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Transcriptional activity of human endogenous retroviruses is higher at birth in inversed correlation with gestational age

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ABSTRACT

Human endogenous retroviruses (HERVs) have been studied in relation to the onset and/or progression of several diseases. However, increasing evidence highlights that they also have important physiologic roles, for instance they are involved in preimplantation embryonic growth and in placentation. We assessed the transcriptional activity of HERVs in PBMCs of healthy newborns, infants and children to gather further information on their potential physiological roles. mRNA expression of HERV-H, −K and −W was evaluated in PBMCs of 63 preterm newborns, 47 term newborns, 38 infants (1–24 months of age), and 36 children (25–131 months of age) using a PCR real time Taqman amplification assay and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression levels of HERV-H, −K, and −W were significantly higher at birth than in infancy and childhood. Furthermore, HERV activation was highest in preterm newborns and a significant inverse correlation was found between HERV transcripts and duration of pregnancy. The overexpression of HERVs at birth in inversed correlation with gestational age are further clues of their potential involvement in early life events.

1. Introduction

Human endogenous retroviruses (HERVs) constitute about 8% of the human genome. They were integrated into the germ cell DNA of primates over 30 million years ago following infections by exogenous retroviruses (Sverdlov, 2000). During evolution, the accumulation of mutations, deletions, and recombinations rendered them no longer infectious (Hohn et al., 2013). While most copies of HERVs are inactive, some are transcribed and encode original viral proteins which can generate new insertions able to stimulate or block the transcription of nearby cellular genes. For instance, inserted long terminal repeats (LTRs) can act as promoters to trigger expression of oncogenes or inactivation of tumor suppressor genes (Gotzinger et al., 1996; Katoh and Kurata, 2013). On the other hand, transcription of HERV elements can be suppressed by epigenetic factors, such as DNA methylation and heterochromatin-silencing by histone modifications. Given their potential pathologic effects, HERVs have been widely studied and proposed as possible cofactors in the aetiology of various diseases, such as cancer, autoimmune or neurodegenerative disorders (Banki et al., 1992; Moyes et al., 2007). However, increasing evidence documents that HERVs can also have important physiological roles, especially in the preimplantation embryonic growth (Grow et al., 2015) and in placentation (Gotzinger et al., 1996; Blond et al., 2000; Blaise et al., 2003; Katoh and Kurata, 2013). Information about HERV activation over time in the general population is limited, in particular in the first periods of life.

There are several families of HERVs; among these HERV-H, HERV-K and HERV-W are those most widely studied (Balestrieri et al., 2015). In order to gather targeted information on the activation of HERVs in the first periods of life, we evaluated the transcriptional activity of HERV-H, HERV-K, and HERV-W in peripheral blood mononuclear cells (PBMCs) from newborns of different gestational age and from healthy infants and children.

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Table 1
Characteristics of the study population with mean values and standard deviations (SD) of transcriptional activity of HERV-H, HERV-K and HERV W in PMBCs from four groups of subjects.

Number of subjects	M/F	Median gestational age (range) [days]	Median age (range) [months]	Groups	ΔCt (HERV H-GAPDH) mean value ± SD	ΔCt (HERV K-GAPDH) mean value ± SD	ΔCt (HERV W-GAPDH) mean value ± SD
63	33/30	249 (171–258)		A Preterm newborns	5.43 ± 0.52	6.15 ± 0.54	3.34 ± 0.42
47	19/28	276 (260–295)		B Full-term newborns	3.93 ± 0.63	4.52 ± 0.74	1.62 ± 0.45
38	19/19		5 (1.15–21.34)	C Infants	3.53 ± 0.63	3.68 ± 0.65	1.22 ± 0.67
36	23/13		68.42 (30.87–141.6)	D Children	3.52 ± 0.59	3.64 ± 0.65	1.34 ± 0.63

See text for statistical differences among means.

Fig. 1. HERV-H, HERV-K, and HERV-W transcriptional levels in PBMCs from cord blood of preterm and term newborns and from peripheral blood of infants and children. *Footnote*: Data are represented as box plot, depicting mild (black dot). Relative pol levels were assessed by real-time PCR and represented by ΔCt. Two-way Anova test was performed.

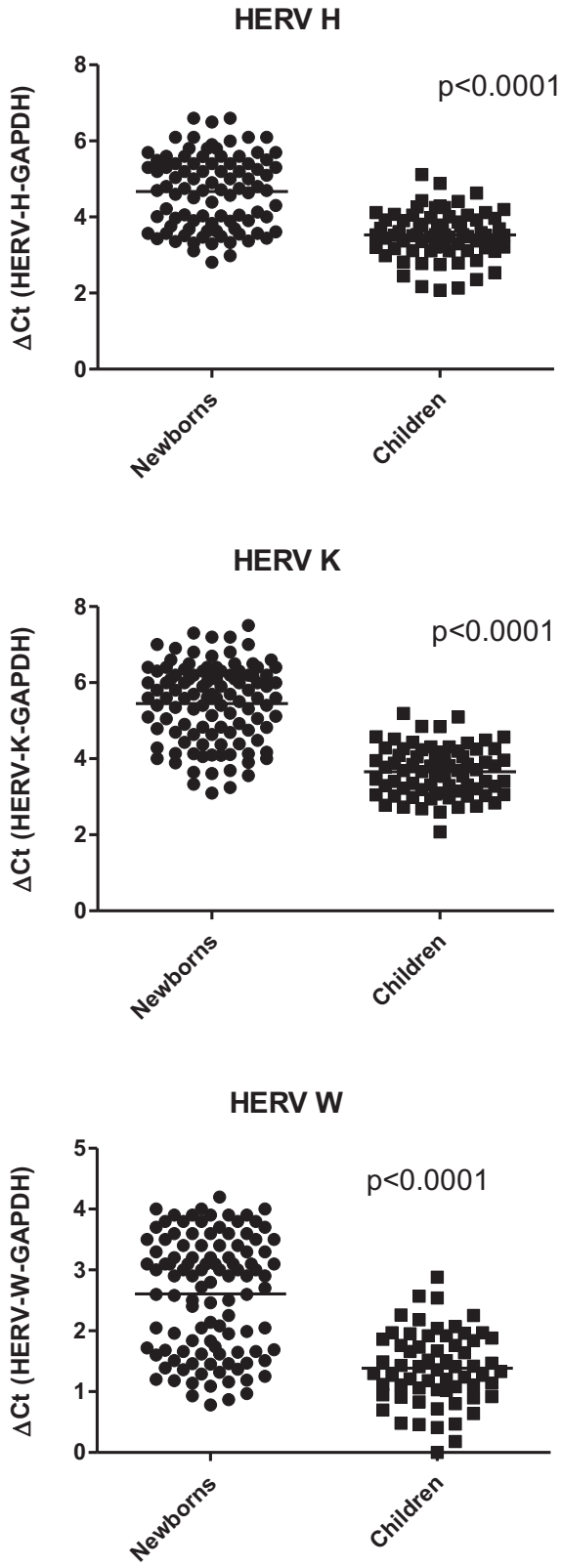

Fig. 2. Transcriptional levels of HERV-H, HERV-K, and HERV-W in PBMCs from newborns (irrespective of gestational age) and children (from 1 to 131 months of age).

2. Results

2.1. Transcriptional activity of HERVs

As detailed in Table 1, the study population consisted of 184 children (94 males, 90 females), with 63 Group A preterm newborns; 47 Group B term newborns; 38 Group C infants; and 36 Group D children.

HERV-H, HERV-K, and HERV-W were always transcriptionally active in the entire study population.

As illustrated in Fig. 1, the expression levels of *pol* genes of HERV-H, –K, and −W were significantly different in the four groups of subjects studied ($p < .0001$, by two-way ANOVA test). In particular, the activation of every HERV family was significantly higher at birth (Group A + Group B newborns) than in the subsequent periods of life (Group C infants + Group D children, ($p < .0001$; Fig. 2). Furthermore, preterm newborns (Group A) had transcriptional levels of every HERV family significantly higher than term newborns (Group B, $p < .0001$); with the latter showing transcriptional levels significantly higher than in infancy and childhood ($p = .0028$, $p < .0001$, and $p = .0294$ for HERV-H, HERV-K and HERV-W, respectively; Fig. 3). In contrast, postnatally no significant differences emerged between Group C infants and Group D children ($p = .8796$, $p = .9396$ and $p = .6392$ for HERV-H, HERV-K and HERV-W, respectively; Fig. 3).

The Spearman correlation analysis showed a significant inverse correlation between the HERV-K, −W, and −H transcription levels and gestational age: the higher the transcriptional levels the shorter the gestational age ($p < .001$, Fig. 4). Conversely, the HERV activation remained substantially stable during the first decade of life, with no significant correlations between transcriptional levels of HERV-K, −W, and −H and child's age ($p = .7347$, $p = .3075$, and $p = .5288$ for HERV-H, HERV-K and HERV-W, respectively; Fig. 5).

3. Discussion

The results of our study document that the *pol* genes of HERV-H, HERV-K, and HERV-W are always transcriptionally active in PBMCs, beginning from birth throughout the first decade of life. The expression levels of all the three HERV families were significantly higher in newborns; in particular, their activation was further increased in preterm newborns as compared to term newborns. This is accounted for by the significant inverse correlation between the HERV transcriptional activity and gestational age, demonstrating that HERV expression is higher in earlier phases of fetal life. Conversely, postnatally HERV activation decreased and remained substantially stable during infancy and childhood. This is consistent with minor variations of transcriptional activity of HERV-H, −K and −W observed by Balestrieri et al. (Balestrieri et al., 2015) testing children from the second year of life (apart higher levels of HERV-H in preschool age).

Most studies on HERVs have been focused on the their expression (and thus possible involvement) in a large array of diseases. Our results of enhanced HERV transcriptional activities during the intrauterine life, mainly in the earlier phases of pregnancy, further support the hypothesis that HERVs play important physiological roles during the fetal development. Indeed, some retroviral genes have been preserved from the mutational degeneration and their expressions are crucial during embryogenesis and placenta formation. For instance, syncytin-1 and syncytin-2 are envelope proteins, respectively of HERV-W and HERV-FRD (ERV-3), that mediate the fusion of the villous cytotrophoblast in the human placenta to form the syncytiotrophoblast layer (Gotzinger et al., 1996; Blond et al., 2000, Blaise et al., 2003, Katoh and Kurata, 2013). The syncytin genes are thus typical examples showing how host genome has co-opted retroviral genes for its own biological functions. In addition, Growth et al. demonstrated that HERV-K, the most recently acquired retroviral family, is transcribed during human embryogenesis beginning at the 8-cell stage, continuing through the pre-implantation blastocysts, and ceasing during the blastocyst outgrowths. Recently, a

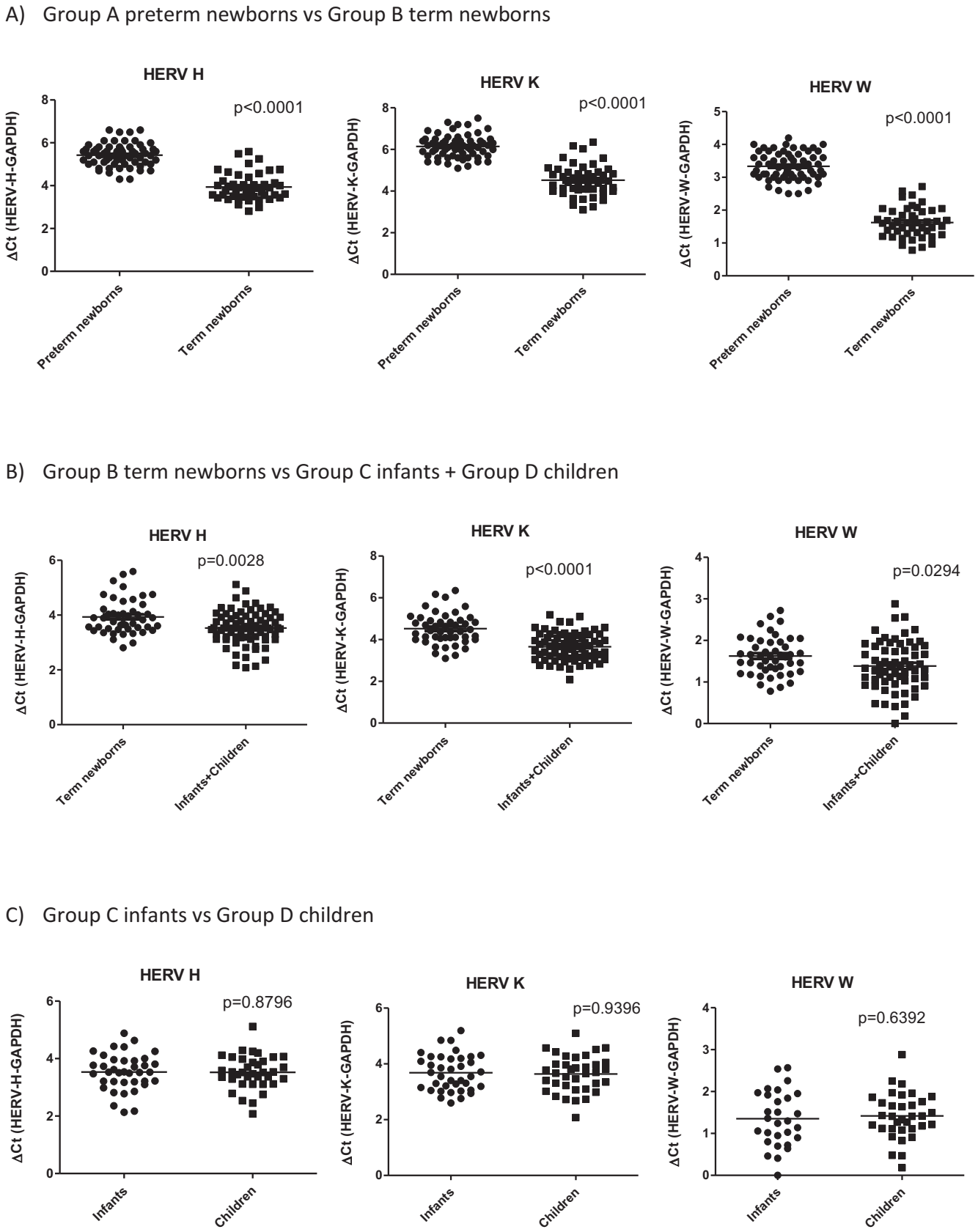

Fig. 3. Comparison of HERV-H, HERV-K, and HERV-W transcriptional levels in PBMCs of Group A preterm newborns, Group B term newborns, Group C infants, and Group D children.
Footnote: Statistical analysis by Mann-Whitney test.

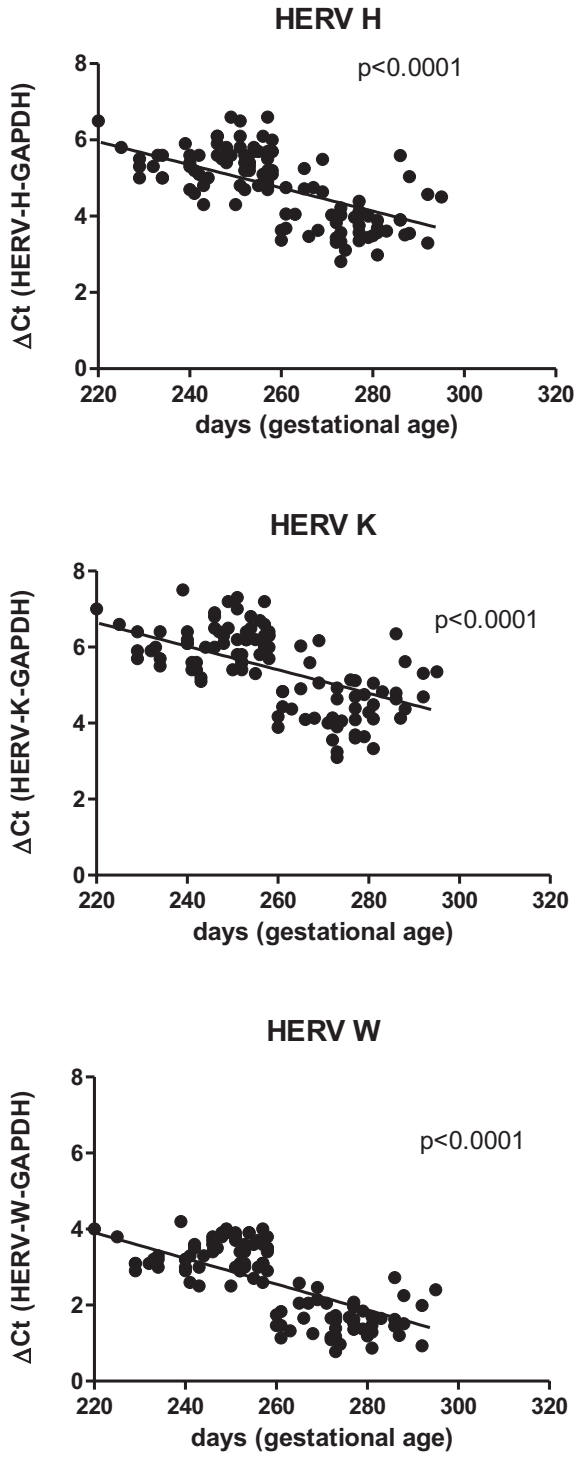

Fig. 4. Correlation of HERV-H, HERV-K, or HERV-W pol levels in PBMCs from cord blood according to gestational age.
Footnote: See Results session for correlation Spearman analysis details. Pol levels are represented by ΔCt.

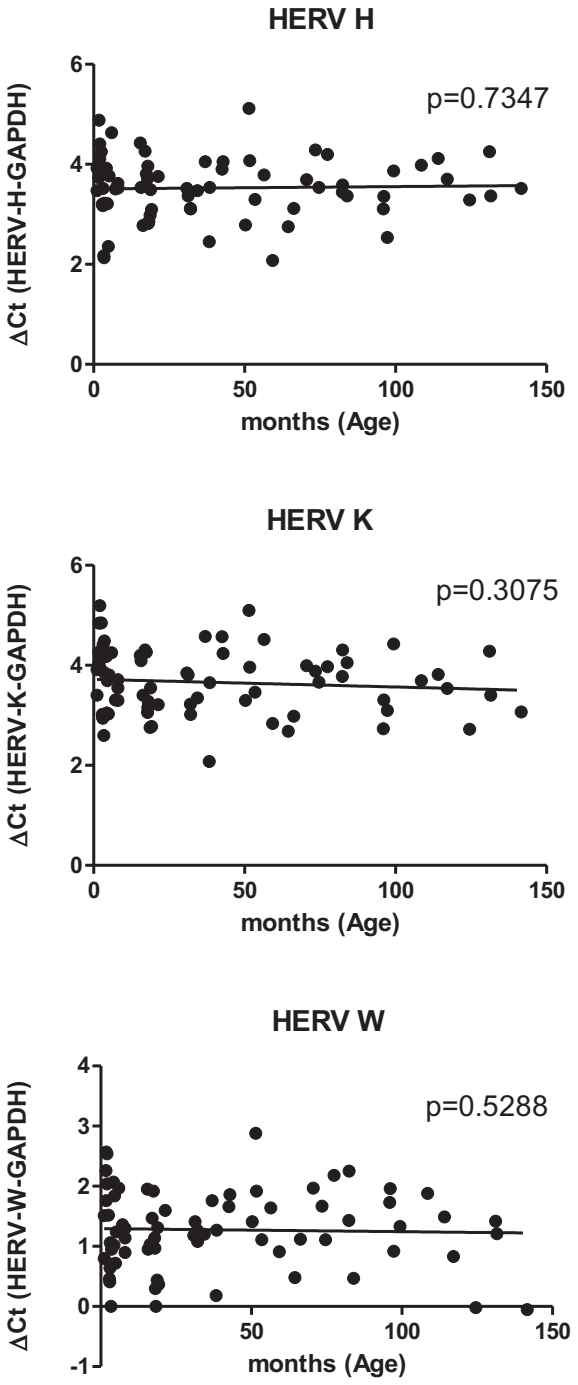

Fig. 5. Correlation of HERV-H, HERV-K, or HERV-W pol levels in PBMCs of infants and children according to age.
Footnote: See Results session for correlation Spearman analysis details. Pol levels are represented by ΔCt.

new tamed retroviral envelope produced from the fetus and then shed extracellularly into the maternal blood has been identified (Heidmann et al., 2017). Furthermore, the expression of several HERV genes is highest in the placenta (Sugimoto et al., 2013; Meyer et al., 2017), presumably due to the marked DNA hypometylation that characterizes the placenta tissues (Cotton et al., 2009; Hon et al., 2013; Schroeder et al., 2015). In addition, some HERV proteins have immunosuppressive properties (Mangeney et al., 2007; Meyer et al., 2017): the down regulation of the maternal immune response may thus favor the tolerance of the semi-allogenic fetus.

Provided that HERVs have important physiological roles during the intrauterine life, alterations of their activity might result in failures or abnormalities of pregnancy or in disturbaces of offspring development. A reduced expression of HERV envelope genes with fusogenic properties and putative placental functions have been found in patients with intrauterine growth restriction (Ruebner et al., 2010). In addition, as seen the syncytins regulate the development and mantainance of the syncytiotrophoblast that is responsible of the secretion of

corticotrophin releasing hormone, a hormone linked to gestational length determination. There is general agreement that HERV expression is regulated by epigenetic mechanisms. The different hormonal patterns occurring throughout pregnancy may represent typical temporary factors conditioning HERV activation. Indeed, among the epigenetic mechanisms modulating HERV transcription special emphasis has been put on the repressive effects of DNA methylation (Groh and Schotta, 2017). Knight et al. (2016) found that the DNA methylation levels in cord blood directly correlate with gestation age. Therefore, one can speculate that the DNA hypomethylation in the earlier phases of pregnancy is responsible for the higher transcription of endogenous retroviral elements observed in our study.

It is worth mentioning that the expression of HERV genes discloses enhanced levels in germ cells and pluripotent stem cells (Heidmann et al., 2017). In fact, accumulating evidence highlights that endogenous retroviruses can modulate stem cell biology with relevant effects in maintaining their pluripotency circuit and cell fate reprogramming (Yin et al., 2018). We assessed the HERV transcriptional levels in peripheral blood mononuclear cells (PMBCs) from cord blood, that is very rich of CD34+ hematopoietic and progenitor stem cells (HPSCs), and the concentration of these cells is significantly higher in preterm newborns than in term newborns (Wisgrill et al., 2014). Therefore, the higher proportion of CD34+ HPSCs in cord blood might explain, at least in part, the marked HERV activation in PBMCs at birth, most of all in preterm newborns, and its postnatal decrease in healthy infants and children.

In conclusion, after initial specific studies it became clear that endogenous retroviruses are not just junk DNA or passive bystanders. Subsequently, a number of targeted researches documented their activation in several diseases and drew attention on their possible pathologic effects. More recently an increasing evidence underlines that HERVs may also play important physiological roles. Our findings of overexpression of HERV genes in PBMCs of healthy newborns in inversed correlation with the duration of pregnancy are further clues of their potential involvement in early life events whose impact on the destiny of every individual might be critical (Magiorkinis et al., 2017).

4. Materials and methods

4.1. Study population and blood sample collection

The study population was arbitrary subdivided in 4 groups: Group A: preterm newborns (< 37 weeks of gestational age), Group B: full-term newborns (≥37 weeks of gestational age), Group C: infants (1–24 months of age), and Group D: children (25–131 months of age).

PBMCs were obtained from cord blood of healthy newborns of different gestational age with normal laboratory findings or from asymptomatic children who were tested at the Regina Margherita Children's Hospital, Turin, Italy, for routine laboratory examinations and whose results were all within the normal reference range. Children with any confirmed or suspected disease, such as infections, cancer, autoimmune disorders, inflammatory diseases, neurological disturbances, or abnormal laboratory results were excluded from the study. The tests were performed using leftovers of laboratory samples after informed parent's consent; data were gathered anonymously.

4.2. Reverse transcription

Total RNA was extracted from PBMCs using the automated extractor Maxwell (Promega, Madison, WI) using simply RNA Blood Kit protocol without modification. This kit provides treatment with DNase during the RNA extraction process. Four hundred nanogram of total RNA was reverse-transcribed with 2 µl of buffer 10×, 4.8 µl of $MgCl_2$ 25 mM, 2 µl ImpromII (Promega), 1 µl of RNase inhibitor 20 U/l, 0.4 µl random hexamers 250 µM (Promega), 2 µl mix dNTPs 100 mM (Promega) and dd-water in a final volume of 20 µl. The reaction mix was carried out in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems, Foster City, CA, USA) under the following conditions: 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C for the inactivation of enzyme; the cDNAs were stored at −80° until use. About control for genomic DNA contamination we amplify directly RNA extract without reverse transcription.

4.3. Relative quantification of HERV activities by real-time PCR

Relative quantification of mRNA expression of HERV-H, −K and −W was achieved by means of PCR real time Taqman amplification and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was chosen as a reference gene, using the ABI PRISM 7500 real time system (Life technologies, Texas, USA). 40 ng of cDNA were amplified in a 20 µl total volume reaction containing Go-Taq mastermix probe (Promega), 500 nmol of specific primers and 200 nmol of specific probe. The following primers and probe for *pol* genes were used: HERV-K primers (KPOLF-5'-CCACTGTAGAGCCTCCTAAACCC-3′) (KPOLR-5′-TTGGTAGCGGCCACTGATTT-3′) and probe (KPOLP-6FAM-CCCACACCGGTTTTTCTGTTTTCCAAGTTAA-TAMRA); for HERV-H primers (HPOLF-5′- TGGACTGTGCTGCCGCAA-3′) (HPOLR-5′-GAAGS-TCATCAATATATTGAATAAGGTGAGA-3′) and probe (HPOLP-6FAM-TTCAGGGACAGCCCTCGTTACTTCAGCCAAGCTC-TAMRA); HERV-W primers (WPOLF-5′-ACMTGGAYKRTYTTRCCCCAA-3′) (WPOLR-5′-GTAAATCATCCACMTAYYGAAGGAYMA-3′) and probe (WPOLP-6FAM-TYAGGGATAGCCCYCATCTRTTTGGYCAGGCA-TAMRA); GAPDH primers (GAPDHF-5′-CCAAGGTCATCCATGACAAC-3′) (GAPDHR-5′- GTGGCAGTGATGGCATGGAC-3′) and probe (GAPDH-6FAM- TGGTATCGTGGAAGGA-3′ MGB). The established assays use probes and primers designed by Primer Express™ software version 3.0 (Applied Biosystems, Foster City, USA). Basic Local Alignment Search Tool (BLAST) analysis confirm not cross-reaction between HERVs primer designed.

The amplifications were run in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Furthermore, in order to confirm that there was no DNA genomic contamination, control PCR was performed with RNA before reverse transcription using the same primers and probe described above. Each sample was run in triplicate. Relative quantification of target genes expression was performed with the ΔCt method. All HERVs pol expression data were normalized with GAPDH (housekeeping) expression. Using 40 ng of cDNA in amplification we obtained Ct value from 26 to 30.4. These Ct value correspond to a good performance of real time PCR. Since we measured Ct for all target in all the samples tested, we argued that ours methods is suitable for HERV detection and quantification.

4.4. Statistical analysis

Two-way ANOVA test and Mann-Whitney test were used to compare the transcriptional levels of every HERV family in different age groups. Spearman's rho correlation coefficient was calculated to determine any correlation between HERVs transcriptional levels and newborn's gestational age or, postnatally, child's age. Statistical analyses were done using the Prism software (GraphPad Software, La Jolla, CA). In all analyses, $P < .05$ was taken to be statistically significant.

Author contributions

MB and PT designed the study. VD, PM, AP and IG performed laboratory work. EB, FL and AC analyzed the data. All authors contributed to the writing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans, http://www.wma.net/en/30publications/10policies/b3/index.html., and informed consent was obtained in each case. As healthy controls (HC) 20 young blood donors were tested for each assay.

Informed consent

Informed consent was obtained from all individual participants included in this article.

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