

Maturation of preterm newborn brains: a fMRI–DTI study of auditory processing of linguistic stimuli and white matter development

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Abstract To evaluate brain development longitudinally in premature infants without abnormalities as compared to healthy full-term newborns, we assessed fMRI brain activity patterns in response to linguistic stimuli and white matter structural development focusing on language-related fibres. A total sample of 29 preterm newborns and 26 at term control newborns underwent both fMRI and DTI. Griffiths test was performed at 6 months of corrected age to assess development. Auditory fMRI data were analysed in 17 preterm newborns at three time points [34, 41 and 44 weeks of post menstrual age (wPMA)] and in 15 controls, at term. Analysis showed a distinctive pattern of cortical activation in preterm newborns up to 29 wPMA moving from early prevalent left temporal and supramarginal area activation in the preterm period, to a bilateral

temporal and frontoopercular activation in the at term equivalent period and to a more fine-grained left pattern of activity at 44 wPMA. At term controls showed instead greater bilateral posterior thalamic activation. The different pattern of brain activity associated to preterm newborns mirrors their white matter maturation delay in peripheral regions of the fibres and thalamo-cortical radiations in subcortical areas of both hemispheres, pointing to different transient thalamo-cortical development due to prematurity. Evidence for functional thalamic activation and more mature subcortical tracts, including thalamic radiations, may represent the substantial gap between preterm and at term infants. The transition between bilateral temporal activations at term age and leftward activations at 44 weeks of PMA is correlated to better neuropsychological results in Griffiths test.

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Introduction

In industrialized countries, preterm delivery is responsible for 70 % of mortality and 75 % of morbidity in the neonatal stage (Challis et al. 2001; Repka 2002; Wen et al. 2004). Advances in perinatal and neonatal care over the last 30 years have contributed to improve survival amongst preterm newborns. As preterm delivery occurs at a time when the foetal brain is maturing rapidly and is at high risk of cerebral lesions (Hack et al. 2002; Hope et al. 1988; Kuban and Leviton 1994; Marlow 2004), a significantly high number of early preterm babies have neurological disabilities (Aylward 2002). Several studies have proven that about 50 % of early preterm infants will develop long-

term cognitive and neuropsychological deficits becoming evident later in childhood, even in the absence of brain lesions detectable by conventional magnetic resonance imaging (MRI) (Yu 2000; Tin et al. 1997; Draper et al. 1999; Stoelhorst et al. 2005; Volpe 2003, 2005; Barre et al. 2011). Moreover, it has been observed with functional MRI (fMRI) (Peterson et al. 2002; Ment et al. 2003; Rushe et al. 2001; Wilke et al. 2014; Myers et al. 2010) that healthy premature babies, investigated during childhood and compared to term control subjects, seem to develop compensatory systems for processing language by engaging alternative neural systems. Therefore, even in the absence of overt focal lesions, the brain in very preterm infants presumably undergoes some modifications in normal cortical and white matter maturation processes. In this regard, during the early preterm period, from 26 to 36 weeks of post menstrual age (wPMA), transitory thalamo-cortical connections are growing by means of the subplate, a transient laminar brain compartment. This structure is believed to be critical for definitive cortical organization and the physiological development of thalamo-cortical connections and in the growth of long cortico-cortical axons (Kostovic and Rakic 1990; Kostovic and Jovanov-Milosevic 2006; Kostovic and Judas 2010; Judas et al. 2013). Thus, to understand early cortical functional organization, this very specific period of brain development must be investigated.

Functional MRI and diffusion tensor imaging (DTI) are two advanced non-invasive MR techniques enabling to investigate the neural correlates engaged by specific brain activity on the one hand, and the structural organization of white matter (WM) fibre tracts on the other hand. Previous papers have demonstrated that DTI and fMRI allow the study of microstructural architecture and the gradual development of the brain, by showing the variability in WM and gray matter (GM) of structural and functional maturation rates and patterns in different phases of life (Ball et al. 2013; Huppi and Dubois 2006; Olesen et al. 2003; Mukherjee et al. 2002; Neil et al. 1998, 2002; Partridge et al. 2004; Fransson et al. 2007, 2009, 2011; Smyser et al. 2010).

Some attempts to characterize neurodevelopmental milestones using both structural and functional MRI have been made in adults born preterm through the investigation of memory processes or executive functions coupled with their specific neuroanatomical underpinnings (Lawrence et al. 2010; Narberhaus et al. 2009; Nosarti et al. 2009). All these studies revealed that, despite task behavioural outcomes fall in a range of normality, cognitive processing measured through fMRI and its underlying neural substrates may be quite different between adults born preterm and normal controls, which may point to different neurodevelopmental patterns. All the above-mentioned studies

investigated structural–functional relationships in terms of the association between BOLD signal and gray matter. However, the relationship between white matter structural connectivity through DTI and neurodevelopmental changes in brain activation induced by preterm birth still need to be studied. Salvan et al. (2014) have recently investigated the differences between preterm born adults and controls in terms of the relationship between functional activation patterns during learning and fractional anisotropy (FA) microstructural differences in fibre tracts known to connect brain areas subserving memory processes. Notwithstanding the absence of behavioural differences between the two groups, a different pattern of functional activation emerged involving the right anterior cingulate cortex, the hippocampus and the thalamus which mirrored reduced FA in preterm born adults in several WM structures, connecting posterior to anterior brain areas and passing through both the thalamic/hippocampal structures.

Previous fMRI and DTI studies have investigated infants aged a few months or newborns at term with auditory stimulation (Altman and Bernal 2001; Anderson et al. 2001; Perani et al. 2010; Dehaene-Lambertz et al. 2002, 2006; Smith and Gutovich 2011; Liu et al. 2010) but both techniques have not been systematically used before to investigate brain function in preterm infants and, at the same time, to assess longitudinal white matter changes.

Auditory processing of linguistic stimuli is a crucial landmark for the developmental of language functioning for both mechanisms of comprehension and production. The milestones of language processing seem to be affected by preterm birth (Bhutta et al. 2002); thus, we hypothesized that preterm infants, already since their birth, should show altered patterns of functional activation with respect to normal ones in response to linguistic stimuli in a language-related network. This network is defined by prefrontal, occipital and temporal brain areas as well as subcortical structures connected through WM tracts (Catani and Thiebaut de Schotten 2008) which could result being disrupted by preterm birth. In addition, we focused on exploring longitudinal brain activation changes that might be somehow mirrored by FA maturational changes or alterations in different WM tracts such as the arcuate fasciculus, which subserves auditory processing of linguistic stimuli and represents the neurodevelopmental grounding for language comprehension.

The aim of our study is thus to specifically highlight the functional landmarks of development associated to auditory processing of linguistic stimuli in a group of premature infants without focal or diffuse brain abnormalities as well as the neural signature of WM development by means of a longitudinal structural DTI investigation. A group of term neonates was recruited and served as a control population in both fMRI and DTI studies. All infants underwent a

neurodevelopmental assessment at 6 months of corrected age, by Griffiths scales, a neuropsychological test used for clinical and research purposes in several Italian Units of Neonatology and Paediatrics.

Materials and methods

Subjects

Twenty-nine infants (13 females/16 males) born prematurely (median PMA 29 weeks + 6 days (29w6d PMA); range 24w4d, 33w1d) were selected from a cohort of 45 premature newborns undergoing MR examination, following exclusion criteria such as congenital infections, multiple congenital anomaly syndrome, focal intra-cerebral

parenchymal lesions, hydrocephalus, congenital brain malformations, metabolic disorders, intraventricular haemorrhage, punctate T1 WM hyperintensities, periventricular leukomalacia or other pathological conditions visible on an MRI scan. All MR examinations were reviewed and fully reported by a neonatal neuroradiologist. For the excluded pathological infants any resultant clinical implications were discussed with parents and the clinical team.

Clinical information for preterm newborns is listed in Table 1. A sample of 26 healthy full-term newborns (14 females/12 males) was also recruited, as a normal control group.

The 29 preterm neonates were scanned three times: (1) between 29 and 34 weeks of PMA (hereinafter called *Pre1s*); (2) at term equivalent, between 38 and 41 weeks of PMA (hereinafter called *Pre2s*); and (3) between 44 and

Table 1 Clinical information of preterm newborns

Subject	PMA (weeks)	Birth weight	AV (yes/no)	Surfactant (yes/no)	Intubation (yes/no)	RDS (yes/no)	ROP (yes/no)	Jaundice (yes/no)	Sepsis (yes/no)	BDP (yes/no)	Hypoglycemia (yes/no)
1	32	1,150	N	N	N	N	N	Y (96 h)	N	N	N
2	30 + 5	925	Y	Y	N	N	Y	N	N	N	N
3	32 + 1	1,680	N	N	N	N	N	Y (48 h)	N	N	N
4	32 + 1	1,570	N	N	N	N	N	Y (96 h)	N	N	N
5	32 + 1	1,635	Y	Y	Y	N	N	Y (48 h)	N	N	N
6	28 + 3	1,269	Y	Y	Y	Y	N	Y (24 h)	N	N	N
7	32 + 4	1,930	N	N	N	N	N	N	N	N	N
8	29 + 1	1,225	Y	N	N	N	N	N	N	N	N
9	29	1,330	Y	N	Y	N	N	Y (72 h)	N	N	N
10	29	1,045	Y	Y	N	N	N	Y (144 h)	N	N	N
11	28	850	Y	Y	Y	Y	Y	Y (48 h)	N	Y	N
12	25	755	Y	Y	Y	Y	N	Y (48 h)	N	N	N
13	32 + 5	2,025	Y	N	Y	N	N	Y (48 h)	N	N	N
14	29	1,300	Y	Y	Y	N	N	Y (48 h)	N	N	N
15	31 + 1	1,120	Y	Y	Y	N	Y	Y (48 h)	N	N	N
16	33 + 1	1,980	Y	N	N	N	N	Y	N	N	Y
17	32 + 2	1,300	N	N	N	N	N	Y (48 h)	N	N	N
18	31 + 4	1,720	N	N	N	N	N	Y	N	N	N
19	27 + 5	865	Y	Y	Y	Y	N	N	N	Y	N
20	27 + 5	910	Y	Y	Y	Y	N	Y	N	Y	N
21	29 + 2	1,170	Y	Y	Y	Y	N	Y (96 h)	N	N	N
22	29 + 2	1,160	Y	N	N	N	Y	Y (96 h)	N	N	N
23	31 + 1	1,800	N	N	N	N	N	Y (72 h)	N	N	Y
24	32 + 2	2,440	N	N	N	N	N	Y (48 h)	N	N	N
25	24 + 4	790	Y	Y	Y	Y	Y	Y	Y	Y	N
26	29 + 6	1,090	N	N	N	N	N	Y (72 h)	Y	N	N
27	28	1,260	Y	N	Y	Y	Y	Y (150 h)	N	N	N
28	30	975	N	N	N	N	Y	Y (48 h)	N	N	N
29	28 + 6	1,460	Y	N	N	N	N	Y (96 h)	N	N	N

PMA post menstrual age, AV artificial ventilation, RDS respiratory distress syndrome, ROP retinopathy of preterm, BDP bronchopneumo dysplasia

45 weeks of PMA (hereinafter called *Pre3s*). The 26 at term newborns (hereinafter called *Cnts*) were scanned once at 2–3 days after birth.

Visual inspection and quality assessment of all fMRI datasets for both preterm newborns (i.e. 77 fMRI datasets = 29 preterm neonates \times 3 time points) and at term newborns (i.e. 26 fMRI datasets) were carried out before entering SPM analysis. FMRI datasets for both preterm and control newborns were excluded from analysis if EPI images showed large movement artefacts after realignment (head movement above 2 mm in one direction and rotations higher than 2°) or if the number of images in the dataset was not sufficient for successive analysis due to scanning interruption (<70 % of the entire data acquisition).

After image quality assessment for each dataset, a final sample of 17 *Pre1s* (median 33w2d PMA; range 29w4d, 33w6d), 17 *Pre2s* (median 39w6d PMA; range 38w5d, 41w2d), 17 *Pre3s* (median 44w5d PMA; range 44w, 46w + 1d) (i.e. 51 fMRI datasets) and 15 *Controls* (median 40w3d PMA; range 38w3d, 41w5d) entered the fMRI single-subject and group analyses.

Given that DTI data were collected at the end of the examination, many subjects woke up just before or during the DTI acquisition so that a smaller number of datasets were available with respect to fMRI data (i.e. 21 preterm datasets = 7 subjects \times 3 time points; 7 control datasets). After visual inspection and quality assessment of DTI datasets, only six *Pre1s* (median 33w1d PMA; range 29w4d, 33w5d) six *Pre2s* (median 40w PMA; range 39w, 40w5d), six *Pre3s* (median 44w4d PMA; range 44w2d, 46w1d) and six controls (median 38w5d PMA; range 37w6d, 40w4d) entered the white matter DTI analyses.

The protocol was reviewed and approved by our Institute Ethical Committee (San Raffaele Hospital). Parents of all study infants provided written permission for the protocol.

Neurodevelopmental assessment

All infants underwent neurodevelopmental assessment using the Griffiths Mental Development Scales (Revised) (Huntley 1996) performed at 6 months of chronological age in controls and 6 months of corrected age (=chronological age reduced by the number of weeks born before 40 weeks of gestation) in preterm newborns. The Griffiths Mental Development Scales provide an index of global developmental quotient (DQ) with subscales assessing skill areas (locomotor development, personal–social development, hearing–language, hand–eye coordination and performance). The mean (\pm SD) global developmental quotient score for the general population is 100 (\pm 12), and for the five subscales it is 100 (\pm 16).

The assessment was always performed by the same psychologist.

Neuropsychological data were analysed using a non-parametric test. The comparison between the two groups was performed using the Mann–Whitney test ($p < 0.05$).

Magnetic resonance imaging acquisitions

The MRI exams were performed at the Neuroradiology Unit of San Raffaele Hospital using a Philips Intera 3 Tesla scanner with an 8-channel phased-array head coil. All studies were conducted after feeding the children in a warm, quiet and dark environment to allow undisturbed sleep and thus reduce their movement during the acquisitions. No pharmacological sedation was used. Children's heart rate and arterial oxygen saturation were monitored throughout the exam and a neonatologist was present.

Anatomical MRI, DTI and fMRI acquisition protocol

All children underwent anatomical MRI, DTI and fMRI acquisitions using the following protocol. Anatomical MRI consisted in axial and sagittal T1-weighted images, and axial and coronal T2-weighted images to check for brain lesions and/or abnormalities. A high-resolution axial 3D T2 sequence (TR = 2,000 ms, TE = 160 ms, voxel size = $0.625 \times 0.750 \times 1 \text{ mm}^3$) was also acquired to allow post-processing spatial realignment and normalization. Diffusion tensor images were acquired using an SE diffusion EPI sequence with the following parameters: TR = 6,183 ms, TE = 84 ms; voxel size = $1.4 \times 1.4 \times 2 \text{ mm}^3$; 21 directions of diffusion gradients, $b = 700 \text{ s/mm}^2$, SENSE factor = 2, for a total acquisition time of 2 min and 41 s. Functional MRI was performed with a GE EPI pulse sequence having the following acquisition parameters: TR = 2,200 ms; TE = 40 ms; voxel size = $2.8 \times 2.8 \times 4 \text{ mm}^3$, SENSE factor = 2.

fMRI: study design

Functional MRI consisted in a block-design language paradigm, including 3 sessions of 70 scans each, alternating 5 volumes of stimulation and 5 volumes of rest condition for a total acquisition time of 3 min and 33 s. During the activation blocks, infants were stimulated with a passive language task consisting in listening to a fairytale (Little Red Riding Hood), whilst no stimulation was given during rest blocks. The natural voice of the same female operator was always used as language source of stimulation and was transmitted through home-made MR compatible headphones adapted for small infant ears which attenuated the EPI noise. Throughout the entire acquisition, the sound delivered through the headphones was constantly

monitored by an operator for infants' hearing, to exert the maximum degree of control possible over auditory stimulus presentation during each EPI scanning.

fMRI and DTI data processing and analysis

fMRI preprocessing

Functional MRI data were analysed using SPM5 (Wellcome Dept. Cogn. Neurol., London, <http://www.fil.ion.ucl.ac.uk/spm>). Each EPI volume was visually examined to exclude any severe head movements. Preprocessing consisted in the following three steps. First, for movement correction, all EPI volumes of each subject were realigned to one particular EPI volume, which was devoid of artefacts using a six-parameter linear transformation. If head movements were greater than 2 mm in one direction, and rotations higher than 2°, all the dynamic scans from that volume onwards were excluded from analysis. The remaining data were included in the analysis if at least the 70 % of the entire acquisition was still acceptable. Second, the EPI volumes of each subject were co-registered with their original T2-weighted 3D images. Third, the co-registered images were normalized to standard templates for group-based analyses. For this purpose, two ad-hoc T2-weighted templates were created. For *Pre1s* data, the first template (Atlas1) was obtained by co-registering the 3D T2-weighted image of each *Pre1* individual to the image of one specific *Pre1* subject, then averaging the co-registered 3D T2-weighted images together. The second template (Atlas2) was created with the data of *Pre2s*, *Pre3s* and *Controls*. This was obtained by co-registering the high-resolution T2-weighted 3D images of each subject to a T2-weighted paediatric atlas (Dehaene-Lambertz et al. 2002) then averaging the co-registered 3D T2-weighted images together thus creating the Atlas2. The co-registered EPI volumes of *Pre1s*, *Pre2s*, *Pre3s* and *Controls* were then normalized to the corresponding templates (*Pre1s* to Atlas1, *Pre2s*, *Pre3s* and *Controls* to Atlas2). First, the subjects' 3D T2-weighted images were normalized and then the same transformation parameters were applied to each co-registered EPI volumes.

The creation of two templates instead of one was necessary because the size and shape of the brain change dramatically from a premature to a mature stage so it was difficult to use the same reference for the three time points of preterm newborns and the *Controls*.

The quality of the normalization was visually inspected for each subject and the normalized EPI images were then spatially smoothed using an 8-mm Gaussian kernel (Friston et al. 2011).

fMRI analyses

At the single-subject level, statistical analysis of the smoothed normalized EPI images was implemented according to the general linear model (GLM). High-pass temporal filtering was applied to fMRI data using the cut-off of 128 s, and response to blocks of passive language listening alternating with blocks of rest was convolved with a box-car hemodynamic response function (HRF). To accommodate for motion in newborn EPI data, the six movement realignment parameters (3 translations, 3 rotations) were entered in the analysis as regressors of no-interest.

The second-level random effect group analysis was performed on the contrast images coding for passive listening vs rest independently in *Pre1s*, *Pre2s*, *Pre3s* and *Controls* (1-sample *t* test, $p < 0.001$ uncorrected, minimum cluster size 10 voxels). Since deactivations have been observed in single-subject analyses, as previously shown in newborns by other authors (Anderson et al. 2001), we have coded contrasts for both activations and deactivations to passive listening at the first level to perform the second-level group analysis depending on the pattern of activation.

To compare the *Pre2s* (40 weeks of PMA) and *Pre3s* (44 weeks of PMA) with the *Controls*, two independent two-sample *t* tests ($p < 0.001$ uncorrected, minimum cluster size 10 voxels) were also performed using the contrast images relative to subjects in each specific group.

Positive and negative correlations between fMRI contrast images (the *con_*.img*) for passive listening and the Griffiths Global Scale were investigated with SPM5 multiple regression analyses. Whole-brain correlations were assessed at a more liberal threshold ($p < 0.05$ uncorrected minimum cluster size 10 voxels) exclusively with the intent to explore the link between brain activity and functional mental growth in relation to the findings emerging from both one-sample and two-sample *t* tests.

For localization of brain areas, activations were superimposed to Atlas1 and Atlas2 images.

DTI: preprocessing

First, each subject's raw DTI data were visually inspected to exclude any severe head movements and DTI artefacts. All DTI processing steps were then performed using SPM8 (<http://www.fil.ion.ucl.ac.uk/spm>) (Friston et al. 2011) and the "Artefact Correction in Diffusion MRI (ACID)" SPM toolbox (<http://www.diffusioontools.com>).

All DTI images were effectively realigned and corrected for eddy currents and head movements (Mohammadi et al. 2010).

A custom brain mask was created for each group (i.e. *Pre1s*, *Pre2s*, *Pre3s*, *Cnts*) from the sum of prior tissue

probability maps of four structures—cortex, white matter, subcortical gray matter and cerebellum (<http://www.brain-development.org/>) (Serag et al. 2012; Kuklisova-Murgasova et al. 2011)—at a time point close to the median age of the group (*Pre1s*: median 34w PMA, range 31w, 34w; *Pre2s*: median 40w PMA, range 39w, 41w; *Pre3s*: median 45w PMA, range 44w, 46w; *Cnts* median 39w PMA, range 38w, 41w). Each group mask was co-registered to each subject to remove the non-brain tissue using the “Make Brain Mask” function in ACID.

After masking, the fractional anisotropy (FA) maps were estimated by ordinary least squares tensor-fitting method (Koay et al. 2006). At this point, two different atlases were created prior normalization step: a FA template representative of *Pre1s*, *Pre2s* and *Pre3s* groups (*Longitudinal Atlas*) and a FA template representative of *Pre2s*, *Pre3s* and *Cnts* groups (*At Term Atlas*).

To do this, first, the EPI images measured without diffusion gradients ($b = 0$ images) and FA maps were co-registered to age-appropriate T1 templates (Serag et al. 2012; Kuklisova-Murgasova et al. 2011) using affine transformations. Second, a preliminary template, average of FA registered maps, was calculated. Third, the original FA images were normalized to this preliminary template using nonlinear transformations. Fourth, a final template was recalculated using the normalized images until the influence of the template on the registration procedure was negligible. The normalized FA images are then finally obtained. The template generation procedure is part of the FA-VBS normalization toolbox in ACID (Mohammadi et al. 2012). The normalized FA images were further smoothed with a 6-mm (FWHM) Gaussian kernel.

The combination of improved eddy current correction and iterative multi-contrast registration in a customized DTI pre- and post-processing ACID pipeline as implemented here may substantially reduce the risk of potential image registration insufficiencies between neonatal FA images due to fast structural changes occurring between gestational weeks. We considered these image processing steps optimal for neonatal whole-brain voxel-based statistics (VBS) to enable the detection of very focal FA alterations as those that may be highlighted between neonatal preterm and control brains at statistical thresholds used in VBS analyses.

FA-VBS statistical analyses

First, for the longitudinal exploration of FA changes in preterm newborns (*Pre1s*, *Pre2s* and *Pre3s*), we applied voxel-based statistics (FA-VBS) using statistical parametric mapping (SPM8, <http://www.fil.ion.ucl.ac.uk/spm/>) by means of a repeated measures ANOVA model. We tested the null hypothesis $H_0 =$ “no significant FA

changes between 1, 2, and 3 s” on a voxel-by-voxel basis computing an F-contrast ($p < 0.05$ FWE corrected at the voxel level) assessing the differences between time points. Given that we wanted to compare FA measured at the three DTI time points in a very limited sample of preterm newborns, no specific hypothesis was formulated concerning the direction of the differences, and thus we used a more stringent threshold. We then explored which parameters were driving the difference by plotting effects of interest.

Second, we evaluated FA differences between preterm newborns (*Pre2s* vs *Controls* and *Pre3s* vs *Controls*) by means of a two-sample t test modelling the factor gestation week as a covariate to account for the age dependency of FA. Differences of FA values between these groups were statistically evaluated by T-contrasts in line with the fMRI group analysis ($p < 0.001$, uncorrected; minimum cluster size 10 voxels).

An absolute FA threshold of 0.1 was used in all analyses to exclude low FA values and further constrain misregistration effects whilst estimating true potential FA changes as well as to reduce the gray matter and cerebro-spinal fluid influence, where an explicit age-appropriate brain mask was used (i.e. mean weeks PMA of all subjects included in each analysis).

Results

Neurodevelopmental outcome

All infants underwent clinical follow-up and at 6 months objective evaluations were normal. Table 2 lists the scores on the Griffiths Developmental Scale performed at 6 months of corrected age for preterm infants (median 6m, range 6m, 6m3w3d) and 6 months of chronological age for controls (median 6m2w, range 6m, 7m1w4d). Preterm infants presented Griffiths global mean scores within the normal range but significantly lower compared to controls (97.65 vs 103.69; $p < 0.001$). A similar trend was also present in all Griffiths subscales, except for “personal-social development”.

fMRI results

In single-subject analyses, the cerebral activation to the linguistic stimulus was detected in preterm newborns as of 29w PMA. Cerebral activations or deactivations in response to the stimulus were detected in 7/17 (41.1 %) of *Pre1s*, in 15/17 (88.2 %) of *Pre2s*, in 15/17 (88.2 %) of *Pre3s* and in 14/15 (93 %) of *Controls*. A cerebral activation was found with negative HRF contrast in three *Pre1s*, in three *Pre2s*, in two *Pre3s* and in two *Controls*.

Table 2 Griffiths neurodevelopmental test mean score

	Age (days)	Griffiths					
		Global score	Locomotor development	Personal–social development	Hearing—language	Coordination eye–hand	Performance
Preterm subjects	185.46 ± 7.8	97.65 ± 6.4***	94.12 ± 8.8**	98.92 ± 7.1	100.12 ± 6.8**	97.00 ± 8.7*	97.73 ± 9.0**
Control subjects	195.7 ± 15.6	103.69 ± 5.05	101.46 ± 8.0	102.39 ± 7.494	104.87 ± 4.9	102.21 ± 8.16	104.609 ± 6.5

Results of preterm infants and controls groups at 6-month follow-up. Comparison preterm subjects vs controls with Mann–Whitney test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$

Age corrected age for preterm subjects and chronological age for control subjects

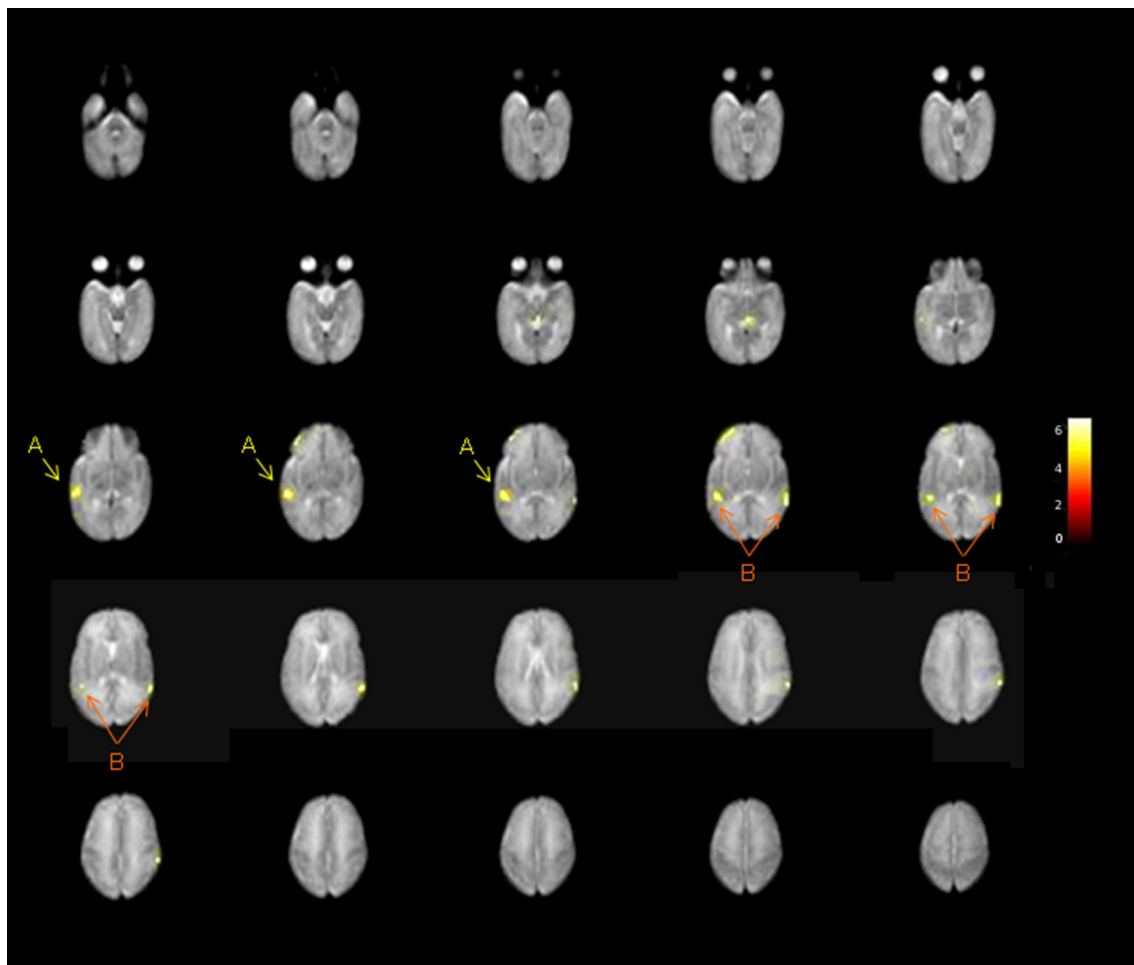


Fig. 1 Preterm 1. Results of the fMRI second-level random effect analysis ($p < 0.001$ uncorrected, 10 voxels minimum extent) for *Pre1s* group overlaid to axial slices of *Atlas1* obtained averaging all 3D-T2 normalized images of *Pre1s* subjects. Images are shown in

neurological convention (*right side of the image* right side of the brain). The *colour scale* represents the level of significance of the activation areas. *A* Left superior temporal gyrus, *B* left supramarginal gyrus cortex

Results of fMRI group analyses are shown in Figs. 1, 2, 3 and 4. A significant HRF BOLD signal response was found in the posterior superior temporal gyrus (STG) and supramarginal gyrus cortex with left prevalence in *Pre1s*. In *Pre2s* the HRF BOLD response was located in language

areas, specifically the superior temporal gyrus (STG) and supramarginal gyrus cortex (SMG), the inferior frontal gyrus (IFG) bilaterally, and the left temporo-occipital cortex. The cluster mass of language areas of activation was 4,784 mm³ on the right side and 5,544 mm³ on the left

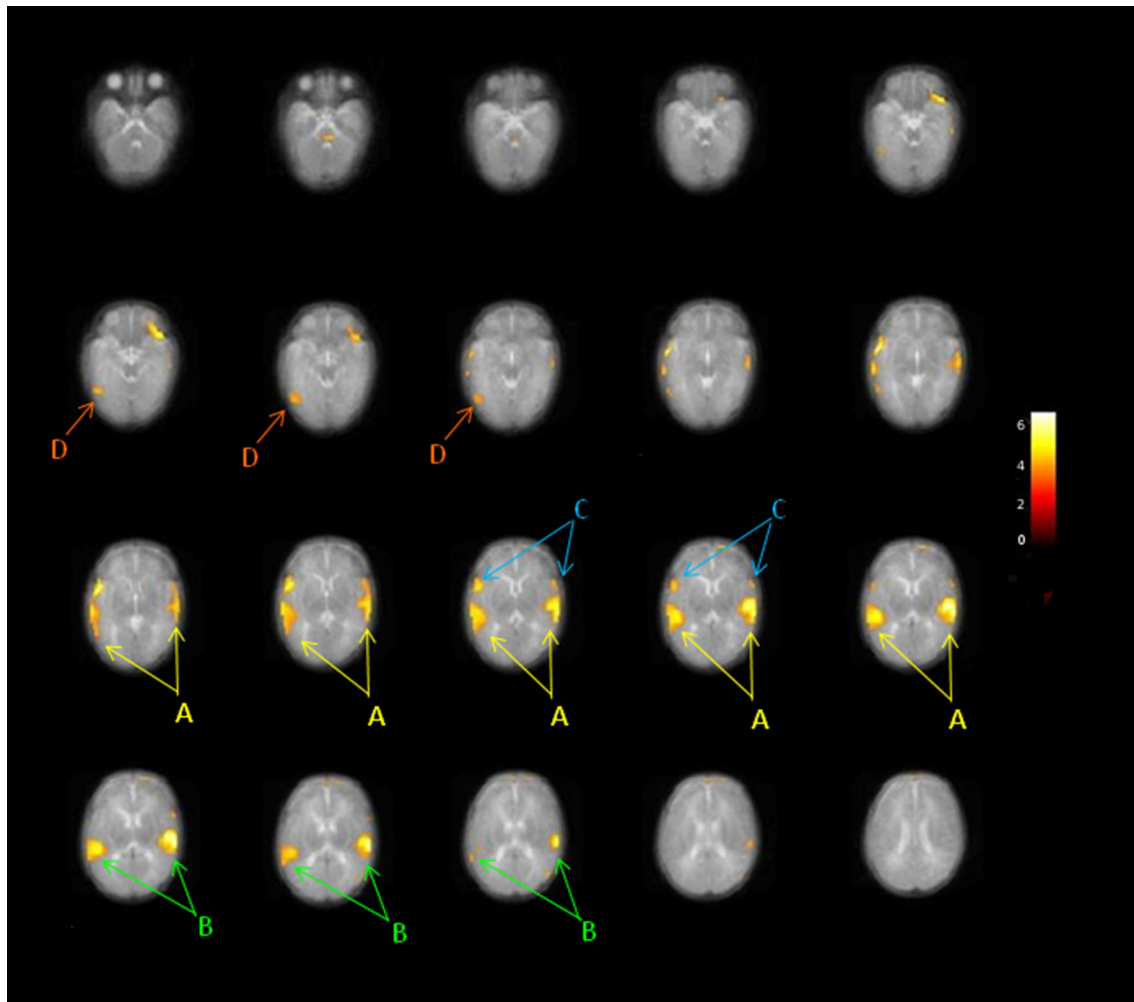


Fig. 2 Preterm 2. Results of the fMRI second-level random effect analysis ($p < 0.001$ uncorrected, 10 voxels minimum extent) for *Pre2s* group overlaid to axial slices of *Atlas2* obtained averaging all 3D-T2 normalized images of *Pre2s*, *Pre3s* and *Cnts* subjects. Images are shown in neurological convention (right side of the image right side of the brain). The colour scale represents the level of significance

of the activation areas. *A* Bilateral superior temporal cortex, *B* bilateral supramarginal cortex, *C* bilateral inferior frontal cortex, *D* left temporo-occipital cortex. The volumes of perisylvian language areas of activation are 4,784 and 5,544 mm³ in right and in the left side, respectively

side. In *Pre3s* the BOLD response to stimuli was detected in the STG and SMG cortex bilaterally. The cluster mass of language areas of activation was 4,504 mm³ on the right side and 4,584 mm³ on the left side. *Pre3s* showed a smaller extent of activation area in STG than *Pre2s* and no BOLD signal change was evident in the IFG. Both groups showed a leftward prevalence in the SMG. In *Controls*, bilateral activations were observed in the STG and supramarginal gyri, as well as in the areas of the postero-inferior thalami. Activation in this last position was not detectable in preterm newborns at any developmental stage (Figs. 1, 2, 3, 4). The two-sample *t* test second-level random effect analysis, performed for the group comparison between the *Pre2s* and *Controls*, showed activation in the area of the thalami, detectable only in *Controls* (Fig. 5).

fMRI correlations with neurodevelopmental outcome

Results of correlations between fMRI signals and the Griffiths Global Scale showed that in *Pre1s* neither a positive nor a negative correlation was found between the BOLD signal and the neuropsychological assessment. In *Pre2s* a positive correlation regarding the bilateral STG and the supramarginal gyrus was present between the BOLD signal and the Griffiths Global Scale, whilst no negative correlations were observed. In *Pre3s* a positive correlation between the BOLD signal and the Griffiths Global Scale was evident in the left inferior and middle temporal gyrus, and in the thalami with a leftward asymmetry. A negative correlation was present in the STG bilaterally. In controls no significant correlations were found.

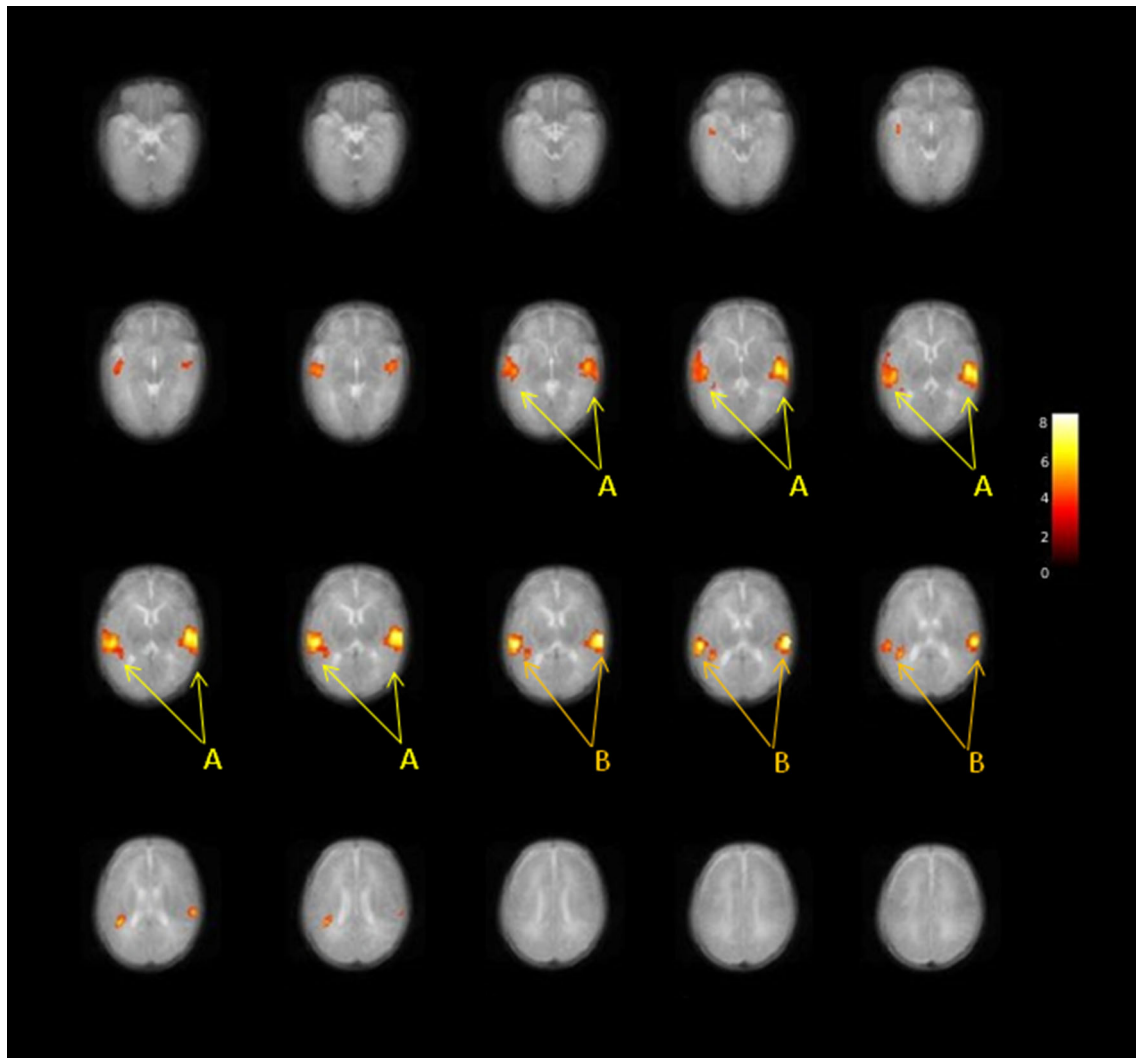


Fig. 3 Preterm 3. Results of the fMRI second-level random effect analysis ($p < 0.001$ uncorrected, 10 voxels minimum extent) for *Pre3s* group overlaid to axial slices of *Atlas2* obtained averaging all 3D-T2 normalized images of *Pre2s*, *Pre3s* and *Cnts* subjects. Images are shown in neurological convention (*right side of the image*

right side of the brain). The *colour scale* represents the level of significance of the activation areas. *A* Bilateral superior temporal cortex, *B* bilateral supramarginal cortex. The volumes of perisylvian language areas of activation are 4,504 and 4,584 mm³ in *right* and in the *left* side, respectively

FA-VBS: results

Significant longitudinal FA increases were observed bilaterally encompassing mainly the deep white matter, as well as the cortico-spinal tract at the level of the posterior arm of internal capsule, in particular the corona radiata and thalamic radiations. Moreover, the analysis showed deep portions of association fasciculi such as the inferior longitudinal fasciculus (ILF) and superior longitudinal fasciculus (SLF) including the arcuate fasciculus.

In Fig. 6, plots of parameter estimates of FA values for the three time points are represented for the left portion and right portion of corona radiata and the thalamic radiations (i.e. bars with standard errors from left to right refer to

Pre1s, *Pre2s*, *Pre3s* showing the significant FA increase from time 1 to time 3). The greatest FA increase was observed in the transition between *Pre1s* and *Pre2s*, rather than between *Pre2s* and *Pre3s*. Significant longitudinal FA increases were also observed in right deep occipital white matter, referring to deep portions of optic radiations.

Substantial increases of FA were observed in controls relative to *Pre2s*, affecting mainly peripheral WM tracts in both hemispheres. An asymmetric pattern of increases arose with a right prevalence in superior temporal, frontal and inferior parietal regions, whilst increases of FA appear to be symmetric in posterior parietal regions. Significant increases of FA were also observed in fornix bilaterally (see Fig. 7).

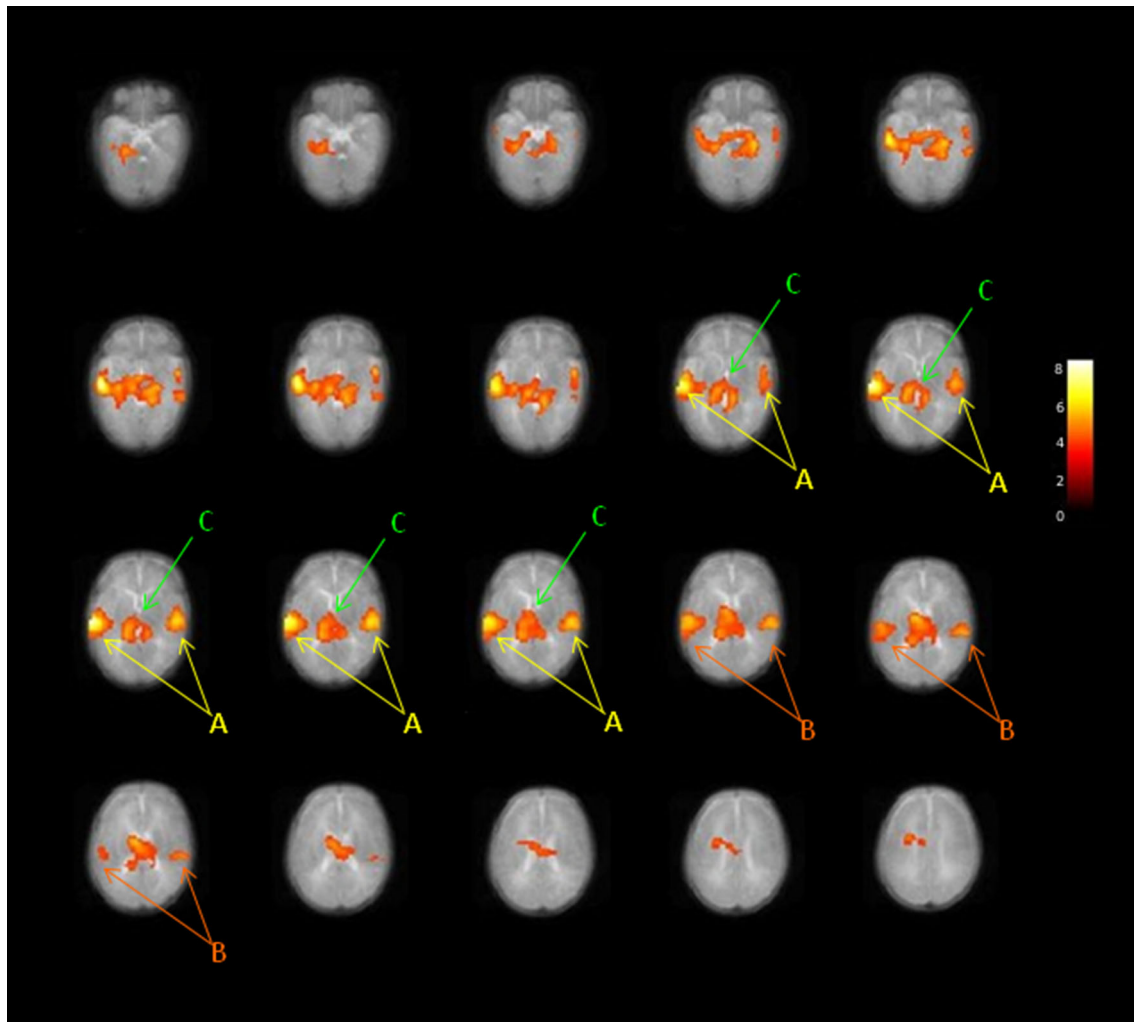


Fig. 4 Controls. Results of the fMRI second-level random effect analysis ($p < 0.001$ uncorrected, 10 voxels minimum extent) for *Cnts* group overlaid to axial slices of *Atlas2* obtained averaging all 3D-T2 normalized images of *Pre2s*, *Pre3s* and *Cnts* subjects. Images are

shown in neurological convention (*right side of the image* right side of the brain). The *colour scale* represents the level of significance of the activation areas. *A* Bilateral superior temporal cortex, *B* bilateral supramarginal cortex, *C* postero-inferior thalami

As for the comparison between *Pre3s* and controls, significant increases in FA were evident for the *Pre3s* only in small clusters (fornix bilaterally, left posterior arm of internal capsule and right subcortical posterior frontal area), whilst no FA reduction was instead detected in *Pre3s* compared to controls.

Discussion

fMRI

First-level single-subject analyses of passive listening to linguistic stimuli showed superior temporal and parietal cortical activation in response to language stimulation as of the 29th week of PMA. Previous fMRI studies have

reported cortical activations to auditory stimuli in foetal human brains at 33 weeks of GA (Jardri et al. 2008) and in newborns as of 33 weeks of PMA (Anderson et al. 2001). Moreover, the neurophysiological cortical response of sensory systems was previously detected in the early pre-term phase, before 32 weeks of PMA (Graziani et al. 1974).

In our study the percentage of subjects showing preterm cortical activation increases from *Pre1s* to *Pre2s*: single-subject fMRI analyses show a rate of activation of 41.1 % in *Pre1s* (<34w PMA), significantly increasing at 88.2 % in *Pre2s* (aged 40w PMA), and then confirming in *Pre3s* (aged 44w PMA). The rate of activation is 93 % in *Cnts*.

A low rate of cortical activation in infants was reported in another fMRI study (Dehaene-Lambertz et al. 2002). Moreover, it has been previously demonstrated that the percentage

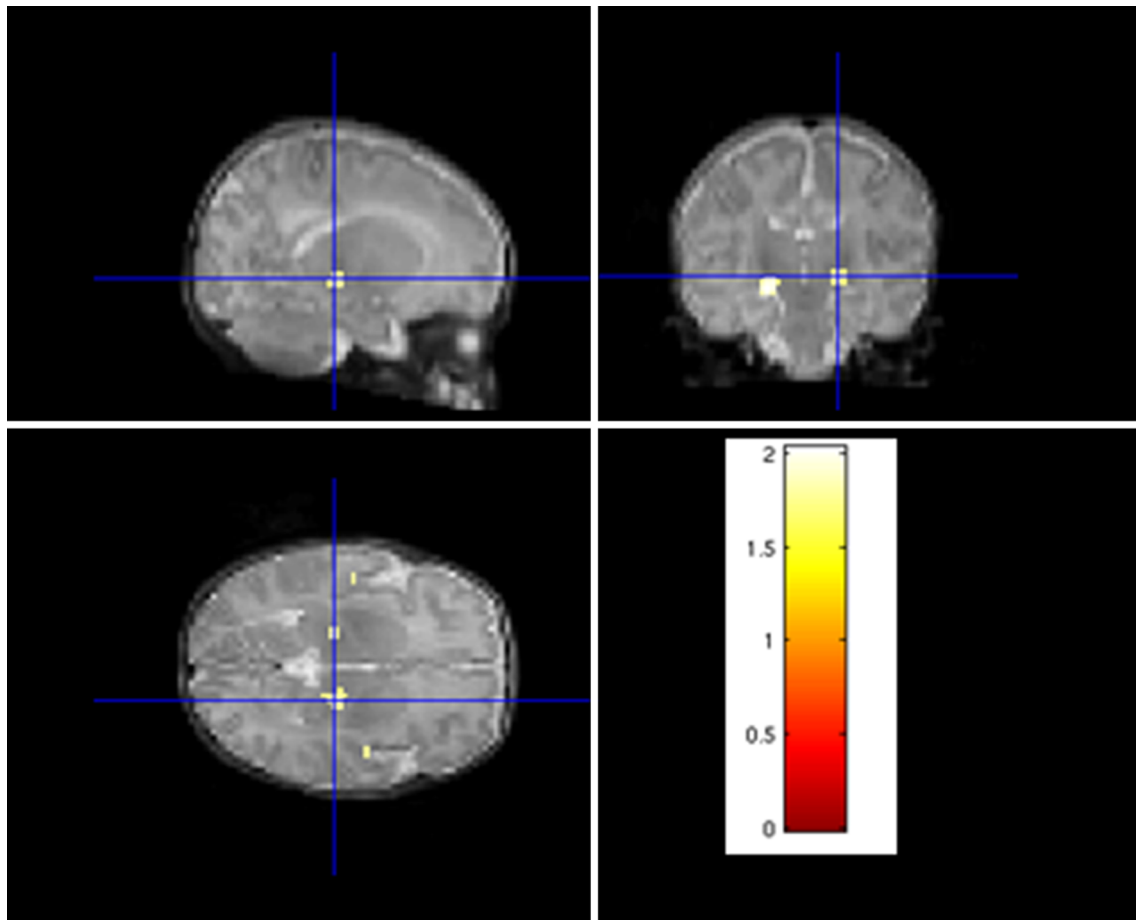


Fig. 5 The two-sample t test second-level random effect analysis with t -maps performed for a group comparison between *Pret2s* and *Cnts* shows the different pattern of activation in the area of medial geniculate body and auditory thalamus, detectable only in *Cnts*.

of brain activation increases with age, suggesting a developmental trend from infancy to adulthood (Schapiro et al. 2004). Concerning our study population, the absence of cortical activation in a few preterm subjects and the progressive increment of activation rate may be at least partly due to the immaturity of the cortex and of the brain connections in the first acquisitions. In fact, according to neuropathological studies, during the early preterm phase (24–32 weeks PMA) the thalamo-cortical axons are still establishing connections through the subplate zone. Cortico-cortical and transcallosal connections are yet to be fully established. Vascular system is still immature. Moreover, there is a coexistence of transient and permanent sensory-driven circuits and the first evoked potentials measured in the foetal brain seem to be generated by this transient circuit, which involves both the subplate and the cortical plate (Kostovic and Jovanov-Milosevic 2006; Judas et al. 2013). In addition, after 24 post conceptional weeks, synapses (which are present from 8.5 weeks of gestation, but until 18 weeks, only outside the cortical plate) rapidly

Results are overlaid to *Atlas2* images and are in neurological convention (*right side of the image* right side of the brain). The colour scale represents the level of significance of the difference in activations between the two groups

accumulate in the cortical plate together with the growing of thalamo-cortical axons, suggesting the development of direct thalamo-cortical synapses (Molliver et al. 1973). From 29 to 32 weeks thalamo-cortical connections with layer IV represent an anatomical substrate for a sensory-expectant cortical activation by somatosensory, auditory and visual stimuli (Kostovic and Judas 2010).

Thus, the difference in registered BOLD signal of *Pre1s* subjects with respect to BOLD signal in *Cnts* may be the consequence of these differences in structural, metabolic and functional connectivities.

Finally, in single-subject analyses a negative brain activation was estimated only in a few subjects both in preterm and control subjects. The prevalent positive activation agrees with other previous reports (Yamada et al. 1997; Morita et al. 2000) in which preterm newborns and infants younger than 2 months show positive activation whilst infants between 2 months and 1 year old may have a negative activation.

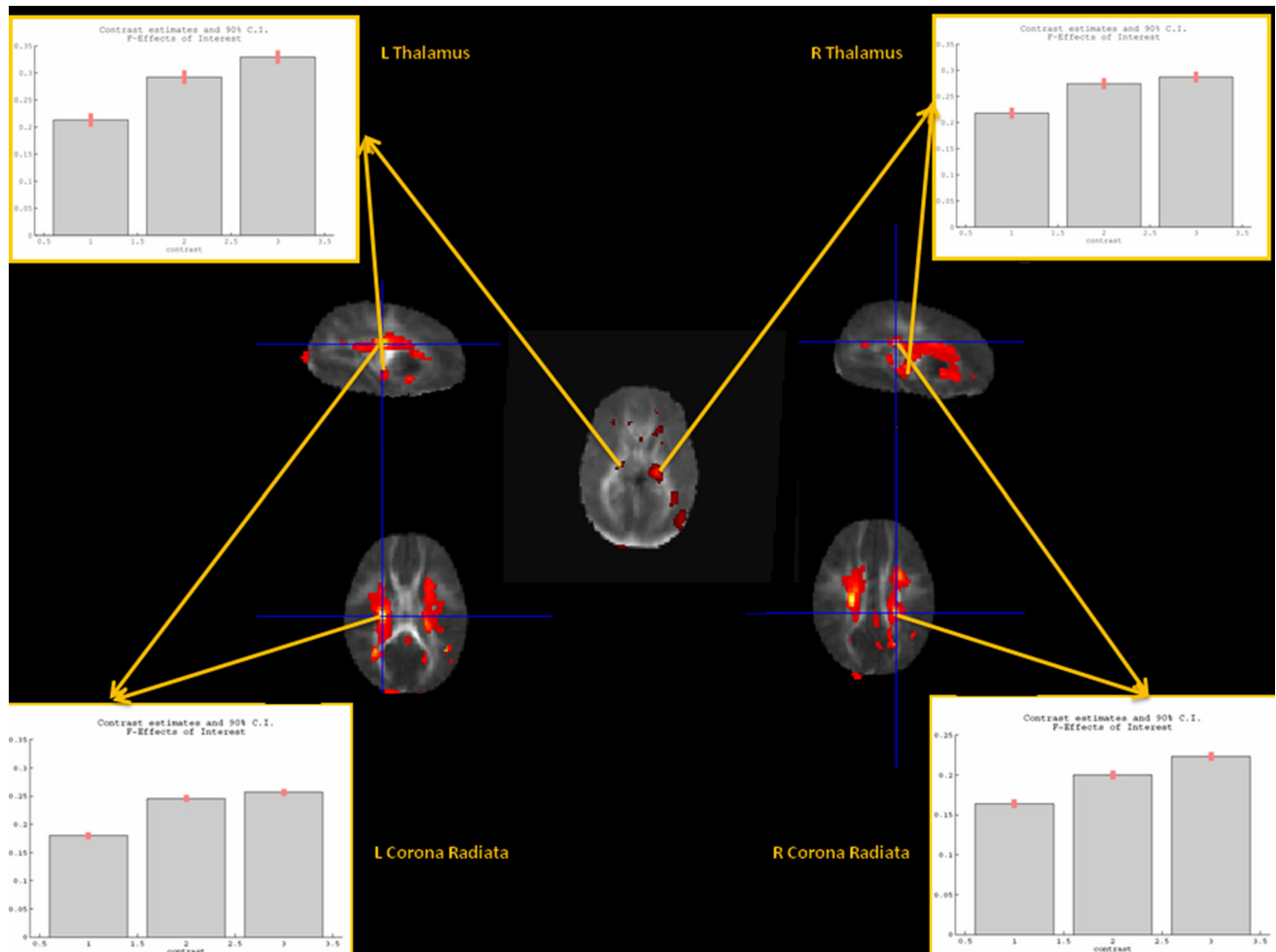


Fig. 6 Plot of the F-contrast, resulting from the one-way ANOVA analysis of FA values for the three time points represented for the left portion and right portion of corona radiata and the thalamic

radiations. Bars with standard errors from *left to right* refer to *Pre1s*, *Pre2s*, *Pre3s* show the significant FA increase from time 1 to time 3. Significance level: $p = 0.05$ FWE corrected at the voxel level

Arichi et al. (2012) showed that a negative activation on group analysis may be the result of a non-optimized (i.e. adapted for age) modelling of HRF. A negative activation in our case was evident only in some subjects homogeneously distributed across all age groups; however, it was not significantly evident in any group analyses, not even in the *Pre1s* group. On the other hand, the complexity of the BOLD signal should be considered, especially the one in newborns (Gaillard et al. 2001; Anderson et al. 2001; Chugani et al. 1987; Seghier et al. 2006). Kozberg et al. (2013) have recently demonstrated that neonatal responses to stimulus may be susceptible to stimulus-evoked systemic blood pressure increases which may resemble adult positive BOLD responses. So, as our main interest was the comparison amongst groups, and given that the negative apparent activations were equally distributed in the groups, we did consider either positive or negative contrast images to be included into the group analysis of our samples.

Regarding the group analyses, to our knowledge our study is the first fMRI study with passive listening to linguistic stimuli in preterm infants in the first months of life acquired at several time points. One previous fMRI study explored brain development longitudinally in the same subjects by applying the resting-state technique but without any sensorial stimulation (Smyser et al. 2010).

In our group analyses, a distinctive pattern of cortical activation was observed in preterm newborns within the first few months of PMA: from early prevalent left temporal activation to bilateral temporal activation at term equivalent and then a further slightly left prevalence. These results are in accordance with a previous fMRI study showing unilateral activation in the sensory motor cortex in preterm infants before 32 weeks of PMA whilst bilateral activation was depicted in newborns at term PMA (Arichi et al. 2010).

Specifically, in *Pre1s* (below the 34th week of PMA) a leftward asymmetry in activation was found in the posterior STG and supramarginal gyrus cortex. Early brain

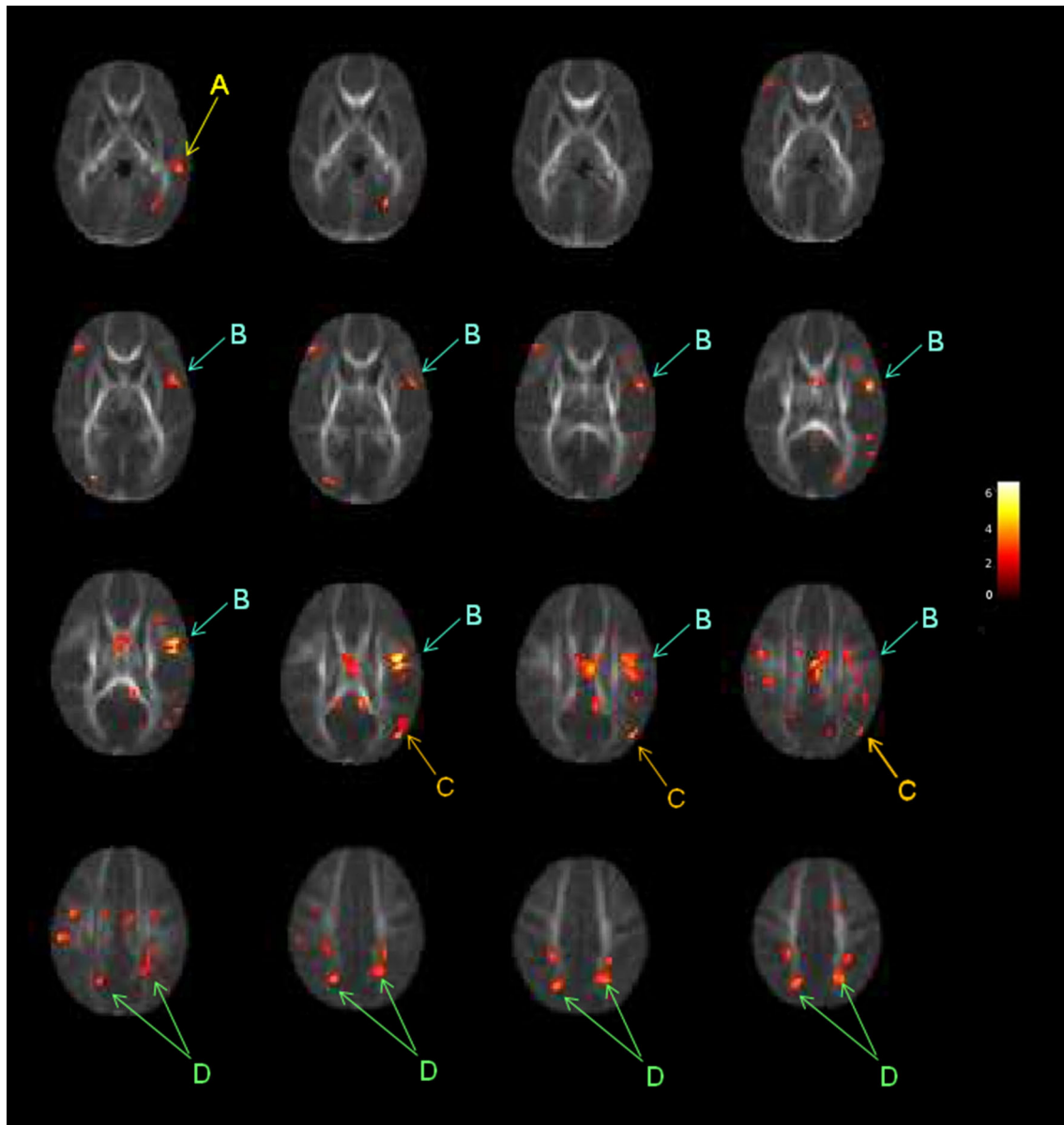


Fig. 7 Two-sample t test FA comparison between *Cnts* and *Pre2s* ($p < 0.001$, uncorrected; minimum cluster size 10 voxels). The figure shows areas of significant FA increases in *Cnts* with respect to *Pre2s* overlaid to *At term Atlas* obtained on averaging all the normalized *Pre2s*, *Pre3s*, *Cnts* FA maps. The colour scale represents the level of

significance of the difference between FA maps of controls and *Pre2s*. Images are shown in neurological convention (*right side of the image* right side of the brain). *A* Right superior temporal regions, *B* right frontal regions, *C* right inferior parietal regions, *D* bilateral posterior parietal regions

structural asymmetries were found in previous studies: even in foetuses, the sylvian fissure is longer on the left side and is associated with a larger left planum temporale (Chi et al. 1977; Dehaene-Lambertz 2000; Dehaene-Lambertz et al. 2002; Witelson and Pallie 1973). Anatomical and functional hemispheric lateralization originates from differential gene expression and leads to asymmetric structural brain development, which initially appears in the perisylvian regions by 26 gestational weeks. In vivo, a foetal MRI study (Kasprian et al. 2011) demonstrated a predominant pattern of temporal lobe asymmetry in a large

cohort of human foetuses between 18 and 37 gestational weeks: in detail, over two-thirds of the foetuses showed a larger, left-sided TL, combined with the earlier appearance of the right superior temporal sulcus by 23 gestational weeks. Besides, in a recent study (Mahmoudzadeh et al. 2013) based on 14 preterm newborns using near infrared spectroscopy, a posterior temporal region showed faster and more sustained responses to syllables in the left hemisphere than in the right one.

In our study a trend towards bilateral activation was observed during brain development: in *Pre2s* and *Pre3s*.

The activation in the language areas was bilateral with a slightly leftward prevalence in size.

We hypothesize that bilateral activation in both *Pre2s* and *Pre3s* groups is due to the maturation of interhemispheric connections between homotopic counterparts, connections that are not yet consistent during the preterm period, as shown by Smyser et al. (2010).

Thus, we suggest that in these preterm infants there are different steps of functional development. First, in the preterm period, the left hemisphere, being more mature than the right one, leads to an “innate” leftward activation even before the acquisition of language (Dehaene-Lambertz et al. 2005, 2006). Second, in the term phase, the activation becomes bilateral with the involvement of both hemispheres due to the development of interhemispheric connections. Third, during the following weeks, the slightly leftward prevalence observed in these infants is probably due to early developmental modifications as a consequence of language exposition. These modifications may reflect the first preverbal steps of the well-known process of language left lateralization as a function of age, lasting many years until adolescence (Brauer et al. 2008, 2011; Friederici et al. 2011; Holland et al. 2007; Szafarski et al. 2006; Perani et al. 2011).

Furthermore, larger clusters of brain activation in *Pre2s* with respect to *Pre3s*, in STG, supramarginal areas and in bilateral IFG and left temporo-occipital, point to a more extensive recruitment of these language-driven brain areas (Vannest et al. 2009). This largely transient pattern of cortical activation may call for the presence of an immature redundant brain response involving many additional areas in this period, not fully specialized, however affected by the progressive exposure to language. Then, once a further developmental stage has been rapidly reached, such as in *Pre3s*, the activation pattern involves less areas and appears to be reduced in extension (i.e. lesser extent of activation in STG and a lack of activations in the IFG and the left temporo-occipital area) likely reflecting a more fine-grained and focalized activity.

In *Controls*, a distinctive pattern of thalamic activation was found, whilst no thalamic activation was present in the preterm groups at any stage of development. A recent study of resting-state functional MRI (Smyser et al. 2010) performed on preterm and at term infants found a significant difference between the two groups in these areas. In their study, the authors showed more robust localized and interhemispheric connections in term control infants than in preterm infants at 40 weeks of PMA. In addition, functional connections between the thalamus and sensorimotor cortex were prominent in the term infants. Our findings are in agreement with this study’s hypothesis that the thalamo-cortical connections may be less mature and/or

organized differently in preterm infants than in term newborns, at least in the first few months of life.

Several factors may play a role in the different patterns of thalamic activation in preterm infants. Firstly, during the third trimester of gestation the foetal brain undergoes crucial changes for future cortical development. At this stage, both the permanent thalamo-cortical fibres and the transient subplate circuit (subcortical nuclei–subplate neurons–cortical plate) are present (Kostovic and Jovanov-Milosevic 2006). Preterm delivery, even in the absence of macroscopic brain injury, may alter the subplate thus affecting the proper growth of thalamo-cortical connections leading to a different pattern of cortical and thalamic activation (Ghosh and Shatz 1994; Judas et al. 2013).

fmMRI correlations with neurodevelopmental outcomes

At 6 months of corrected age preterm subjects without major neurosensory impairment showed scores on the Griffiths test within the normal range, but significantly lower than controls. These differences were particularly marked, in the global index and in several domains, as locomotor, hearing–language and performance. These data confirmed the cognitive difficulties described in preterm children during childhood (Caravale et al. 2005; Dall’oglio et al. 2010).

Statistically, the Griffiths test presents good reliability coefficients as internal consistency, but critical test–retest reliability coefficient in the first 2 years of life, like other standardized scales. In fact, some studies have demonstrated that caution is needed in the interpretation of data obtained from several standardized neurodevelopmental examinations during the first 2 years of life in preterm infants (Chaudhary et al. 2013; Grimmer et al. 2010; Walch et al. 2009) because in this period, there is a high variability in development and only after the second year changes become more constant. Nevertheless, our intent is to study these infants with a long-term neuropsychological follow-up so that our assessment can therefore be a starting point for future evaluations, and further studies of these children at school age are required.

Taking into account the limits of an evaluation at such an early age, the correlation analysis between BOLD signal and Griffith measures showed that in preterm newborns at 40 weeks of PMA (*Pre2s*) bilateral activations in STG correlate with better scores on neurodevelopmental evaluations, whilst in preterm infants at 44 weeks of PMA (*Pre3s*) the better neurodevelopmental outcome was linked with a leftward temporal activation.

These correlations, although interesting, need to be confirmed by future longitudinal studies after the second year of age and with more stringent thresholds. Therefore,

these results should not be generalized and interpreted with caution considering the above-mentioned limitations.

DTI-FA VBS

The neurodevelopmental characteristics of WM microstructural changes induced by premature birth are of great interest and many studies have been conducted in this direction (Hermoye et al. 2006; Huppi et al. 1998, 2001; Miller et al. 2003; Mukherjee et al. 2001, 2002; Neil et al. 1998; Partridge et al. 2004; Schneider et al. 2004; Liu et al. 2010; van Kooij et al. 2012; Serag et al. 2012; Kuklisova-Murgasova et al. 2011). To trace the structural precursors of altered brain activation patterns through lifespan and in adulthood, it remains substantial to address as closely as possible to birth and auspiciously in a longitudinal fashion, which surely represents the best possible way to investigate maturational changes.

We have carried out a longitudinal DTI study on babies born preterm, observed in three time periods, and an FA comparison between babies born preterm and at term at a comparable PMA. Specifically to both these aims, we have designed and implemented a novel voxel-based FA normalization approach, which implies the creation of a customized template based on the characteristics of the samples (i.e. PMA) to be compared and allows fine-grained alignment between WM structures of each subject for a more accurate comparison between time periods and groups.

Our intent was on the one hand to trace the first stages of WM development after preterm birth and identify the fibres most sensitive to development in the earliest stages of life, and on the other to parallel an FA measure of WM structural connectivity with BOLD activation in infants born preterm and at term at a comparable PMA in the attempt to shed light on potentially different functional structures responsible for a different functional architecture (Thomas and Karmiloff-Smith 2002).

An in vitro DTI study (Huang et al. 2009) pointed out early evidence of association tracts. In particular, the authors showed that for 19-week-old brains, inferior frontal occipital, inferior longitudinal fasciculus, and uncinatus can be traced, but the superior longitudinal fasciculus is not prominent, even at birth.

In our DTI longitudinal evaluation, we found, as expected, a progressive increase of FA values in preterm brain maturation from 29 to 44 weeks of PMA. Only few studies (Huppi et al. 1998, 2001; Miller et al. 2002) have demonstrated these modifications, as in our study, in serial evaluations of the same patients, studied at different ages in the first months of life. In these studies, the authors reported that anisotropy increases with age in widespread white matter regions in preterm normal newborns and

observed a higher FA in the internal capsule than in the rest of central WM.

In our longitudinal results the increase of FA is significantly evident overall in profound white matter, in particular involving corona radiata fibres and thalamic areas, including thalamic radiations. Furthermore, we observed a significant FA increase also in more peripheral portions of some of these fibres, namely in optic radiations and considerably in fronto-temporal radiations along the deep part of “arcuate fasciculus” (anterior part of superior longitudinal fasciculus). A so precocious development of fibres for connection between frontal and temporal areas involving the language relevant areas is a very intriguing finding. This early structural connectivity seems to be the structural basis of the functional cortical activity already present from 29 weeks PMA, detected in temporal areas with fMRI. These FA increments are stronger for *Pre1s* to *Pre2s* and less evident from *Pre2s* and *Pre3s* highlighting an early maturation of these fibres.

In the comparison between control infants and preterm infants evaluated at the same PMA (Control vs *Pres2s*) we observed, in accordance with Huppi et al. (1998), lower white matter FA values in the latter, which means later maturation compared the former. This is mainly evident in peripheral portions of white matter tracts, including thalamo-cortical radiations in subcortical areas of both cerebral hemispheres. Conspicuous differences were observed in subcortical superior temporal, frontal and inferior parietal regions with a right prevalence, and bilaterally in posterior parietal regions. Thus, it appears that significant differences between controls and preterm newborns emerge strikingly in subcortical areas, where, from the 20th to the around 45th week of PMA, the subplate is known to be located in the form of a transient laminar brain compartment (Kostovic and Jovanov-Milosevic 2006; Kostovic and Judas 2010; Judas et al. 2013). This structure is essential for cortical development and is known to be selectively vulnerable in preterm birth. If the subplate is in any way altered at this stage of development, and somehow even premature birth itself can interfere with the normal development of this framework, the cortical afferent axons may undergo a maturational delay or follow different paths to the cortical connections.

These different patterns of maturation involving thalamo-cortical connections and consequently cortico-cortical connections might be the basis of alternative networks for processing linguistic stimuli in children born prematurely.

For example, functional connectivity measured in preterm born children highlights different neural pathways for lexical semantic processing with respect to term controls, suggesting that the dynamic nature of neural connectivity is involved for recovery in the preterm brain (Schafer et al. 2009). Finally, we have observed a right prevalence of FA

increases in controls, overall observed in the subcortical superior temporal, frontal and inferior parietal regions supporting the idea that the right hemisphere is involved in more sudden developmental changes with respect to the left one which, on the contrary, seems to be provided with an innate genetic promptness.

It should be underlined, however, that our DT analyses are limited by the very small subject groups, 6 vs 6, and so our results should be interpreted with caution.

Conclusions

In preterm infants, auditory processing of linguistic stimuli is already evident since very early stages of development (in our study since 29 wPMA) in the form of BOLD activation in left superior temporal and supramarginal areas. The pattern of functional cortical activity quickly evolves with the recruitment of the same areas in right hemisphere added with crucial areas for successive language processing as fronto-opercular areas bilaterally, in the at term equivalent period. After 4–6 weeks, functional cortical activation appears in a more tuned and lateralized way in the left hemisphere, thus possibly reflecting the first pre-verbal steps of the future lateralized pattern of activation linked to progressive language exposition. The transition between a bilateral temporal activation at term age and a leftward activation at 44 weeks of PMA correlates positively with neuropsychological outcome and seems to suggest a link between these two results. Further longitudinal studies over the first 2 years should be performed to confirm this evidence.

The observed cortical activity is combined with a very precocious maturation of the deep part of thalamic radiation and of association fibres such as SLF, including immature arcuate fasciculus, and ILF. The evidence of functional thalamic activation and more mature subcortical tracts of the fibres, including thalamic radiations, may represent the substantial gap between preterm and at term newborns. These results might be in part related to the different developmental trajectory of the subplate and thus of the transient thalamo-cortical connections in infants born prematurely even in the absence of focal brain lesions.

In conclusion, fMRI and DTI may provide insights on the impact of premature birth on microstructural brain injury by adding microstructural and functional information to morphological MRI to support the latest Neonatal Intensive Care Unit rehabilitative programs with intensive sensory stimulation dedicated to preterm newborns.

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