Research paper

Grandmaternal cells in cord blood

Karlin R. Karlmark[a], Marina El Haddad[a], Xavier-Côme Donato[b], Gabriel V. Martin[a],
Florence Bretelle[c], Nathalie Lesavre[d], Jean-François Cocallemen[c], Marielle Martin[a],
Christophe Picard[e,f], Tiffany Albentosa[a], Jean Roudier[a,g], Raoul Desbriere[b],
Nathalie C. Lambert[a,*]

[a] INSERM UMRs 1097 Arthrites Autoimmunes, Aix Marseille Université, Marseille, France
[b] Department of obstetrics and gynecology, St Joseph Hospital, Marseille, France
[c] Department of Gynaecology and Obstetrics, Pôle Femme Enfant, AP-HM, Assistance Publique-Hôpitaux de Marseille, AMU, Aix-Marseille Université, France
[d] CIC1409, AMU, AP-HM, Marseille, France
[e] Centre National de la Recherche Scientifique (CNRS) UMR7268 (ADES), "Biologie des Groupes Sanguin", Marseille, France
[f] Etablissement Français du Sang (EFS), Marseille, France
[g] Service de Rhumatologie, Hôpital Sainte Marguerite, AP-HM, Marseille, France

ARTICLE INFO

ABSTRACTS

Background: During pregnancy a feto-maternal exchange of cells through the placenta conducts to maternal microchimerism (Mc) in the child and fetal Mc in the mother. Because of this bidirectional traffic of cells, pregnant women have also acquired maternal cells in utero from their mother and could transfer grandmaternal (GdM) cells to their child through the maternal bloodstream during pregnancy. Thus, cord blood (CB) samples could theoretically carry GdMMc. Nevertheless this has never been demonstrated.
Methods: Using Human Leukocyte Antigen (HLA)-specific quantitative PCR assays on three-generation families, we were able to test 28 CB samples from healthy primigravid women for GdMMc in whole blood (WB) and isolated cells (PBMC, T, B, granulocytes, stem cells).
Findings: Five CB samples (18%) had GdMMc which could not be confounded with maternal source, with quantities 100 fold lower than maternal Mc in WB and PBMC. Risk of aneuploidies and/or related invasive prenatal procedures significantly correlated with the presence of GdMMc in CB ($p=0.024$). Significantly decreased HLA compatibility was observed in three-generation families from CB samples carrying GdMMc ($p=0.019$).
Interpretation: Transgenerational transfer of cells could have implications in immunology and evolution. Further analyses will be necessary to evaluate whether GdMMc in CB is a passive or immunologically active transfer and whether invasive prenatal procedures could trigger GdMMc.
Funding: Provence-Alpes-Côte d'Azur APEX grant # 2012_06549E, 2012_11786F and 2014_03978) and the Foundation for Medical Research (FRM Grant #ING20140129045).

1. Introduction

More than sixty years ago, maternal cells were proven, with radio-active elements, to cross the placenta and were found in infant's peripheral blood or in cord blood (CB) units [1]. Since 1995, the presence of nucleated maternal cells in CB samples has been studied with non-invasive methods, such as Fluorescence in situ hybridization (FISH) or Polymerase Chain reaction (PCR) assays, with increasingly performant quantitative assays [2−10].

These technical feats made it possible to determine that maternal microchimerism (MMc) in a CB sample is not the only result of passive feto-maternal exchange of cells during pregnancy that will die quickly in the host. Maternal cells engraft and persist long-term in immunocompetent offspring and may have long term effect on the host's health [11]. Flow cytometry sorting cell strategies further showed that MMc could persist in different cell subsets such as T cells, B cells, monocytes, Natural Killer (NK) cells in the adult recipient [12]. Similarly, maternal cells can be found in different immune cell subsets from CB samples and in sorted hematopoietic progenitor cells (CD34+) with levels reaching up to 1.5% [4,5,13]. Allogeneic

* Corresponding author: Nathalie C. Lambert, Address: INSERM UMRs1097 Arthrites Autoimmunes, 163 avenue de Luminy, case 939, 13288 MARSEILLE, Cedex 09, Phone: +(33) 491-828-768
E-mail address: nathalie.lambert@inserm.fr (N.C. Lambert).

Research in Context

Evidence before this study

We reviewed the literature reporting on maternal cells in cord blood (CB) samples. From PubMed search realized until august 18th, 2021, date of submission of the current study, using the search terms "*maternal microchimerism*", AND "*cord blood*" in the title and/or the abstract, we could find 17 results with full text available. Six were excluded as they did not correspond to our query. To complete the search of the 11 remaining articles, we used the terms "*maternal cells*" AND "*cord blood*" and, to avoid redundancy, we excluded "*microchimerism*", giving 33 new results from which 23 were not appropriate and were excluded. Thus, at least 21 studies clearly confirm the presence of maternal microchimerism (Mc) in CB units.

Moreover, maternal microchimeric cells persist throughout postnatal development into adulthood. We searched PubMed using the terms "*maternal microchimerism*" in the title and/or the abstract and excluded "*cord blood*" to only have studies showing persistence in children or adults. We also filtered the results by selecting only *human* studies. We found 92 articles, from which 65 were excluded as they were related to fetal Mc (because of the keywords "feto-*maternal microchimerism*") or were reviews. The remaining 27 are pillar studies in the field of maternal Mc, showing either that maternal Mc is commonly present in healthy conditions, in fetuses, in infants or in adults, or proving that maternal cells can be of different cell types and can form an integral part of certain tissues/organs. The long term survival of maternal microchimeric cells and their differentiated phenotypes decades after delivery in the offspring suggests a passage of maternal stem cells. Maternal stem cells are likely stored in a host's biological niche, as it has been demonstrated for fetal Mc in the mother's bone marrow.

Starting from both accepted fact that i) maternal cells are commonly acquired in any individual through pregnancy and persist for decades, ii) maternal cells are present in cord blood, we hypothesized that persistent maternal Mc in pregnant women could be transferred to the next generation as grandmaternal Mc. Thus, cord blood should theoretically carry vertically-transferred grandmaternal cells. Nevertheless, although it had been suggested that grandmaternal cells could be found in grandchildren and/or CB, it had never been demonstrated as when we searched PubMed and Google Scholar using the terms "*grandmaternal*" AND "*chimerism*"; "*grand-maternal*" AND "*chimerism*" or, "*grandmaternal chimerism*" as well as with the search term "*cell*" instead of "*chimerism*", we did not find any matches.

Added value of this study

For the first time, we have detected grandmaternal Mc (GdMMc) in CB samples with specific and sensitive methods. We have quantified the equivalent number of grandmaternal cells present per million of CB cells. We have shown that the presence of GdMMc in CB samples correlated with i) the risk of aneuploidies and/or related invasive prenatal procedures and ii) decreased Human Leukocyte Antigen (HLA) compatibility in three-generation families.

Implications of all the available evidence

For the last three decades the use of banked umbilical cord blood units has increased access to hematopoietic stem cell transplantation for individuals who did not have a matched HLA donor available and needed such a curative treatment for cancer or inherited non-malignant diseases. Having found significant quantities of maternal cells in CB turned out to be beneficial for the outcome of the transplant. Finding now grandmaternal cells definitely changes our vision of CB samples. It remains to determine what the presence of grandmaternal cells means. Finding them in a particular family HLA relationship pleads in favor of an immunological role that will still have to be demonstrated.

hematopoietic stem cells from CB samples are increasingly used as a curative treatment for many cancers and inherited non-malignant diseases [14], and the beneficial role of maternal cells in the fate of the CB transplant is increasingly evidenced [15].

Because of the bidirectional exchange of cells during pregnancy, pregnant women have acquired maternal cells during their fetal life from their mother and could transfer grandmaternal (GdM) cells to their child through the maternal bloodstream during pregnancy. Thus CB samples could theoretically carry GdM cells. Nevertheless this has never been demonstrated.

In the current study, we tested the hypothesis that GdM cells could be found in CB samples from healthy primigravid women. To test this hypothesis we used a panel of HLA-specific real-time quantitative PCR assays to specifically target GdM-specific HLA sequences and quantify GdMMc. HLA-A, B and DR typing was realized from CB, mother and grand-mother's DNA samples to identify a non-shared, non−inherited GdM HLA locus. Twenty-eight CB samples could satisfy the criteria of a three generation-HLA typing informative for unequivocal GdMMc research. Genetic, biological, anthropometric and obstetrical parameters of mothers and/or babies were further studied for their influence on GdMMc frequencies.

2. Methods

2.1. Familial collection of samples and HLA genotyping

Ninety-two healthy primigravid women with singleton pregnancy were recruited from 2014 to 2017 from three maternities in Marseilles, France. Obstetrician gynecologist and midwives asked them through a questionnaire whether they would like to participate to a multigenerational study of microchimerism monitoring during and after pregnancy. The inclusion criteria were: not to have had a pregnancy before this one, not to have had a blood transfusion, not to have an autoimmune disease, nor to have parents, brothers or sisters with autoimmune diseases, and not to have a cancer. No screening was realized for any disease and the healthy status was self-reported at the first trimester visit. It is to note that probands' primigravity is based on their pregnancy history declarations, but incomplete and unnoticed pregnancies could have occurred and could disqualify some women from being considered primigravid.

Out of the 92 women, three were excluded as they did not fulfill criteria for primigravidity, two dropped and one had a spontaneous abortion at first trimester, leaving to the number of 87. We could not obtain cord blood samples at delivery for 21 babies. Out of the 66 remaining mothers, 11 mother/child pairs were not informative for HLA-QPCR assays for maternal Mc detection. So, a total of 55 CB could be tested for MMc as detailed in El Haddad et al [13]. Among them 30 maternal grandmothers participated to the study, from whom 29 were informative for HLA-QPCR assays for grandmaternal Mc detection.

The two main maternities which recruited primigravid women are the two biggest of the area, one is located in the North and the other one in the South East of Marseilles. Together they cover over 60% of deliveries in Marseilles and have a population representative of the wider Marseille population. Most of the non-inclusions were due to the fact that the women did not meet the conditions of

primigravidity. Thus, the final sample of women included is representative of the wider primigravid population of Marseille.

Samples were obtained, as previously detailed, by double clamping the umbilical cord segment and drawing CB (~15mL) by venipuncture into lithium heparin tubes [13]. An aliquot of $350\mu L$ of CB collected at delivery and a similar aliquot of blood collected from each primigravid woman at first trimester of pregnancy were kept frozen at- 40°C for genomic DNA extraction (EZ1 DNA blood kit, Qiagen, Hilden, Germany) using a Biorobot EZ1 system according to the manufacturer's instructions. The remaining CB was processed within 24 hours from delivery for cell sorting and chimerism studies as described below.

Three-generation family collection with participation of maternal grandmothers was possible for 29 CB samples out of the 55 previously collected. None of the 29 primigravid women had a twin. Furthermore 19 fathers of the 29 three-generation families participated also. DNA samples from grand-mothers and fathers were extracted from Eludril solution mouthwash samples (Pierre Fabre Laboratories, France) using High Pure PCR Template Preparation kit (Roche, Indianapolis, IN) as previously described [13].

All DNA samples were HLA-A, B and DRB1 genotyped at Etablissement Français du Sang, Marseilles France, to further investigate GdMMc in CB samples by HLA-specific PCR.

2.2. Ethics

The study has received the approval of the ethics committee (CPP Sud-Méditerranée II) and is registered at the INSERM (Biomedical Research Protocol RBM-04-10) and as a collection (DC-2008-327). All participants signed an informed consent form according to the Declaration of Helsinki [16].

2.3. Obstetrical, anthropometric and clinical characteristics

Characteristics of mothers and children from whom the 29 CB samples were collected for the present study are detailed in Table 1. In our study mothers were 29.9 years old when they gave birth to their first child, which is very comparable to the age of the general population in 2015 (28.5 years old) according to the National Institute of Statistics and Economic Studies (INSEE). Pregnancy Associated Plasma Protein A (PAPP-A) and free beta human chorionic gonadotropin (β-hCG) were measured for 22 of the 29 primigravid women at first trimester (12 ±2 weeks of amenorrhea). The level of each serum marker was measured and expressed as the multiple of the median (MoM) of the expected normal median for women with pregnancies of the same gestational day using values established in a previous study [17]. As a standard practice in France, PAPP-A and β-hCG concentrations are combined with the mother's age and ultrasound examination to measure nuchal translucency to assess the risk of Down syndrome or other fetal aneuploidies (N=23). Patients participating to the study reflect the general population and do not have higher risk of complications.

2.4. Cord blood cell separation for chimerism analyses

CB samples were processed to isolate peripheral blood mononuclear cells (PBMC) by gradient centrifugation with Histopaque 1077 (Sigma-Aldrich, MO, USA) and B cells, T cells, granulocytes and hematopoietic progenitor cells (HPC) were respectively sorted by immuno-magnetic cell sorting (RoboSep^{TM}-S, STEMCELL^{TM} Technologies, Canada) as previously described [13]. Cell fractions with purity higher than 95% were kept for microchimerism analysis.

Table 1
Obstetrical, anthropometric and clinical characteristics of the 29 primigravid mothers and children from whom cord blood samples were tested for grandmaternal microchimerism

Obstetrical, clinical parametersN=29	Details
History of pregnancies	29 pregnancies for which no fetal abnormalities were observed
	23 normal pregnancies*
	1 IVF (gamete donation: unknown)
	1 endometriosis/ IVF (ova donation and intracytoplasmic sperm injection) / mild high blood pressure
	1 polycystic ovary syndrome
	1 placental abruption
History of deliveries	29 single lived fetus delivered
	3 delivered a baby with birth weight above the 10th centile of normal for gestational age
	3 delivered before 37 complete weeks of gestation
PAPP-A (MoM), N=22	0.96 (range: 0.13 – 2.21)
βhCG (MoM), N=22	1.17 (range: 0.48- 4.12)
Risk of aneuploidies, N=23	2
Type of deliveries	25 vaginal, 4 caesarian
Number of girls	9
Number of boys	20
Body Mass Index of the mother (prior to pregnancy), N=22	21.6 (range: 16.2-38.0)
Mean weight of baby males (kg)	3.30 (range : 2.58-4.20)
Mean weight of baby females (kg)	3.33 (range: 2.62-3.73)
Mean weight of all babies (kg)	3.30 (range: 2.58-4.20)
Mean number of gestational weeks	39.9 (range: 35.9 - 42.0)
Mean maternal age	29.9 (range: 25 - 40)

* A normal pregnancy was defined as a pregnancy in which a single live fetus was delivered after 37 complete weeks of gestation with birth weight above the 10th centile of normal for gestational age and for which no fetal anomalies were observed. IVF: in vitro fecundation; PAPP-A: Pregnancy Associated Plasma Protein A; βhCG: beta human chorionic gonadotropin; MoM: multiple of the median.

2.5. Quantification of grandmaternal Mc by HLA-specific Real Time Polymerase Chain reaction

Family HLA genotyping determined which HLA-specific PCR assay should be chosen to test GdMMc (See Figure 1 for strategy). DNA concentration of each CB was calculated by using a reference β-globin standard curve, as previously described [13,18]. All results of DNA concentration are expressed in genome equivalent of Mc cells (gEq), with the consensus correspondence of 1 cell = 1 gEq = 6.6 pg.

HLA specific primer and probe sets were synthetized by TIB Mol-Biol (Berlin, Germany). PCR assay sensitivity per reaction-well was of 1 gEq of microchimeric cell in 20,000 gEq of host cells (0.005%), thus DNA samples were adjusted to ~20,000 gEq (132ng) per well as higher concentrations could reduce the sensitivity and were tested for Mc in ten replicate wells (for a combined final maximal sensitivity of 0.0005%). Some samples, as for example CD34+ cell samples, were tested at lower concentration than 20,000 gEq/ well, as this type of cell is rare. Results of Mc are given in genome equivalent of donor cells (maternal or grandmaternal) per million of genome equivalent of host cells (gEq/10^6). Quantitative PCR assays were done using Light Cycler FastStart DNA MasterPLUS reaction kits on a LightCycler®480 instrument (Roche Diagnostics) as previously described [18].

2.6. Statistics

Statistical analyses were conducted using GraphPad Prism 6 software (La Jolla, CA, USA). The non-parametric Mann-Whitney test was used to compare anthropometric or obstetrical parameters (baby's weight, between CB positive or negative for GdMMc. Fisher's exact test was used to determine whether type of deliveries, prenatal procedures could influence the presence or absence of GdMMc in CB
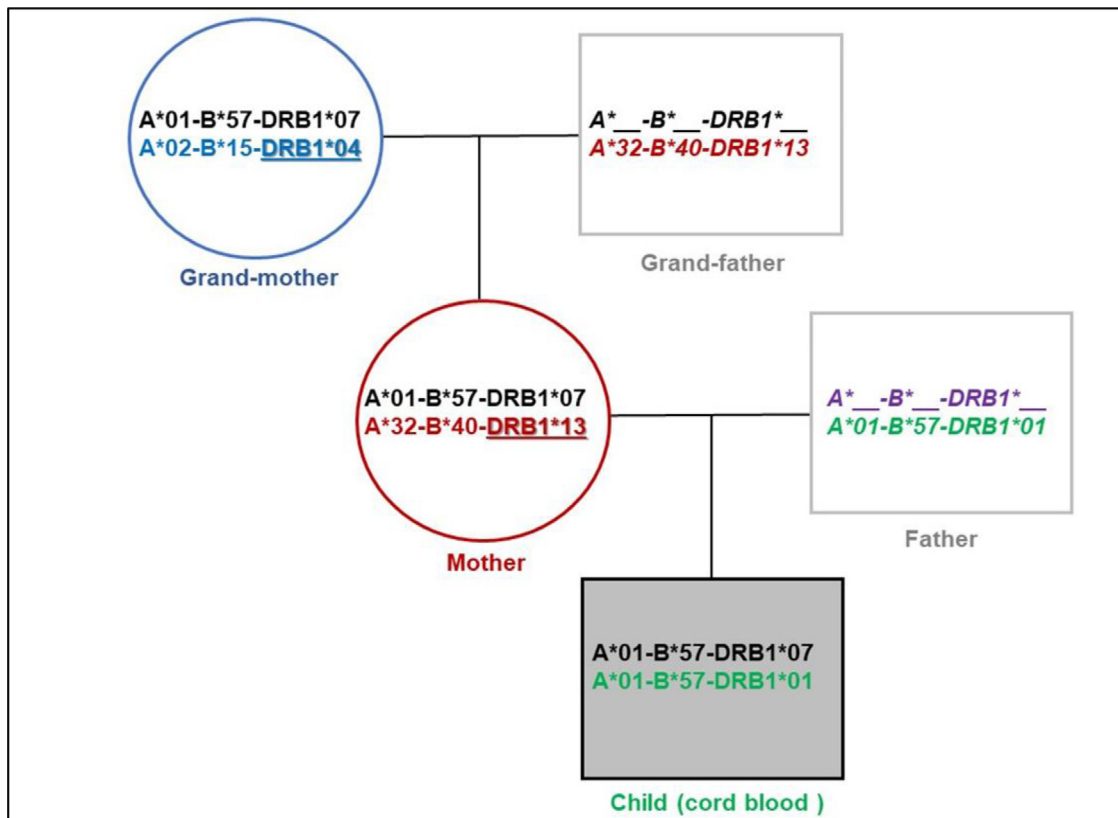
Figure 1. Example of a three- generation family and strategy to quantify maternal and grandmaternal Mc by HLA-specific PCR in a cord blood sample. HLA-A, B, and DRB1 typing was realized on grandmaternal, maternal and cord blood (child) DNA. In the current example the grandmaternal HLA-DRB1*04 (DR4) allele is non-inherited and unshared with the child's mother and the child, thus doing a DR4 specific PCR on cord blood DNA will indicate whether there is or not the presence of grandmaternal DNA. To quantify maternal Mc in the cord blood sample a DRB1*13 PCR (non-inherited maternal antigen and unshared) will indicate whether there is or not the presence of maternal DNA. Other unshared non-inherited HLA-A or B alleles could have been used to (ie. HLA-A*02 for grandmaternal Mc research).

samples. Fisher's exact test was used to determine whether HLA compatibility was different in three generation families between CB positive and CB negative for GdMMc. Only families for whom complete HLA typing for all loci was available could be counted into statistical analyses, which corresponds to 5 families having a CB positive for GdMMc and 16 having a negative CB.

2.7. Role of funding source

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

3. Results

3.1. Grandmaternal Mc in 18% of cord blood samples

Familial HLA typing on three generations on the maternal side was realized for 29 CB samples to evaluate the best strategy to quantify grandmaternal Mc (example in Figure 1). In four CB samples (#53, 61, 65 and 90, Table 2) non-inherited maternal and grandmaternal HLA-DRB1 alleles were identical and undistinguishable. One (CB#53) was thus excluded as we could not discern whether the amplification was from maternal or grandmaternal origin, both origin being HLA-DRB1*11 positive (Table 2). Results from the three others were kept as they were negative, thus negative for maternal as well as grandmaternal Mc.

We could then unequivocally detect GdMMc in 5 CB samples out of 28 (18%) by GdM HLA-specific QPCR. This is three times less than the frequency of MMc which, on the same 28 samples, is of 57%.

3.2. Low quantities of GdMMc

The highest quantities of GdMMc in WB and/or PBMC did not reach the highest quantities observed for MMc in similar type of samples. They were ∼100 fold lower (Table 2).

Of note, one CB sample (CB#80) had high levels of GdMMc in the hematopoietic progenitor cell subset, while absent from whole blood or PBMC, but the total quantity of DNA obtained for the CD34+ subset was at a very low yield. Thus only a small quantity of CD34+ gEq could be tested for GdMMc (∼5,500 gEq) and results may be overestimated when given in gEq per million of host cells. Nevertheless, this quantity was still 12 fold lower than the highest quantity of MMc observed in similar CD34+ subset (258 gEq/10^6 compared to 3,021 gEq/10^6).

3.3. GdMMc not systematically observed in samples positive for maternal Mc

Out of the five CB positive for GdMMc, three (CB#20, #31 and #80) were also positive for MMc, while two (CB#12 and #47) had discordant results for MMc (Table 2). Nevertheless it is to note that CB#12 and #47 could not be extensively tested in cell subsets for both chimerisms due to insufficient volume of blood collected.

Moreover, CB#14, #45, #48 were highly positive for MMc and had a mother who had maternal Mc during pregnancy (data not detailed, indicated in Table 2), thus grandmaternal Mc would be highly expected but was not observed.

3.4. Excluding the possibility of Mc from a vanished twin

Another possible source explaining foreign but non-maternal HLA Mc in a CB sample could be a dizygotic vanished twin. For example

Table 2
Detection of maternal and grandmaternal Mc in CB samples

CB ID	Child's sex	mother's age at delivery	MMc in the mother	Maternal Mc in cord blood (gEq/10^6)							Grandmaternal Mc in cord blood (gEq/10^6)						
				HLA-QPCR detector	WB	PBMC	CD3	CD19	CD66	CD34	HLA-QPCR detector	WB	PBMC	CD3	CD19	CD66+	CD34+
CB-02	M	28	+	DR07	9	0	0			0	DR01	0	0	0			
CB-09	F	29	+	DR07	27	0				8	DR02	0	0				
CB-11	M	29	0	DR04	0	3					A*01	0	0			0	0
CB-12	M	34	0	A*02	0	0					DR11	4	3				
CB-14	M	28	+	B*44	192	169					DR13	0	0				
CB-16	M	30	0	DR11	0	0					DR07	0	0				
CB-19	M	25	0	DR14	0	0					DR10	0	0				
CB-20	M	25	+	DR01	197	41	0		0		DR02	12	0	0		0	
CB-23	M	26	0	DR07	449						A*02	0					
CB-27	F	33	+	A*11	0	0	0	0	0	0	DR01	0	0	0	0	0	0
CB-31	M	27	+	DR13	0	0	1	0			DR04	0	5	0	0	0	0
CB-32	F	31	+	DR11	0	166					DR03	0	0				
CB-34	M	28	+	DR02	0		0	0	0	0	DR02	0		0	0	0	0
CB-35	M	30	0	DR02	20	0	51	0	0	0	DR13	nd	0	0	0	0	0
CB-38	F	25	0	DR08	1199	517	329	798	1276	0	DR13	0	0	0	0		
CB-39	F	40	nd	DR02	0	0	85	0	0	0	DR13	0	0	0	0	0	
CB-41	F	33	0	DR07	0	0	0	0	0	0	A*03	0	0	0	0	0	0
CB-45	F	31	+	DR14	1240	502	452	171	1096	0	DR01	0	0	0	0	0	0
CB-47	M	41	+	DR10	0	0					DR02	5	0				
CB-48	M	29	+	DR08	550	426	0	0	909	3021	DR07	0	0	0	0	0	0
CB-51	F	28	0	DR01	0	22	27	0	11	0	A*03	0	0	0	0	0	0
CB-53*	M	27	0	DR11*	0	0	1*	0	0	0	DR11*	0	0	1*	0	0	0
CB-61	M	26	nd	DR03	0	0	0	0	0	0	DR03	0	0	0	0	0	0
CB-65	M	30	nd	DR03	0	0	0	0	0	0	DR03	0	0	0	0	0	0
CB-80	F	32	+	A*11	0	0	3	0	2	0	DR02	0	0	0	0	0	258
CB-86	M	28	0	DR02	0	2	0	0	0	0	DR13	0	0				
CB-88	M	28	0	DR01	0	0	0	0	0		DR07	0	0	0	0	0	0
CB-89	M	29	+	DR12	0	0					DR07	0					
CB-90	M	37	+	DR02	0	0	0	0	0	0	DR02		0	0	0	0	0

Three-generation families (maternal grandmother, mother and child) were HLA-A, -B and DRB1 typed. CB samples were tested for maternal microchimerism (MMc central columns) and grandmaternal Mc (GdMMc right columns) by non-transmitted and unshared HLA-specific quantitative PCR assays. Whole blood samples (WB) were tested for MMc and GdMMc, as well as cell subsets: peripheral blood mononuclear cells (PBMC), T lymphocytes, B lymphocytes, granulocytes and hematopoietic stem cells (HSC). Results are expressed in genome equivalent of Mc cells per million of cord blood cells (gEq/10^6). nd: not done.
Four CB samples (#53,61,65 and 90) had similar maternal and grand-maternal non-inherited HLA-DRB1 alleles and could not be tested for other HLA loci. Three were negative for both chimerism, and one (*CB #53) was positive and excluded because of confounding maternal and grandmaternal Mc sources.
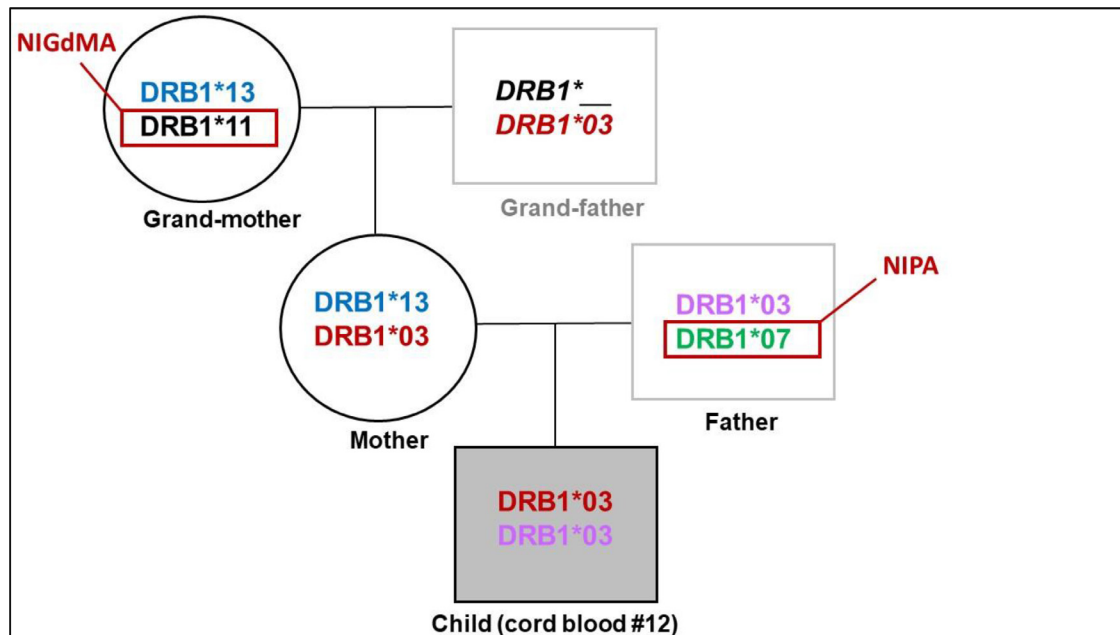
Figure 2. Distinct HLA genes between maternal grandmother and father In the case the father carries the same HLA than the maternal grandmother, GdMMc would be undistinguishable from Mc cells from a vanished twin. In such case, the non-inherited paternal HLA (NIPA) would be identical to the non-inherited grandmaternal antigens (NIGdMA). Three CB positive for GdMMc for whom we obtained father's HLA typing were analyzed for such relationship. Here is the example of CB#12 for whom NIGdMA were different than NIPA, so were the two other CB (#20 and #80).

CB#12 had a HLA-DRB1*03/*03 genotype, had a mother DRB1*13/*03 and a grand-mother DRB1*13/*11, thus the presence of DRB1*11 Mc could come from GdMMc but also from a dizygotic vanished twin under the condition that the father carried also a DRB1*11 allele. We thus verified whether CB positive for GdMMc had the same non-inherited paternal human leukocyte antigens (NIPA) than non-inherited grandmaternal antigens (NIGdMA). We obtained father's DNA of three out of the five cord blood samples positive for GdMMc, and none of the three had such a family HLA pattern, ruling out the possibility of Mc from a dizygotic vanished twin (Figure 2).

3.5. No influence from the type of delivery or anthropometric parameters

We tested whether grandmaternal cell traffic could be influenced by differences in collection procedures in the different hospital maternities, delivery type (cesarean versus vaginal), baby's gender, baby's weight, baby's conception (in vitro fertilization or not), number of gestation weeks.

Grandmaternal Mc was not influenced by any of those factors (data not shown). Grandmaternal Mc was also not influenced by the age at which the grandmother had her daughter (current mother), nor by the body mass index of the mother before pregnancy (data not shown), although the analysis was underpowered due to the small sample size of CB being positive.

3.6. Risk of aneuploidies and/or related invasive prenatal procedures correlated with GdMMc in CB

The risk of Down syndrome or other fetal aneuploidies was available for 23 women. Two out of the four positive CB for GdMMc (and for which such measures were available) had a high risk for fetal aneuploidies of respectively 1/22 and 1/96 and had a trophoblastic puncture. Of note one of the two had had an in vitro fecundation with ova donation and intracytoplasmic sperm injection. None of the 19 CB negative for GdMMc for which these data were available had a high risk for Down syndrome. The difference between the two groups

was significant (2/4 versus 0/19, *p=0.024,* Fisher's exact test two tailed).

3.7. Decreased HLA compatibility in three-generation families from CB positive for GdMMc

As feto-maternal HLA-A and/or −DR compatibility is predictive of the presence of maternal Mc in CB [13], we analyzed whether transgenerational HLA compatibility could influence grandmaternal Mc. grandmother/mother or mother/child or grandmother/child HLA relationship were evaluated and classified into either HLA compatible or incompatible from the child's perspective for HLA-A, -B or -DR loci (Table 3). Significantly decreased HLA compatibility was observed in three-generation families from CB samples carrying GdMMc (*p=0.019,* see details in Statistical section and Table 3). When comparing the three pairs, only the mother/child's pair had a significant decreased HLA compatibility in CB samples carrying GdMMc (*p=0.034,* Fisher's exact test two tailed, Table 3).

4. Discussion

Feto-maternal bidirectional exchange of cells occuring during pregnancy creates the so-called maternal and fetal microchimerism in respective hosts and Mc persists decades after delivery. Pregnant mothers could transfer to the fetus not only their cells but theoretically also cells from their mother, conducting to GdMMc in the offspring. Nevertheless, to our knowledge, GdMMc has never been demonstrated in second generations, probably for technical and practical reasons as three-generation family collection with participation of maternal grandmothers are difficult to obtain and detection of rare cells require very sensitive methods. For the first time, we have detected unambiguous grandmaternal Mc in five out of 28 CB samples with HLA-specific PCR assays based on non−inherited, non-shared HLA polymorphisms between grandmothers and grandchildren. Moreover, we have eliminated the possibility of a dizygotic vanished twin for three CB by verifying that the non-inherited paternal HLA allele of the child (CB) was not similar to the GdM HLA allele

Table 3
HLA compatibility across generations

	Grandmother/Mother			Mother/Child			Grandmother/Child			GdMMc	MMc
	A	B	DR	A	B	DR	A	B	DR		
CB-12										+	-
CB-20	red									+	+
CB-31										+	+
CB-47										+	-
CB-80	red									+	+
CB-02	nd	nd					nd	nd		-	+
CB-09		nd		red				nd		-	+
CB-11			red	red	red					-	+
CB-14		red	red	red	red	red	red	red		-	+
CB-16	nd	nd		red	red		nd	nd		-	-
CB-19		nd						nd		-	-
CB-23			red	red						-	+
CB-27					red	red				-	-
CB-32				red						-	+
CB-34		red						red		-	-
CB-35										-	+
CB-38	nd	nd					nd	nd		-	+
CB-39	nd	nd					nd	nd		-	+
CB-41		red						red		-	-
CB-45				red	red					-	+
CB-48					red					-	+
CB-51			red							-	+
CB-61			red							-	-
CB-65	red		red	red	red		red			-	-
CB-86										-	+
CB-88										-	-
CB-89					red					-	-
CB-90	red		red							-	-
p values					0.019						
		0.7			0.034			0.58			

■ HLA compatibility from child's perspective
■ HLA incompatibility from child's perspective

The HLA compatibility for HLA-A, B and DR loci is evaluated between grandmother/mother (GdM/M), mother/child (M/C) or grandmother/child (GdM/C) pairs from the child's perspective for each cord blood (CB) sample. Boxes colored in red correspond to a situation where HLA compatibility is observed in the pair and locus analyzed. Boxes colored in grey where HLA incompatibility is observed. Positivity for grandmaternal microchimerism (GdMMc) or maternal Mc (MMc) is indicated in the last two columns. The five first lines correspond to the five CB being positive for GdMMc, while the 23 lines below correspond to CB negative for GdMMc. nd: not done.

The first P value is calculated by comparing the number of any locus for which there is an HLA compatibility in any pair (grandmother/mother, mother/child or grandmother/child) from the chid's perspective in the first group (CB positive for GdMMc) compared to the second group (CB negative for GdMMc). Only CB for which all family members could be typed at all loci were included in the count (2/45 versus 30/153, p=0.019, Fisher's exact test).

Similar calculation was done for each pair and complete lines (GdM/M: 2/15 versus 11/51, p=0.7; M/C: 0/15 versus 16/66, p=0.034; GdM/C: 0/15 versus 5/51, p=0.58, Fisher's exact test).

used to test GdMMc. Another confounding source of Mc could come from an older sibling of the primigravid woman, either full born or from an incomplete pregnancy, leaving fraternal cells in the mother who would pass them on to her child (here CB) as previously shown by Müeller et al [19]. But in the current study this option remains very unlikely since i) none of the primigravid had a twin, ii) the five women for whom CB were positive for GdMMc did not have more often an older sibling than women for whom CB were negative for GdMMc (60% versus 53%, data not shown).

The question now is to know whether transgenerational passage of cells is a passive or active phenomenon. Is there a particular condition conducting to the presence of GdMMc in grandchildren? We have observed levels of GdMMc 100 fold lower than commonly described levels of MMc in CB samples. This suggests passive passage of GdM cells diluted through maternal flow. Indeed, GdMMc has previously been identified in peripheral blood during normal, uncomplicated pregnancy, with a peak concentration in the third trimester [20]. Nevertheless, a few arguments contradict the hypothesis of a passive passage. If such was the case, CB samples for which maternal Mc was highly concentrated would have been the most microchimeric with grandmaternal cells, which was not observed. A passive traffic of cells would have allowed a random distribution of GdM cells in all cell subsets, instead one CB had very high levels of GdMMc in the hematopoietic stem cell subset (CD34+) while it was absent from all other subsets. Another argument is that a significant increased HLA incompatibility was observed in three-generation families from CB samples carrying GdMMc, suggesting an immune process, favoring the passage of GdMMc. Increased HLA incompatibility from the child's perspective leads to a capacity of GdM and maternal (donor) lymphocytes to be recognized as foreign by the child (host), as well as the ability of donor lymphocytes to recognize and react to host tissues. Why this peculiar HLA relationship would

conduct to transgenerational passage of cells remains unknown. Kinder et al. have recently demonstrated that overlap between NIMA and fetal antigen during pregnancy (increased three-generation HLA compatibility) accentuates fetal tolerance and improves reproductive fitness [21]. Could it be that decreased HLA compatibility through three-generations might conduct to more reproductive failure? Remarkably, one of the children (CB) positive for GdMMc was from an ova donation and intracytoplasmic sperm injection, highlighting reproductive failure in the couple. The significance of the presence of grandmaternal cells in such conditions remains to be elucidated.

If the passage is not passive through the maternal bloodstream then, what could specifically conduct grandmaternal cells to migrate to CB? Interestingly, out of the CB samples positive for GdMMc and for whom we had information for Down syndrome or other fetal aneuploidy risks (N=4), two had a high risk and one had a trophoblastic puncture. Since the first trimester screening comprising measurement of nuchal translucency and assay of serum markers (PAPP-A and free beta hCG) is carried out routinely on all pregnant women without distinction in all French maternity hospitals, women participating to the study reflect the general population and did not have higher risk of complications. Thus, results are not biased due to a high risk in the study population. However the small number of samples, where GdMMc is found, is a limit to our study and consequently to the statistical analyzes. Therefore, further analyses will be necessary to evaluate whether invasive prenatal procedures could increase GdMMc.

A concrete example that few microchimeric cells can have a biological function is illustrated by the study from Wrenshall et al. showing that IL2-KO mice born from heterozygous mothers (IL2+/-) have maternal cells expressing IL2 in amounts detectable by conventional means. [22] Nevertheless here the number of GdM cells observed is about 1/100 less than maternal cells and calls for caution as to any biological relevance. But it should be noted that grandmaternal cells may populate tissues in the growing child at greater density than what is observed in CB. Whether a few cells contribute to a biological effect on future generations is an open debate which will need to be settled in animal models to be answered.

Transgenerational effects have been demonstrated for epigenetic and metabolic changes that occur because of environmental exposures and may be transmissible to future unexposed generations. [23,24] Children have for example an increased risk of asthma in the first 6 years of life if their grandmothers smoked during early pregnancy, independent of maternal smoking [25].

Other limitations of our study not mentioned above are i) the potential bias of self-reported healthy status of women ii) the impossibility to adjust for confoundings (due to small numbers) and finally iii) incomplete data for some variables which could influence the presence of GdMMc. Smoking during pregnancy could for example plausibly influence GdMMc, especially since Mads Kamper-Jørgensen et al. report an association between baseline smoking and fetal (male) microchimerism positivity [26]. However our study is not appropriate to address this issue, as our records are not precise enough to tell whether it was baseline smoking or smoking during pregnancy, nor does it specify the pack-years of cigarette smoking.

Thus, as illustrated by this discussion, we have only one certainty, grandmaternal cells are present in 18% of CB with our sensitive detection methods. Whether transgenerational transfer of cells is important in immunology and evolution remains to be elucidated, but our findings advocate changes in our vision on cord blood samples.

Declaration of Competing Interest

The authors declare no conflicts of interests.

Data sharing statement

All the data/analyses presented in the current publication will be made available upon request to the corresponding author.

Contributors

KRK, MEH and NCL conceived and designed the experiments. MEH, KRK, MM, GVM and NCL performed the experiments. KRH and MEH have verified the underlying data. NCL analyzed the data. X-CD, FB, NL, J-FC and RD, contributed to recruitment of healthy primigravid women, their mothers and husbands and to the collection of cord blood samples. X-CD, CP and J-FC contributed to clinical, anthropological and/or obstetrical data. TA contributed to the creation of Table 3. NCL wrote the paper. JR reviewed the manuscript and made corrections. All authors read and approved the final version of the manuscript.

References

[1] Zarou DM, Lichtman HC, Hellman LM. The Transmission of Chromium-51 Tagged Maternal Erythrocytes from Mother to Fetus. American journal of obstetrics and gynecology 1964;88:565–71.
[2] Briz M, Regidor C, Monteagudo D, et al. Detection of maternal DNA in umbilical cord blood by polymerase chain reaction amplification of minisatellite sequences. Bone Marrow Transplant 1998;21(11):1097–9.
[3] Hall JM, Lingenfelter P, Adams SL, Lasser D, Hansen JA, Bean MA. Detection of maternal cells in human umbilical cord blood using fluorescence in situ hybridization. Blood 1995;86(7):2829–32.
[4] Kanaan SB, Gammill HS, Harrington WE, et al. Maternal microchimerism is prevalent in cord blood in memory T cells and other cell subsets, and persists posttransplant. Oncoimmunology 2017;6(5):e1311436.
[5] Kanold AMJ, Westgren M, Gotherstrom C. Cellular Subsets of Maternal Microchimerism in Umbilical Cord Blood. Cell Transplant 2019;28(5):522–8.
[6] Kogler G, Gobel U, Somville T, Enczmann J, Arkesteijn G, Wernet P. Simultaneous genotypic and immunophenotypic analysis of interphase cells for the detection of contaminating maternal cells in cord blood and their respective CFU-GM and BFU-E. J Hematother 1993;2(2):235–9.
[7] Lo YM, Lau TK, Chan LY, Leung TN, Chang AM. Quantitative analysis of the bidirectional fetomaternal transfer of nucleated cells and plasma DNA. Clin Chem 2000;46(9):1301–9.
[8] Opstelten R, Slot MC, Lardy NM, et al. Determining the extent of maternal-foetal chimerism in cord blood. Sci Rep 2019;9(1):5247.
[9] Petit T, Gluckman E, Carosella E, Brossard Y, Brison O, Socie G. A highly sensitive polymerase chain reaction method reveals the ubiquitous presence of maternal cells in human umbilical cord blood. Exp Hematol 1995;23(14):1601–5.
[10] Roh EY, Yoon JH, Shin S, Song EY, Chung HY, Park MH. Frequency of fetal-maternal microchimerism: an analysis of the HLA-DRB1 gene in cord blood and maternal sample pairs. J Matern Fetal Neonatal Med 2017;30(21):2613–9.
[11] Maloney S, Smith A, Furst DE, et al. Microchimerism of maternal origin persists into adult life. J Clin Invest 1999;104(1):41–7.
[12] Loubiere LS, Lambert NC, Flinn LJ, et al. Maternal microchimerism in healthy adults in lymphocytes, monocyte/macrophages and NK cells. Lab Invest 2006;86(11):1185–92.
[13] Haddad ME, Karlmark KR, Donato XC, et al. Factors Predicting the Presence of Maternal Cells in Cord Blood and Associated Changes in Immune Cell Composition. Front Immunol 2021;12:651399.
[14] O'Brien TA, Tiedemann K, Vowels MR. No longer a biological waste product: umbilical cord blood. Med J Aust 2006;184(8):407–10.
[15] Kanaan SBDC, Milano F, Scaradavou A, van Besien K, Allen J, Lambert NC, Cousin E, Thur LA, Kahn E, Forsyth AM, Sensoy O, Nelson JL. Cord blood maternal microchimerism following unrelated cord blood transplantation. Bone Marrow Transplantation 2020.
[16] Vollmann J, Winau R. Informed consent in human experimentation before the Nuremberg code. BMJ 1996;313(7070):1445–9.
[17] Ong CY, Liao AW, Spencer K, Munim S, Nicolaides KH. First trimester maternal serum free beta human chorionic gonadotrophin and pregnancy associated

plasma protein A as predictors of pregnancy complications. BJOG 2000;107 (10):1265–70.

[18] Rak JM, Maestroni L, Balandraud N, et al. Transfer of the shared epitope through microchimerism in women with rheumatoid arthritis. Arthritis and rheumatism 2009;60(1):73–80.

[19] Muller AC, Jakobsen MA, Barington T, et al. Microchimerism of male origin in a cohort of Danish girls. Chimerism 2015;6(4):65–71.

[20] Gammill HS, Adams Waldorf KM, Aydelotte TM, et al. Pregnancy, microchimerism, and the maternal grandmother. PloS one 2011;6(8):e24101.

[21] Kinder JM, Stelzer IA, Arck PC, Way SS. Immunological implications of pregnancy-induced microchimerism. Nat Rev Immunol 2017;17(8):483–94.

[22] Wrenshall LE, Stevens ET, Smith DR, Miller JD. Maternal microchimerism leads to the presence of interleukin-2 in interleukin-2 knock out mice: implications for the role of interleukin-2 in thymic function. Cell Immunol 2007;245(2):80–90.

[23] Sen A, Heredia N, Senut MC, et al. Multigenerational epigenetic inheritance in humans: DNA methylation changes associated with maternal exposure to lead can be transmitted to the grandchildren. Sci Rep 2015;5:14466.

[24] Stegemann R, Buchner DA. Transgenerational inheritance of metabolic disease. Semin Cell Dev Biol 2015;43:131–40.

[25] Lodge CJ, Braback L, Lowe AJ, Dharmage SC, Olsson D, Forsberg B. Grandmaternal smoking increases asthma risk in grandchildren: A nationwide Swedish cohort. Clin Exp Allergy 2018;48(2):167–74.

[26] Kamper-Jorgensen M, Mortensen LH, Andersen AM, Hjalgrim H, Gadi VK, Tjonneland A. Predictors of male microchimerism. Chimerism 2012;3(3):1–8.