cases than in controls. Therefore, we investigated the expression level of its target gene *NFAT*, following the hypothesis of regulation of *NFAT* expression orchestrated by NRON. However, we did not find any significant differences in NFAT expression levels in the MS cases compared (data not shown) and no correlation was found with NRON expression levels. The NRON/NFAT pathway is a complex; hence, other alternative mechanisms could probably be involved in this regulation.

TUG1 is expressed in the developing retina and brain, showing the highest levels in the cortex. It is involved in the regulation of cell cycle and apoptotic processes mediated by p53 [187]. Indeed, *TUG1* expression is activated after DNA damage, and its promoter presents several p53 binding sites.

When it is associated with polycomb repressive complex 2 (PRC2), it acts as repressor for a number of cell cycle genes [109]. Moreover, in the context of the neurodegenerative disease, it was found to be deregulated in patients suffering from the trinucleotide repeat neurodegenerative condition [88].

A robust downregulation of *TUG1* expression levels was observed in MS patients, compared to controls. Interestingly, by stratifying the results according to the disease form, the lowest expression levels were observed in the PP-MS. TUG1, shown to be involved in the apoptotic-p53 pathway, could play a key role in MS disease progression [188].

Despite these results, Santoro et al. showed an opposite trend of TUG1 expression in serum from MS patients [91]. The reason could be the different biological source chosen for the analysis or a possible active role of TUG1 in intercellular communication. Its increased free circulating levels could originate from the release in the cellular milieu by exosomes, an enriched source of non-coding RNA.

GOMAFU, alias *MIAT*, is predominantly expressed in the CNS [119], where it regulates the differentiation of neural stem cells into oligodendrocytes [117]. GOMAFU can bind the splicing factor 1 (SF1) protein through its UACUAAC repeat sequences. This sequence is a much stronger intron branch point sequence than found in most mammalian introns. Moreover, *in vitro* GOMAFU binding to SF1 can inhibit splicing and spliceosomal complex formation, suggesting that it can regulate splicing efficiency [114]. Dysregulation of GOMAFU leads to alternative splicing patterns that resemble those observed in schizophrenia for the archetypal schizophrenia-associated genes *DISC1* and *ERBB4* [189].

Although in the Italian cohort GOMAFU showed a strong downregulation in MS patients, compared to controls, including RR-MS and PP-MS forms, we did not replicate our results in the Belgian population. It could be due to the small number of patients, probably too low to explain the variability of the population.

Moreover, it is interesting to notice that NRON correlates with the disease duration, while ANRIL and TUG1 positively correlate with the disability measured by EDSS. This last aspect can be due to the progression of neurodegeneration. The association of non-coding RNA levels with EDSS and disease duration has been previously reported [85,86]. This prompts not only a possible involvement of these molecules in MS mechanisms, but also an important role as biomarkers for MS progression.

Regarding the study of lncRNA extracted from exosomes, we performed two parallel analyses using two different commercial arrays, Human LncFinder RT^2 array and Human Inflammation response and autoimmunity array (QIAGEN).

The analysis conducted with Human LncFinder RT^2 array showed an overall upregulation of lncRNA in MS patients, in comparison with healthy controls. In particular, 7 lncRNA were significantly deregulated, and some of these could be linked with MS pathology.

The antisense of the insulin-like growth factor 2 receptor (*IGF2R*) non-protein coding RNA (AIRN) overlaps the second intron of the insulin-like growth factor 2 receptor gene on the opposite strand. A CpG island associated with the second intron denotes an active promoter that drives the expression of this gene. The related mouse gene is responsible for silencing the *IGF2R* gene and flanking genes in the imprinted gene cluster of mice [190]. IGF2,

like the other insulin-like growth factors (IGFs), is a neurotrophic factor, and promotes survival and differentiation of neuronal cells. Its receptor, IGF2R, was studied in amyotrophic lateral sclerosis (SLA), where it showed upregulation in reactive astrocytes in the spinal cord of transgenic rats [191].

IGF2R is expressed in murine T lymphocytes. In particular, Yang et al. observed IGF2R expression in more than 90% of murine and human T_{reg} cells but in less than 10% of effector $CD4^+$ T cells. The activation of this receptor induces T_{reg} cell proliferation and the release of TGFβ, with a consequent immune suppressive effect [192].

Geng et al. described the anti-inflammatory role of IGF2R also in B cells. They conducted a study in ovalbumin (OVA)-specific B cells and showed how IGF2 markedly enhanced the expression of IL-10 in these cells [193]. Moreover, a study about the presence of IGF2R in MS astrogliotic plaques revealed that this receptor is not present in them, excluding their involvement in astrogliosis [194].

In MS, B_{reg} and T_{reg} cell functions are altered [195], and microglia-mediated inflammation is a key point in the progression of the disease. We found that upregulation of AIRN was able to repress *IGF2R*, and this suggests that *IGF2R* expression could be downregulated in the target cells of neural exosomes. The immune cells could be the effector cells and their activity could consequently be deregulated, with a persistent inflammatory status. Indeed, it is interesting to note that our data underscored how AIRN is upregulated in the RR-MS form, which is typically characterised by a robust inflammation.
A second lncRNA we found that significantly upregulated not only in all MS patients, but also in the subgroup of RR-MS, in comparison with controls,

was FAS antisense RNA 1 (FAS-AS1). Moreover it showed the same significant upregulation also in the RR-MS group, in comparison with PP-MS. FAS-AS1 might regulate the expression of alternative Fas splice forms through pre-mRNA processing [196]. A recent study showed that *FAS-AS1* is regulated by NF-κB, where decreasing NF-κB activity levels were tracked with increasing transcription of *FAS-AS1* during human erythropoiesis [197].

Fas and its ligand, FasL, are two molecules that belong to the TNF family, and are able to activate the caspase cascade and induce apoptosis in cells. Fas-FasL interaction is important in the regulation of immune homeostasis, and controls the tolerance mechanism. Indeed, it is required for the death of autoreactive T and B cells [198,199]. Moreover, the high expression of FasL in Th1 and its low expression in Th17 regulate the different cell death sensitivity of these immune cells [200]. In the MS contest, when FAS-AS1 is expressed in high levels, the expression of Fas can be downregulated and, finally, the autoreactive lymphocytes, Th1 and Th17 cells, can escape the programmed cell death and persist in inflamed sites.

Fas-FasL could also play a protective role by regulating T_{reg} cells. These immune cells express low levels of FasL, probably because their prolonged survival is important to dampen the immune reaction.

Fas-FasL is important in immune privileged brain tissue. The expression of FasL by BBB cells favours an immune-suppressive environment in the CNS [200]. Therefore, FAS-AS1-mediated alteration of these processes might reduce the inflammatory processes.

In the CNS, MS patients present an increase in Fas, depending on exogenous IL-22. Then, Fas increases phosphorylation of mitogen- and stress-activated protein kinase 1 and activates the nuclear factor-κB pathway in oligodendrocytes, leading to an increase in Fas and oligodendrocyte apoptosis [201]. Since Fas-FasL interaction is also present in neurons and

in oligodendrocytes, they can be other possible target cells of exosomes. In this case, FAS-AS1 could reduce their death, reducing Fas levels.

HOX transcript antisense RNA (HOTAIR) showed an upregulation both in MS and RR-MS patients, in comparison with controls. HOTAIR is located within the Homeobox C (*HOXC*) gene cluster on chromosome 12 and is co-expressed with *HOXC* genes. It functions through an RNA product, which binds lysine-specific demethylase 1 (LSD1) and Polycomb repressive complex 2 (PRC2), and acts as a scaffold to assemble these regulators at the *HOXD* gene cluster, thereby promoting epigenetic repression of *HOXD*. To date several studies have underscored its role in tumours, such as glioma, but not in autoimmune diseases. Therefore, further analyses are required to clarify the involvement of HOTAIR in MS. HOXA cluster antisense RNA 2 (HOXA-AS2) was downregulated in MS patients, in comparison with controls. HOXA-AS2 is located between the *HOXA3* and *HOXA4* genes in the *HOXA* cluster. These genes are expressed in human CD34+ T cells [202] and are involved in erythropoiesis and in cancer. Moreover, a number of genes of the *HOXA, HOXB*, and *HOXC* appear to play a role in lymphoid cells. A recent study showed that HOXA-AS2 is an apoptosis repressor in all trans retinoic acid-treated NB4 promyelocytic leukaemia cells [203], but no data about its connection with autoimmune diseases are reported in the literature.

Likewise, data are scarce on the non-protein coding RNA associated with the MAP kinase pathway and growth arrest (NAMA), which is upregulated in cases and not in controls in our study. It seems involved in thyroid carcinoma [204,205], but it has not been studied in detail so far.

The translation regulatory long non-coding RNA 1 (TRERNA1), which is upregulated in the MS group, was studied in cancer. Indeed, it is involved in metastasis promotion and in invasion by regulating various pro-invasive

proteins. It acts as an enhancer and is able to regulate the expression of SNAI1, its neighbour gene [206]. No data about its link with autoimmunity or neurodegeneration has been published in Literature.

In RR-MS patients AIRN, FAS-AS1, HOTAIR, OIP5-AS1 and TRERNA showed the same deregulation as the total MS group. Only one lncRNA presented a different expression, the DLX6 antisense RNA 1 (DLX6-AS1). Up to now, it has been studied in cancer [207] but there is no significant report about the relationship between lncRNA DLX6-AS1 and MS. Its target gene *DLX6* encodes a member of a homeobox transcription factor gene family. This family comprises at least 6 different members that encode proteins with roles in forebrain and craniofacial development. Mutations in *DLX6* have been described in autism [208].

Instead, Sox 2 overlapping transcript (SOX2-OT) was significantly up-regulated in the PP-MS group. *SOX2OT* is highly expressed in brain and is located in chr3q26.33, which is frequently amplified in cancer tissue [209]. Little is known about its exact role. Recent studies have described it as a transcription regulator. Similar to *SOX2, SOX2-OT* is highly expressed in embryonic stem cells and downregulated upon the induction of differentiation. Indeed, SOX2-OT is involved in neurogenesis [96,210,211]. New data are required to better understand its role in MS.

The analysis conducted using Human LncRNA RT[2] Inflammation response and Autoimmunity array showed an overall downregulation of lncRNA in MS patients, in comparison with healthy controls.

Myeloid Zinc Finger 1 antisense RNA1 (MZF1-AS1) is the regulator of MZF1, a SCAN-Zinc Finger (SCAN-ZF) transcription factor family member, which has been studied for tumours [212]. MZF1 is physiologically involved in early

myeloid lineage differentiation and pro-inflammatory effector function, and pathologically in the aetiology of different solid cancer [212,213]. Interestingly, MZF1-AS1 is downregulated in MS patients. Consequently MZF1 should be upregulated, and this should compete to support the inflammatory processes.

The target of CEP83-AS1 has been studied always in the field of cancer. CEP83 is a centriolar protein that is involved in primary cilium assembly. Not only is it involved in colorectal cancer [214], but also in infantile nephronophthisis and intellectual disability [215]. Our study underscored downregulation of CEP83-AS1 in the MS group, but its biological meaning in MS pathogenesis is not known.

The *RP11-282O18.3* gene encodes pre-mRNA processing factor 31 (PRPF31), a component of the spliceosome complex. Defects in this gene lead to a state of generalised splicing dysfunction. PRPF31 has been associated with retinal dystrophy, a group of diseases characterised by degeneration of the retinal cells [216]. We observed its downregulation in the MS group and in the PP-MS subgroup. Considering PRPF31 involvement in the degeneration of photoreceptor cells, and since deregulation specifically occurs in the PP-MS group, we could theorise a possible role of this lncRNA in neurodegeneration. Further studies are needed to clarify this hypothesis.

As *RP11-282O18.3*, FGF14 intronic transcript 1 (FGF14-IT1) was downregulated in the PP-MS subgroup, in comparison with controls. It is the neighbour of the fibroblast growth factor 14 (FGF14), and probably regulates its expression. FGF14 is expressed in the axon initial segments (AIS) of hippocampal pyramidal neuron structures. It is also located at the AIS in different types of cerebellar neurons. It is involved in the cortico-mesolimbic circuit, in particular in neurogenesis, plasticity and in synaptic transmission

[217,218]. Mutations in this gene have been associated with spinocerebellar ataxia 27 (SCA27), an autosomal-dominant disorder characterised by gait and movement disorders, nystagmus, and cognitive impairment [219,220]. *FGF14* has also been associated with complex brain disorders as a result of its role in neurodegeneration processes [219]. It is interesting to note that we found FGF14-IT1 altered in the PP form. Indeed, inflammatory processes are in the background in this form of the disease, while neurodegeneration is evident. FGF14 expression is deregulated consequently to low levels of FGF14-IT1, and this situation could sustain the neurodegenerative mechanisms.

Small nucleolar RNA host gene 7 (SNHG7) is downregulated in MS patients, in comparison with controls. SNHG7 has a role in the cellular response to radiation-induced oxidative stress [221]. In cancer, it has been reported as promoter of proliferation, migration and invasion, and apoptosis inhibitor [222]. To date, their functions in the autoimmune and neurodegenerative frameworks is unknown.

Also TP73-AS1 presented downregulation in the MS group and in the RR-MS subgroup, compared with controls. It is the antisense of the coding gene tumour protein p73 (*TP73*), which encodes a product that shares structural and functional characteristics with TP53 [223,224]. *TP73-AS1* covers substantial portions of *TP73*, suggesting that *TP73-AS1* may function by post-transcriptional regulation of *TP73* gene expression [224]. *TP73* encodes a transcription factor that belongs to the p53 family and is involved in cellular responses to stress and development. Many transcript variants resulting from alternative splicing and/or use of alternate promoters have been found for the *TP73* gene, but the biological validity and nature of the full length of some variants have not been determined.

Wong et al. described TP73-AS1 downregulation in plasma cells from patients presenting multiple myeloma [225], but its involvement in MS disease needs to be investigated.

lncRNA MALAT1 expression levels are downregulated both in PBMC and in neural-derived exosomes. Conversely, H19, HULC, MEG9, NRON, GOMAFU, TUG1 and XIST expression levels showed opposite directions, although there is no significant correlation between the deregulation of cellular lncRNA and neural-derived exosomal ones. This suggests that there are differences in the mechanisms that underpin the origin of these molecules.

In PBMC, lncRNAs directly regulate gene expression. Dysregulation in their expression levels could primarily contribute to the disease.

Instead, as cargo of neural-derived exosomes, lncRNA plays a different role. Indeed, they are involved in cell-to-cell communication between different target cells. Through endocytosis, they could incorporate lncRNA, which will adjust gene expression and cause an effector cell response. Therefore, the biological meaning of lncRNA levels altered by PBMC or neural-derived exosomes is different. However, the comprehensive investigation of both of them could elucidate the effective role of lncRNA in MS pathogenesis.

6. CONCLUSIONS

Overall these data suggest that epigenetic regulation is carried out in a different way in pathogenic pathways that lead to a pro-inflammatory form rather than to a neurodegenerative one.

This is the first large study about cellular and exosomal lncRNA in the MS framework. We analysed the expression profile of lncRNA derived from PBMC and neural exosomes. Regarding PBMC, we validated and replicated screening data in various populations, using different methods, and we identified 2 mainly deregulated lncRNAs, precisely NRON and TUG1.

Then, we performed neuron-derived exosome extraction and studied their lncRNA content. These acquire remarkable importance in cell-to-cell communication and, as messenger, can support the pathogenic processes. We do not actually know what the target cell is, but neurons and immune cells are the most likely candidates. In this context, AIRN and FAS-AS1 have a biological significance that is linked with MS. The others have to be studied in depth to highlight their role in MS processes. The use of commercial arrays allowed to screen the main lncRNA known to be involved in the inflammatory response and in autoimmunity. A limit of these arrays is sensitivity in detecting small amounts of lncRNA. Indeed, all of our controls showed an "undetermined" measure of Ct for different lncRNAs, and we do not actually know if that molecule was not expressed or whether the method was unable to detect it. In the first case, some of the lncRNA analysed could be expressed only in the pathogenic context and not in the physiological one. In the second case, we need a more sensitive method. Therefore, these data should be validated and replicated in a larger population, using a different

method. In the future, it will be interesting to also study the lncRNA pattern in glial-derived exosomes.

The aim of this study was to identify the possible lncRNA that is deregulated in MS, in order to find a good biomarker of disease progression to be used in a clinical setting. In this context, only after the molecules have been identified, and after seeing their clinical utility would we be interested in their biological functions and in their role in MS pathology. Thus, future studies are required to better clarify the role of deregulated lncRNA in MS. However, lncRNAs profiling could thus represent a new challenge in the search for easily detectable biomarkers of disease susceptibility and progression.

7. APPENDIX 1

Exact p-values of validation and replication analysis

lncRNA	p- values Validation Analysis			p-values Replication analysis
	MSvsCTRLS	CTRLsvsRR	CTRLsvsPP	MSvsCTRLS
ANRIL	9.07E-03	1.79E-03	NS	NA
BACE1-AS	0.0006373	0.0001275	0.0004598	NA
GOMAFU	0.0001312	0.0001401	0.0001383	0.8937
H19	NS	NS	NS	NA
HULC	0.0003072	0.0008527	0.0002085	NA
MALAT1	0.01324	NS	NS	NA
MEG9	0.04426	NS	NS	NA
NRON	0.02134	0.05792	NS	0.01409
NESPAS	NS	NS	NS	NA
SOX2-OT	0.000398	0.000725	NS	NA
TUG1	2.61E-02	1.35E-02	NS	2.75E-04
XIST	0.0000512	0.000169	NS	NA

Table 28. Exact p-values of validation and replication analysis by Wilcoxon-Mann Whitney test. NS=not significant; NA=not available
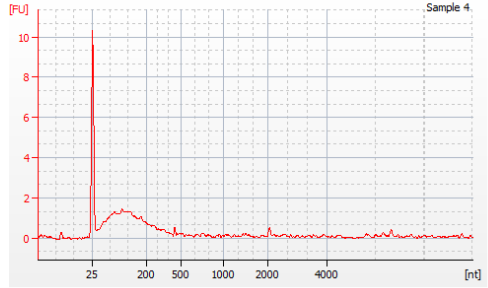
Exosomal RNA analysis by Bioanalyzer (Agilent)

Sample	[RNA] (ng/µL)
1	12.38
2	19.70
3	12.99
4	21.34
5	12.18
6	14.67
7	15.62
8	19.79
9	12.54
10	15.52
11	18.55
12	12.83
13	13.53
14	10.65
15	13.50
16	8.80
17	13.23
18	10.25
19	13.63
20	12.94
21	13.00
22	12.40
23	12.05
24	5.90
mean	**13.67**
SD	**3.50**

Table 29. Exosomal RNA concentration for each sample

155
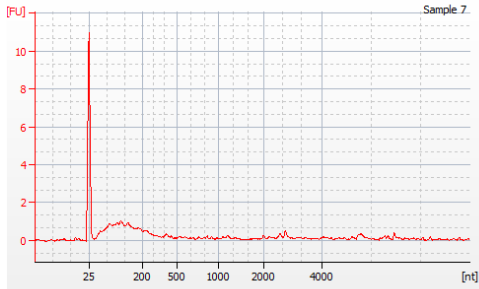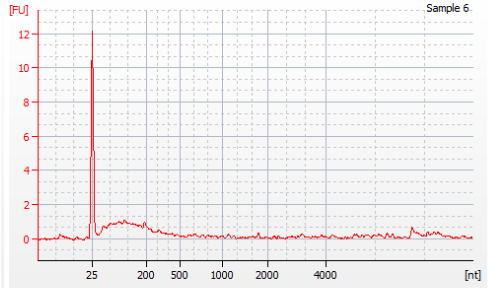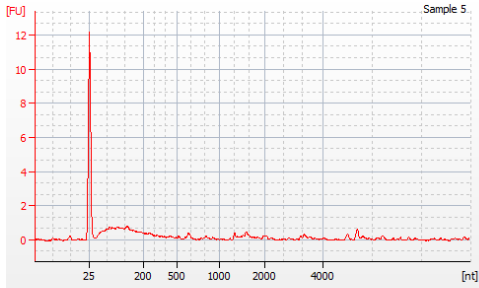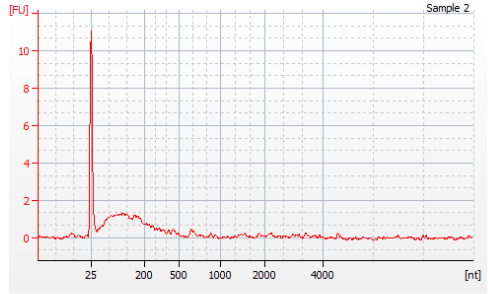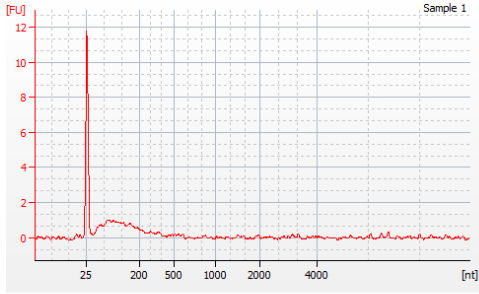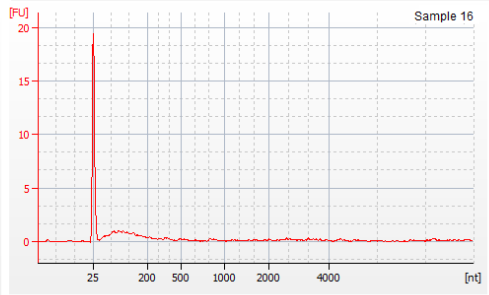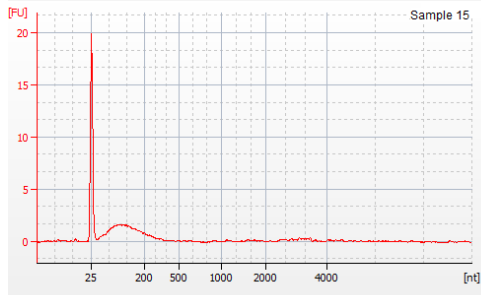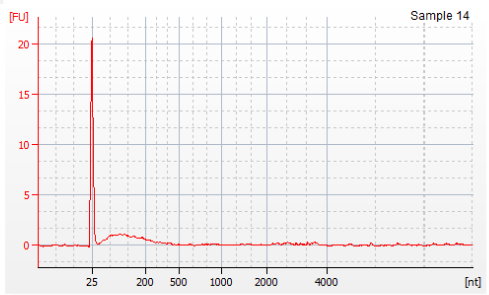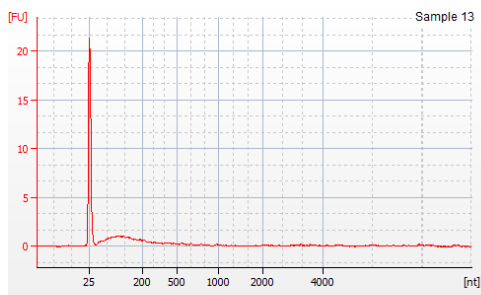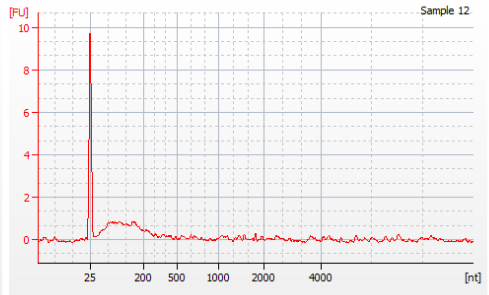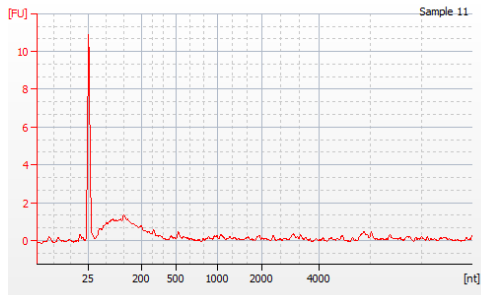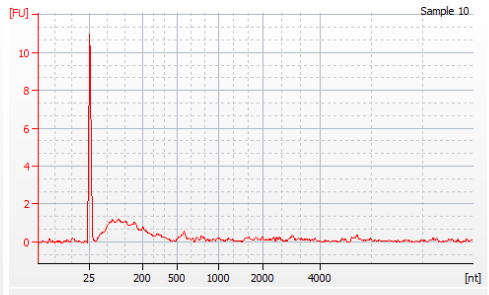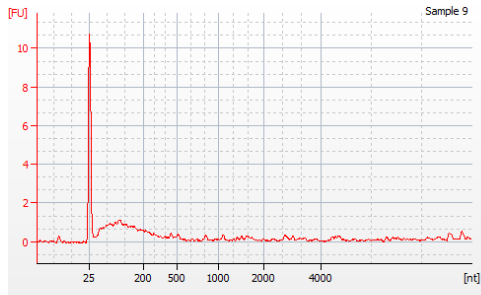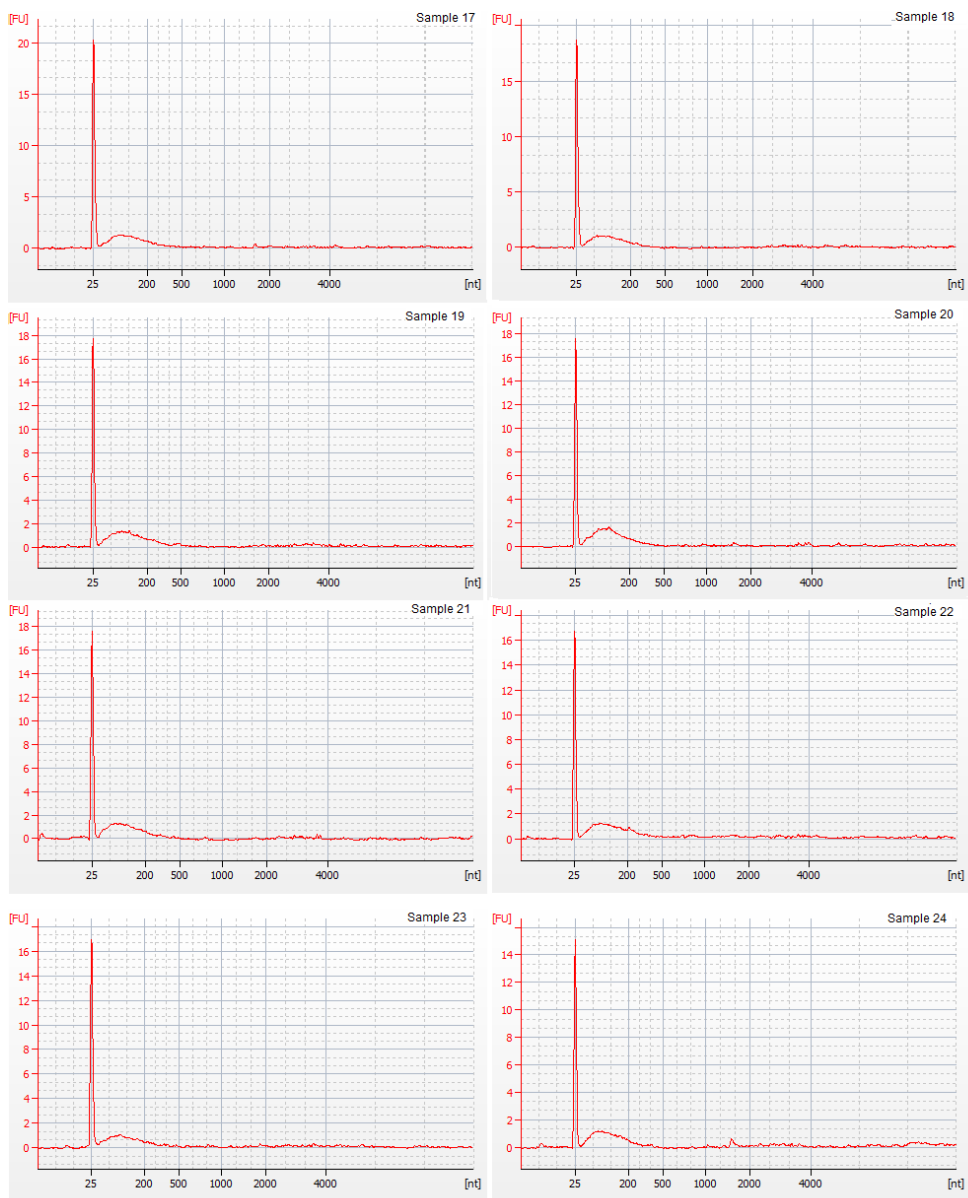
Figure 47 Bioanalyser electropherograms of total RNA isolated from L1CAM-exosomes.
The highest peak is the marker peak, while the lowest are the sncRNA and lncRNA peaks.
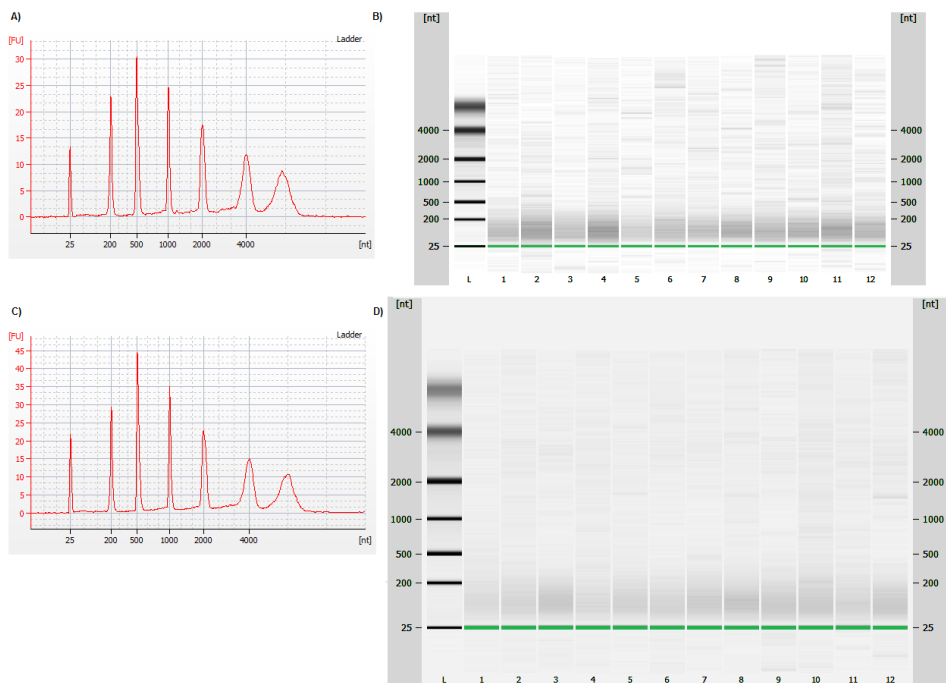
Figure 48 Bioanalyzer analysis of L1CAM-exosomal RNA. A) and C) Ladder electropherogram. We can see the 6 peaks referred to the known-length RNA fragments. B) and D) Gel run of the 24 samples.

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8.2 Web

- ExoCarta: www.exocarta.org

- Long Noncoding RNA Database v2.0:The Reference Database For Functional Long Noncoding RNAs www.lncrnadb.org

- LNCpedia v. 4.1: www.lncpedia.org

- NONCODE: http://www.noncode.org

- Ensembl: www.ensembl.org

- NCBI Gene : www.ncbi.nlm.nih.gov/gene

- GeneCards human gene database v.4.5.0: http://www.genecards.org

9. SCIENTIFIC PRODUCTS

9.1 Publications

Oldoni E, Fumagalli G, Serpente M, Fenoglio C, Scarioni M, Arighi A, Bruno G, Talarico G, Confaloni A, Piscopo P, Nacmias B, Sorbi S, Rainero I, Rubino E, Pinessi L, Binetti G, Ghidoni R, Benussi L, Grande G, Arosio B, Bursey D, Kauwe JS, Cioffi SM, Arcaro M, Mari D, Mariani C, Scarpini E, Galimberti D.
"PRNP P39L Variant is a Rare Cause of Frontotemporal Dementia in Italian Population".
J Alzheimers Dis. 2015;50(2):353-7. doi: 10.3233/JAD-150863.

Fenoglio C, De Riz M, Pietroboni AM, Calvi A, Serpente M, Cioffi SM, Arcaro M, **Oldoni E**, Scarpini E, Galimberti D.
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Pietroboni AM, Schiano di Cola F, Scarioni M, Fenoglio C, Spanò B, Arighi A, Cioffi SM, **Oldoni E**, De Riz MA, Basilico P, Calvi A, Fumagalli GG, Triulzi F, Galimberti D, Bozzali M, Scarpini E.
"CSF β-amyloid as a putative biomarker of disease progression in multiple sclerosis."
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Galimberti D, Cioffi SM, Fenoglio C, Serpente M, Oblak AL, Rodriguez-Porcel F, **Oldoni E**, Hagen MC, Arcaro M, Scarpini E, Ghetti B, Espay AJ.
"Rapidly progressive primary progressive aphasia and parkinsonism with novel GRN mutation."
Mov Disord. 2017 Mar;32(3):476-478. doi: 10.1002/mds.26872.

Calvi A, De Riz M, Pietroboni A, Ghezzi L, Arighi A, Fumagalli G, Basilico P, Scarioni M, Vergari M, Nigro M, **Oldoni E,** Fenoglio C, Galimberti D, Scarpini E and Priori A.
"Neurophysiological Profile, Walking Performance Tests and Self-Reported Questionnaires in Spastic Patients with MS: A Pilot Study"
J Mult Scler (Foster City) 2016, 3:4 DOI: 10.4172/2376-0389.1000191

Vigna L, Solimena G, Bamonti F, Arcaro M, Fenoglio C, **Oldoni E**, Dellanoce C, Rossi P, Gori F, Figliola R, Cighetti G.
"Effects of 1-month R-α-lipoic acid supplementation on humans oxidative status: a pilot study"
Progress in Nutrition 2017; Vol. 19, N. 1: 14-25 - DOI: 10.23751/pn.v19i1.6325

Serpente M, Fenoglio C, Cioffi SMG, **Oldoni E**, Arcaro M, Arighi A, Fumagalli G, Ghezzi L, Scarpini E, Galimberti D
"Profiling of specific gene expression pathways in peripheral cells from prodromal Alzheimer's disease patients"
ACCEPTED in the Journal of Alzheimer's disease (JAD)

Fenoglio C*, **Oldoni E***, Serpente M, De Riz M, Comi C, Cantello R, Lecchi E, Calvi A, Pietroboni A, Arcaro M, D'Anca M, Cioffi SMG, Mallants K, Dubois B, Goris A, Galimberti D, Scarpini E
"LncRNA expression profile in Peripheral Blood Mononuclear Cells from Multiple Sclerosis patients"
SUBMITTED to Molecular Neurobiology

9.2 Abstracts:

E. Oldoni, M. Serpente, C. Fenoglio, S. M. G. Cioffi, M. Arcaro , A. Arighi, G. Fumagalli, M. Mercurio, E. Scarpini, D. Galimberti
P62/SQSTM1 genetic variability in an Italian population of FTLD patients
3rd WINTER SEMINAR ON DEMENTIA: "Recent advances in Clinical and Experimental Research on Dementia and Neurodegenerative Disorders" Brixen, January 21-23, 2015 (Oral Communication)

Oldoni E, Serpente M, Fenoglio C, Bursey D, Cioffi S, Arcaro M, Fumagalli G, Scarioni M, Arosio B, Mari D, Pinessi L, Rainero I, Rubino E, Cappa S, Marcone A, Cerami C, Nacmias B, Sorbi S, Confaloni A, Piscopo P, Bruno G, Benussi L, Ghidoni R, Scarpini E, Galimberti D.
PRNP P39L variant is a rare cause of frontotemporal lobar degeneration in an Italian FTLD population.
Neurological Sciences 36, Suppl.: S37, 2015. (Oral communication)

XLVI Congress of the Italian Neurological Society, October 10-13, 2015, Genova, Italy.

Brixen, January 21-23, 2015 (Oral Communication)

Journal of Alzheimer's Disease, vol. 52, no. s1, pp. S1-S97, 2016

XI Congress of Italian Society of Dementia Neurology (SINDEM), March 17-19, 2016, Italy

Scarioni M, Fumagalli G, Arighi A, Pietroboni A, De Riz M, Basilico P, Calvi A, Leta V, Ferrari M, Fenoglio C, **Oldoni E**, Serpente M, Galimberti D, Mariani C, Scarpini E.

A case report of frontotemporal dementia syndrome associated with recently discoveres N-terminal domain mutation in prion protein gene

Neurological Sciences 36, Suppl.: S103, 2015. (poster)

XLVI Congress of the Italian Neurological Society, October 10-13, 2015, Genova, Italy.

Cioffi S, Espay A, Fenoglio C, Serpente M, Arcaro M, **Oldoni E**, Ardizzoia R, Hagen M, Rodriguez-Porcel M, Scarpini E, Galimberti D.

The novel GRN g.1642_1645delTGAG mutation is associated with language and behavioral impairment, and parkinsonism.

Neurological Sciences 36, Suppl.: S164-165, 2015. (poster)

XLVI Congress of the Italian Neurological Society, October 10-13, 2015, Genova, Italy.

Serpente M, Fenoglio C, Cioffi S, **Oldoni E**, Arcaro M, Arighi A, Fumagalli G, Scarpini E, Galimberti D.

Profiling of specific gene expression pathways in peripheral cells from Alzheimer's disease patients.

Neurological Sciences 36, Suppl.: S312-313, 2015. (poster)

XLVI Congress of the Italian Neurological Society, October 10-13, 2015, Genova, Italy.

M. Serpente, C. Fenoglio, S. Cioffi, M. Arcaro, **E. Oldoni**, A. Arighi, G. Fumagalli, E. Rotondo, M. Mercurio, E. Scarpini, D. Galimberti.

Autosomal dominant and sporadic frontotemporal lobar degeneration: from non-coding RNAS to the identification of preclinical biomarkers and therapeutic targets.

XI Congress of Italian Society of Dementia Neurology (SINDEM), March 17-19, 2016, Florence Italy

M. Arcaro, A. Espay, C. Fenoglio, M. Serpente, S. Cioffi, **E. Oldoni**, R. Ardizzoia, M. Hagen, F. Rodriguez-Porcel, E. Scarpini, D. Galimberti
A novel GRN G.1642_1645 DELTGAG mutation is associated with frontotemporal lobar degeneration with TDP-43 inclusions (FTLD-TDP).

XI Congress of Italian Society of Dementia Neurology (SINDEM), March 17-19, 2016, Florence Italy

Maria Serpente, Chiara Fenoglio, Sara Cioffi, Marina Arcaro, **Emanuela Oldoni**, Andrea Arighi, Giorgio Fumagalli, Emanuela Rotondo, Matteo Mercurio, Elio Scarpini and Daniela Galimberti
Autosomal Dominant and sporadic Frontotemporal Lobar Degeneration (FTLD): from non-coding RNAs to the identification of preclinical biomarkers and therapeutic targets.

10th International Conference on Frontotemporal Dementias. August 31-September 2, 2016
Munich/Germany

C. Fenoglio, M. Serpente, S.Cioffi , M. Arcaro, **E. Oldoni**, G. Fumagalli, A. Arighi, P. Basilico, A. Cattaneo, L. Porretti, E. Scarpini, D. Galimberti
Altered expression of non-coding RNAs in neural-derived serum exosomes in patients with FTD

10th International Conference on Frontotemporal Dementias. August 31-September 2, 2016
Munich (Germany)

E. Oldoni , C. Fenoglio, M. Serpente, M. De Riz, C. Comi, E. Lecchi, A Calvi, AM. Pietroboni, M. Arcaro, SMG. Cioffi, Klara Mallants, Bénédicte Dubois , A. Goris , R. Cantello, D. Galimberti, E. Scarpini.

Long non coding RNA expression profile in Peripheral Blood Mononuclear Cells from Multiple Sclerosis patients: potential biomarkers of disease susceptibility and progression.

MSParis2017 7th Joint ECTRIMS-ACTRIMS meeting, 25-28 October 2017, Paris (France)

9.3 Training courses

Scientific Research Communication

Instructor: Maria Flora Mangano

Milan, June 29-30 July 6-7, 2016

University of Milan

GeneMapper training course

Instructor: Danilo Placenti

Milan, 5th november 2015

Mendeley to bibliographic menagement

Instructor: Elena Bernardini-Chiara Pagani.

Milan, 14th May 2015

University of Milan

General Linear Model and Experimental Design using "R"

Instructor: Prof. Rocco Micciolo

Milan, March 23-27, 2015

Department of Clinical Sciences and Community Health.

Branch of Medical Statistics, Biometry and Epidemiology "G.A. Maccacaro"

University of Milan

10. ACKNOWLEDGMENT