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At First Sight: Fetal Eye Movements Reveal a Preference for Face-Like Configurations From 26 Weeks of Gestation

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ABSTRACT

Previous research indicates that both adults and newborns show enhanced electrophysiological and behavioral responses to schematic face-like configurations (FCs—three dots composing a downward-pointing triangle), as compared to the inverted configurations (ICs). Even fetuses, when exposed to light stimuli projected through the uterine wall, preferentially orient their heads toward FCs rather than ICs. However, when this effect emerges along the third trimester of pregnancy and in relation to the maturation of which brain structures is still unknown. Here, to provide a sensitive measure of fetal preference for FCs along the whole third trimester, fetal lens movements in response to FCs and ICs was monitored with 2D-ultrasound. In a series of three experiments, fetuses were recruited at 26, 31, and 37 weeks of gestational age and were presented with both flashing and continuous light stimuli. Our results showed that significantly more lens movements were observed in response to continuous as compared to flashing light stimuli. Furthermore, lens movements linearly increased within the third trimester and, regardless of the time-point, significantly more lens movements were observed in response to FCs versus ICs. We also found a significant correlation in the first time-point, wherein the greater the FCs versus ICs differential response the larger the thalamic nuclei dimension. These findings suggest that FC preference is already present at the beginning of the third trimester, as soon as thalamocortical projections are established.

1 | Introduction

Faces are highly salient stimuli, representing a crucial source of information and social interaction, for human beings and other animal species (Johnson 2005; Röder et al. 2013). Even from a developmental perspective, paying attention to faces is pivotal for young individuals to grasp conspecifics' intentions and emotions and to support individual recognition (Elsherif, Saban, and Rotshtein 2017; Johnson, Senju, and Tomalski 2015). It is not surprising, therefore, that human faces show an enhanced sensory processing, as compared to other visual stimuli, and dedicated neural circuits (Haxby, Hoffman, and Gobbini 2000; Johnson 2005; Schweinberger and Neumann 2016). Interestingly, schematic face-like configurations (i.e., three dots composing a downward pointing triangle—see Figure 1; from now on FCs[1]) seem to show similar processing advantages as real faces in both adults and newborns. In adults, FCs display an enhanced mismatch detection mechanism as compared to other three-dot geometric configurations (i.e., upward or right/leftward pointing triangles; Galigani, Ronga, Bruno et al. 2021).

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Summary

- Fetuses show a preference for face-like (FCs) versus inverted configurations (ICs): when FC preference emerges and in relation to which brain structures is still unknown.
- Fetal eye-lens movements in response to FCs and ICs were monitored with 2D-ultrasound.
- Our results showed that FC preference is already present at the beginning of the third trimester.
- At 26 weeks, FC preference is significantly correlated with thalamic nuclei dimension.

From a developmental point of view, it seems that human newborns prefer to fixate face-like stimuli as opposed to similar but inverted configurations (from now on ICs; Morton and Johnson 1991; Valenza et al. 1996; Cassia, Turati, and Simion 2004). It has been proposed that such preference in newborns, rather than being specific for FCs, may be supported by general biases toward some physical features systematically (but not selectively) present in FCs, such as the presence of more elements in the upper-visual field (*top-heavy bias*; Turati et al. 2002; Cassia, Turati, and Simion 2004) or positive contrast polarity (Farroni et al. 2005). Farroni et al. (2005) demonstrated that human newborns are sensitive to the phase contrast of the presented face-like stimuli (Farroni et al. 2005). The authors demonstrated that newborns looked longer at the face-like stimuli displaying black elements over a white background (positive contrast polarity), rather than the opposite. This finding suggests that newborns' visual preferences are directed to the stimuli better resembling a real face (where eyes and mouth are usually darker as compared to the skin, due to shading of the eye-area occurring in natural illumination conditions; Farroni et al. 2005; Johnson, Senju, and Tomalski 2015). More recently, Buiatti et al. (2019) demonstrated that an enhanced electrophysiological process (a higher neural entrainment) can be selectively observed for FCs, and not for other top-heavy and non-face-like configurations. Overall, these results confirm the presence of both specific (i.e., selectively related to face-like configurations) and unspecific factors (i.e., related to general attentional biases toward some visual features) supporting the face-like stimulus preference observed in human newborns.

Crucially to the purpose of the present study, a similar finding has been observed in third-trimester fetuses. By monitoring head movements through 4D sonographic scans, Reid et al. (2017) noticed that fetuses preferentially turned their heads toward face-like light stimuli projected through the uterine wall, as opposed to similar light stimuli representing the inverted configuration. More recently, in a group of nine third-trimester fetuses, Reissland et al. (2020) confirmed that a greater amount of head turns can be observed in response to FCs (top-heavy or not) as opposed to different configurations non-resembling a face. However, when this preferential effect for FCs emerges along the third trimester and in relation to the maturation of which brain structures is still unknown.

Here, in a series of three experiments, we capitalized on 2D sonographic scans to record eye-lens movements as a measure of fetal attention orienting to FCs versus ICs stimuli (Figure 1). Importantly, in postnatal studies, gaze behavior is reliably used as an implicit marker of newborns' and infants' attention orienting to salient stimuli (Johnson, Senju, and Tomalski 2015). More relevant to our study, in a previous investigation on newborn preference to FCs and ICs stimuli, a larger number of eye-movements as compared to head-turnings was observed (Johnson et al. 1991). Furthermore, Donovan and colleagues, by analyzing the 2D component of 4D ultrasound scans, demonstrated that fetal eye movements (observable in 2D) can be detected independently of head movements (observable in 4D) (Donovan et al. 2020). Also in the present study, we recorded lens movements (instead of head-turns) to provide a sensitive measure of fetal face-like configuration preference along the whole third trimester. In our view, this approach may overcome the problem that, in the later stages of pregnancy, fetuses' head movements are often limited due to the constrained uterine environment (DiPietro, Costigan, and Voegtline 2015), also providing a translational parameter to bridge the gap between pre- and post-natal investigations. In Experiment 1a, by employing the same visual stimuli of Reid et al. (2017) (Figure 1), in a population of third trimester fetuses (gestational age, from now on g.a.: 37 weeks), we verified whether we were able to replicate the preference for face-like configurations by tracking lens movements instead of head turns. As a result, we expected to observe more lens movements in response to face-like configurations as compared to inverted configurations. In Experiment 1b, we tested different kinds of stimulus delivery, with the aim of comparing the results obtained with a continuous versus a flashing light. In previous fetal studies both static (Eswaran et al. 2002; Eswaran et al. 2004; Kiuchi et al. 2000; Reid et al. 2017) and flashing visual stimuli (Matuz et al. 2012; Sheridan et al. 2010) were exploited. However, to the best of our knowledge, it has never been systematically investigated which visual stimulation modality, that is, continuous versus flashing, is the best in eliciting fetal lens movements and in prompting the differential effect for FCs and ICs. Through this experimental approach we aim at providing novel methodological hints for future research. Verifying whether flashing visual stimuli can elicit consistent responses in healthy third-trimester fetuses seemed as a crucial feasibility testing for future behavioral and fetal-MEG protocols aiming at exploiting moving/flashing visual stimuli, such as those employed in neural entrainment research (see e.g., Buiatti et al. 2019).

Experiment 2 (g.a.: 26 weeks, 31 and 37 weeks) was specifically dedicated to investigate *when* the preference for face-like configurations (again expressed with a greater number of lens movements in response to face-like configurations) emerges within the third trimester and in relation to the maturation of which brain structures. The third trimester represents a crucial period for the neurodevelopment of fetal visual system. While in the second trimester, the connections between the retina and the visual cortices start to develop, within the third trimester, the thalamocortical pathway is established and we assist to the maturation of visual-related cortical and subcortical areas. Through Experiment 2, we checked whether a differential response between FCs and ICs could be observed at three different time-points (26 weeks g.a.—i.e.,—31 and 37 weeks g.a.),

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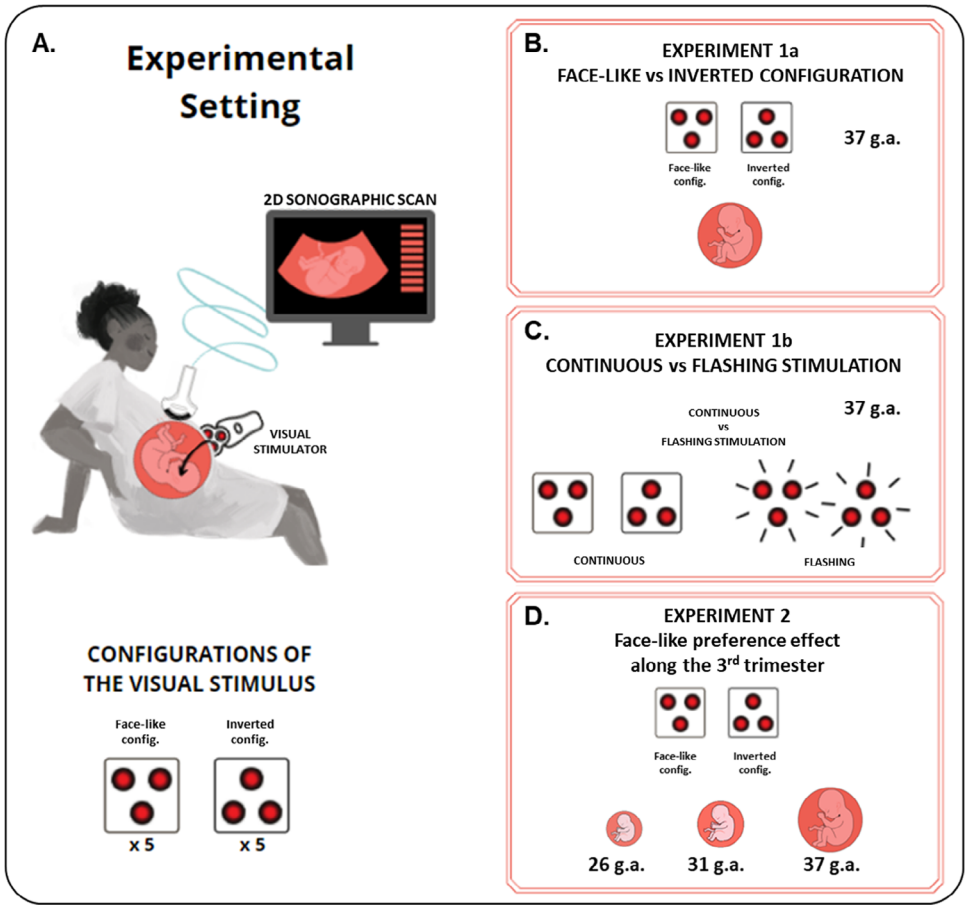

FIGURE 1 | Conceptual illustration of the experimental setting and procedures for Experiments 1–2. (A: Top) Graphical representation of the experimental setting with the pregnant women concurrently subjected to the sonographic exam and to the fetal visual stimulation. An experimenter, blinded to the purpose of the study, manually displaced the visual stimulator in a horizontal direction across the maternal abdomen (5 left-to-right and 5 right-to-left experimental trials). Bottom: experimental visual stimuli delivered to the fetus through the maternal abdomen, depicting either a downward pointing (i.e., FCs) or an upward pointing triangle (i.e., ICs). (B) Experimental procedures for Experiment 1a ($N = 17$; g.a.: 37 weeks), employing continuous visual stimuli. (C) Experimental procedures for Experiment 1b ($N = 8$; g.a.: 37 weeks), with two stimulation modalities (continuous and flashing light). (D) Experimental procedure for Experiment 2, where responses of the fetuses in three time-points of the third trimester were compared ($N = 9$; g.a.: 26; $N = 8$; g.a.: 31; and $N = 9$; g.a.: 37 weeks).

while measuring different neurodevelopmental markers involved in the neurodevelopment of the fetal sensory systems, such as the growth of the thalamic nuclei and the insula and the thickness of the cortical layers. Furthermore, we verified whether, at each time-point, the presence of FCs versus ICs differential response might be related to specific neurodevelopmental parameters among those listed above.

2 | Materials and Methods

2.1 | Participants

For the present study, 60 healthy women with a physiological pregnancy and normal fetal-maternal outcomes were enrolled at Sant'Anna University Hospital, Città della Salute e della Scienza (Torino, Italy). We only recruited women with a normal BMI (between 18.5 and 24.5) at the beginning of the pregnancy. Maternal tissue thickness (as measured from maternal skin to the uterine wall) ranged from 13 to 68 mm (mean ±SD: 31.6 ± 14.5 mm). The gestational age of the fetuses included in the study was always verified by a sonographic scan performed within the first trimester of pregnancy.

A total of 60 fetuses were involved in the study: 20 fetuses of 37 weeks g.a.; 20 fetuses of 31 weeks g.a.; 20 of 26 weeks g.a. (see Table 1, Panel A). The data of the first sample of 37-week-old fetuses were collected in one session and the same measurements for upright and inverted continuous stimulation were included in both Experiments 1a and 1b, and then, in Experiment 2, compared to the data from the other two samples of 31- and 26-week-old fetuses (who took part in Experiment 2 only). In the 37-week-old sample, 3 out of 20 fetuses had to be excluded from data analysis due to poor image resolution (see Table 1, Panel B with the detailed sample's exclusion flowchart). Therefore, the final sample for Experiment 1a consisted of 17 fetuses of 37 weeks of g.a. (260 ± 17 days; mean ± SD). A sub-sample of 37-week-old fetuses ($N = 8$, randomly selected from the sample of participants involved in Experiment 1a), already enrolled in Experiment 1a, was involved also in Experiment 1b. In the other two samples of 31- and 26-week-old fetuses, the number of excluded fetuses was greater, mainly due to greater number of gross movements

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TABLE 1 | Sample size and exclusion criteria.

A. Sample size

	EXPERIMENT 1					
	EXPERIMENT 1a (N=17)			EXPERIMENT 1b (N=8)		
	Involved	Excluded	Final	Involved	Excluded	Final
Group 3 (37 weeks g.a.)	20	3	17	8 (subsample)	0	8

	EXPERIMENT 2 (N=26)		
	Involved	Excluded	Final
Group 1 (26 weeks g.a.)	20	11	9
Group 2 (31 weeks g.a.)	20	12	8
Group 3 (37 weeks g.a.)	9 (subsample)	0	9

B. Study sample exclusion flowchart

Note: Panel A: Sample size for each experiment and group. Notably, Group 3 data were all collected in one session and the same measurements for upright and inverted continuous stimulation were included in both Experiments 1a and 1b, and then, in Experiment 2, compared to Groups 1 and 2 data. Panel B: Flowchart detailing the study's exclusion process (see Section 2.3 for further detail). All the 60 initially involved participants were recruited based on the initial inclusion and exclusion criteria (i.e., physiological pregnancy and normal fetal-maternal outcomes, normal BMI at the beginning of the pregnancy, fetal gestational age eligible for the experiments). After recruitment, 21 participants were excluded from the experiments due to fetal quiescence during the pre-experimental session (without visual stimulation) or poor image resolution, or because experimental (visual) trials were contaminated by fetal gross movements, that prevented a sufficiently adequate image resolution and detection of the target area of acquisition (i.e., transorbital plane, with visible ocular orbits and eye-lens). Finally, five participants were excluded from statistical analyses due to insufficient eligible remaining trials, also considering trial's rejection due to the lack of agreement between judges in the blind offline coding phase.

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of the head or the body (see details below in Section 2.4). In the 31-week-old sample, 12 out of 20 fetuses were excluded, while in the 26-week-old sample, the excluded fetuses were 11 out of 20. Therefore, for Experiment 2 the final sample consisted of 26 fetuses (9 fetuses of 26 weeks: g.a. 179.6 ± 10.5 days, mean ± SD; 8 fetuses of 31 weeks: g.a. 213.5 ± 13.4 days, mean ± SD; data from 9 fetuses of 37 weeks: g.a. 256.2 ± 12.5 days, mean ± SD).

All participants (the mothers of the observed fetuses) gave their written informed consent to participate in the study, which conformed to the standards required by the Declaration of Helsinki and was approved by the Ethical Committee of Sant'Anna University Hospital, Città della Salute e della Scienza, Torino, Italy (prot. n. 0121061; 14/12/2017–14/12/2022).

2.2 | Stimuli

Experimental stimuli were delivered through a custom-made visual stimulator, replicating the same features characterizing the stimulator employed by Reid et al. (2017) and enabling the delivery of fixed visual stimuli to the fetus (refer to Balasubramanian et al. 2022, for a comprehensive comparison between the existing approaches to deliver simple visual stimuli through the maternal abdomen and the new available devices, permitting the presentation of complex shapes and motion patterns to the fetus). It was composed of three dot-shaped (2 mm) and red light-emitting (650 nm) diodes. The three dots composed an equilateral triangle (distance between dots: 15 mm). The power of the emitting light was regulated according to maternal abdomen thickness: 1 mV between 15 and 30 mm; 5 mV above 30 mm of abdomen thickness. This was done to assure that an adequate level of light reached the fetus, independently of maternal abdomen thickness (for detailed calculation of the amount of light projected through the uterine wall, please refer to Reid et al. (2017)).

In all experiments, visual stimuli consisted of three red dots, composing either a downward pointing (i.e., a *face-like configuration*) or an upward pointing triangle (i.e., an *inverted configuration*). Each configuration was presented five times in a fully randomized order. Importantly, different presentation sequences were counterbalanced across fetuses (i.e., different fetuses were presented with different sequences of stimuli), thus to exclude the presence of any presentation order effect in the collected data.

Only for Experiment 1b, which specifically aimed at explore different visual stimulation modalities, the same procedure was repeated either with the continuous stimuli described above (i.e., fixed emission of light), or a flashing light (with a flashing frequency of approximately 1 Hz) delivered through the same visual stimulator.

2.3 | Experimental Procedures

During the 2D sonographic scan, participants (the mothers of the observed fetuses) were usually lying on their back, in a semi-recumbent position, in a dimly lit room. They were asked to relax and not to move during the exam.

As a first step, we collected, at each time-point in which the experimental session was performed, standard biometry parameters (i.e. head circumference, biparietal diameter, abdominal circumference, and femur length) and more specific neurodevelopmental markers, such as the width of cortical layers (transverse plane), the dimension of the thalamic nuclei and the insula (given its involvement in the integration of sensory information and its extensive connections with cortical and subcortical regions relevant for vision, including the thalamus and the superior colliculus). Complete descriptions about Biometrics can be found in the Supporting Information: *Biometrics* (see also Table S1). In the present study, to monitor the maturation of fetal visual system, we decided to focus on the cortical layers, thalamic nuclei, and insula in consideration of their role in the development of the cortical and subcortical visual circuitries in the fetal brain (see also Section 4 for further detail on the development of fetal visual system) and because these structures can be reliably detected with 2D ultrasound during the third trimester of gestation.

Furthermore, to regulate the intensity of the visual stimulator (see Section 2.2), in accordance to Reid et al. (2017), we measured the thickness of the maternal abdomen, from the skin to the uterine wall. Before proceeding with the actual experiment, the exact position of the fetal head was assessed in order to guide the positioning of the visual stimulator. We did not systematically evaluate the level of vigilance of the fetuses, however, before starting the experiment and before delivering any stimulation to the fetus, we observed spontaneous fetal movements for 5 min. Fetuses showing no body movements for 5 min in this pre-experimental phase were not admitted to the experimental phase, that is, no further data were collected in these fetuses and they were excluded from the sample. Indeed, the absence of body gross movements can be considered as an indication of quiescence (Nijhuis et al. 1982; Pillai and James 1990). Notably, random isolated movements were occasionally observed over the 5 min, even in otherwise quiescent fetuses. These movements were interpreted as occasional startles and these fetuses were therefore excluded from the sample.

During the following experimental procedures, the visual stimulator was positioned on the maternal abdomen, laterally with respect to the position of the fetus' head, and was manually displaced by the experimenter in a horizontal direction, across the maternal abdomen, for 5 s (the timing was assessed through a chronometer, visible on a computer screen). The same horizontal movement (i.e., the experimental trial) was repeated 10 times, alternating directions: from right to left with respect to the fetus' head, and from left to right with respect to the fetus' head. This resulted in a total of 10 visual stimulations presented in two different conditions (i.e., FCs and ICs), with five trials per condition (see Section 2.2).

During the experiment, fetal eye-lens movements were tracked through a 2D sonographic scan for the whole duration of the experimental trial. Note that the sonographic operator was instructed to keep the probe still during each trial. Each sonographic scan was recorded to allow the offline analysis of fetal lens movements in response to each configuration. The employed ultrasound machine was a Voluson (GE) with a frame rate of 30 Hz. The sonographic operator and the experimenter delivering the stimulation were blind to the purpose of the experiment

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(i.e., blind to the objectives of the research and to experimental hypotheses, even though the experimenter was not blind to the orientation of the visual stimulation, since they were directly controlling the positioning of the stimulator).

2.3.1 | Coding Procedure

The recording of the sonographic scan of each experimental trial was offline inspected by four medical doctors, expert in sonographic scan image interpretation and blind to the stimulus being presented. Eye movements were coded when the lens moved a sufficient amount in any direction around the eyeball (Donovan et al. 2020). Note that, here, the lens movement direction is not informative because of the continuous visual stimulation lasting 5 s and moving across the maternal abdomen. Since the visual stimulator was manually displaced by the experimenter and the video recording of each scan was manually started and stopped by the sonographer (see above in the Section 2.3), we did not have sufficient accuracy in the stimulation-recording synchronicity to encode the position of the visual stimulus relative to the fetal gaze direction. More specifically, coding the exact fetal gaze direction through ultrasound may be challenging, due to the relatively small range of lens movements and the difficulty in precisely determining gaze focus with respect to visual stimuli external to the ultrasound framing. As common guidelines, to avoid incorrect classifications possibly due to head and/or body movements, the nasion (i.e., the craniometric point, where the top of the nose meets the ridge of the forehead—anytime visible) was taken as a reference point. If the lens moved, while the nasion did not, this was taken as reasonable evidence that the lens was moving independently from the head. This procedure allowed to exclude from statistical analyses trials contaminated by involuntary probe movements or concurrent head/body movements. Note that, as already mentioned in the participants' description (see above), the number of excluded fetuses was greater in 31- and 26-week-old fetuses as compared to 37-week-old fetuses, due to the greater number of gross movements of the head or the body. Furthermore, only trials where three out of four judges agreed on the presence or on the absence of a lens movement were included in the statistical analyses (overall less than 10% of the trial were excluded; among the included trials, judges' agreement ranged from 75% to 100%, on average 87%). For statistical purposes, only fetuses in which the number of remaining trials were equal or greater than 6 (3 for condition) out of 10 trials were included in the final sample.

2.4 | Data Analyses

2.4.1 | Experiment 1a: Verifying the Presence of a Significant Difference Between Lens Movement Numbers in Response to FCs vs. ICs

Experiment 1a was realized to verify the presence of a difference between the number of lens movements elicited by FCs and ICs, in a population of healthy fetuses in the late third trimester of pregnancy, by using lens movements instead of head turns. To analyze the collected data, we performed a two-tailed paired-sample *t*-test, directly comparing the percentage of lens movements in response to FCs and ICs. We also verify such results with a non-Parametric approach, by running a Wilcoxon Matched Pairs Tests *T* and a Mann–Whitney Tests (see Supporting Information: *B. Experiment 1a)*.

Furthermore, as an exploratory analysis, by performing a repeated-measure ANOVA we verified whether any difference in the ability to distinguish between FCs and ICs could be observed between female and male fetuses, since it has been observed that the early development of neural structures might be slightly different between male and female fetuses (Gaglioti, Oberto, and Todros 2009), thus possibly influencing gaze behavior (see details in Supporting Information: *Experiment 1a)*.

2.4.2 | Experiment 1b: Exploring Different Visual Stimulation Modalities to Obtain the Greatest Number of Lens Movements

Experiment 1b aimed at comparing continuous versus flashing light as the stimulation modality able to induce the greatest number of lens movements. Experimental stimuli were presented into two different conditions (i.e., FCs and ICs), five trials per condition. Importantly, the same procedure was repeated either with a continuous or a flashing light (see Section 2.2).

To verify which stimulation modality granted the highest number of lens movements, we performed a repeated-measure ANOVA with lens movements as dependent variable and two within-subject factors: "Stimulus Orientation" (two levels: FCs; ICs); and "Stimulation Condition" (two levels: continuous; flashing). The presence of a possible interaction between the factors was then explored.

Statistical threshold was set at $p < 0.05$. The same analyses were also repeated with a non-parametric Friedman ANOVA and Wilcoxon Matched Pairs Tests *T* (details on the non-parametric analyses and results are reported in Supporting Information).

2.4.3 | Experiment 2: Pinpointing the Emergence of the Difference Between FCs and ICs

Experiment 2 aimed at investigating the emergence of the preference for face-like configurations along the third trimester by comparing three different time-points, by contrasting differential responses between FCs and ICs in 26-, 31- and 37-week-old fetuses (g.a.). To verify the presence of such an effect, as a preliminary analysis, we verified through an *F* test whether the variance between the three groups was comparable (i.e., not significantly different—see Supporting Information: *B. Experiment 2* for results). Then, we performed a repeated-measure ANOVA with lens movements as dependent variable: one within-subject factor "Stimulus Orientation" (two levels: FCs; ICs); and one between-subject factor "Gestational Age" (three levels: Group 1, 26 weeks; Group 2, 31 weeks; and Group 3, 37 weeks). In addition, to corroborate these main analyses, we also conducted non-parametric comparison employing a Wilcoxon Matched Pairs Tests *T* and Kruskal–Wallis tests (see Supporting Information: *B. Experiment 2*). The presence of an interaction between factors would indicate that the possible differential response between

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configurations is different across time-points. Conversely, a main effect of stimulus orientation without a significant interaction between factors would indicate the presence of a similar differential response between FCs and ICs across time-points. On the other hand, a main effect of gestational age would indicate the presence of different amounts of lens movement across time-points, irrespective of the configuration type (either FCs or ICs). To better explore the main effect of gestational age, we calculated the g.a. in days of each subject and conducted a correlation analysis to explore the relationship between the percentage of observed lens movements (irrespective of the configuration type, either FCs or ICs) and the fetuses' age in days.

Finally, to verify whether the presence versus absence of this differential response might be related to the development of specific developmental markers, we performed an ANCOVA model. First, we computed a Face-like preference Index expressed as the number of movements in response to face-like *minus* the number of movements in response to inverted configurations. Then we ran the ANCOVA on the whole sample, with the Face-like preference Index as the dependent variable, the Gestational Age (i.e., the specific time-point) as categorical predictor and the width of cortical layers, the dimension of the thalamic nuclei and the insula as continuous predictors (covariates).

Possible significant interaction effects between the gestational age and the developmental markers were explored by correlational analyses (Pearson's correlation coefficients) separately for each time-point.

3 | Results

3.1 | Experiment 1a: Verifying the Presence of a Significant Difference Between Lens Movement Numbers in Response to FCs vs. ICs

Results of the paired sample *t*-test show that, in the late third trimester of pregnancy, the percentage of lens movements are significantly greater in response to FCs (66 ± 6.7%), as compared to ICs (52 ± 7.6%), $t_{16} = 3.26$, $p = 0.005$, $d_z = 1.95$ (see Figure 2). This result is also supported by non-parametric analysis using a Wilcoxon Matched Pairs Tests *T* (Supporting Information: *B. Experiment 1a*). This finding is crucial since it confirms the results obtained by Reid et al. (2017) extending this observation to fetal lens movements. The exploratory analysis on possible gender differences in the preference for face-like configurations did not show any significant effect (see details on the parametric and non-parametric results in section A and B of the Supporting Information: *Experiment 1a*).

3.2 | Experiment 1b: Exploring Different Visual Stimulation Modalities (Continuous vs. Flashing) to Obtain the Greatest Number of Lens Movements

The ANOVA (factors: *Stimulus Orientation*: FC vs. IC; and *Stimulation Condition*: continuous vs. flashing) shows a main effect of Stimulus Orientation ($F_{(1,7)} = 27.339$; $p = 0.001$; $\eta_p^2 = 0.80$), with overall significantly greater lens movements in response to FCs (mean ± SEM: 66 ± 9%) rather than ICs (48.6 ± 11.06%), and a

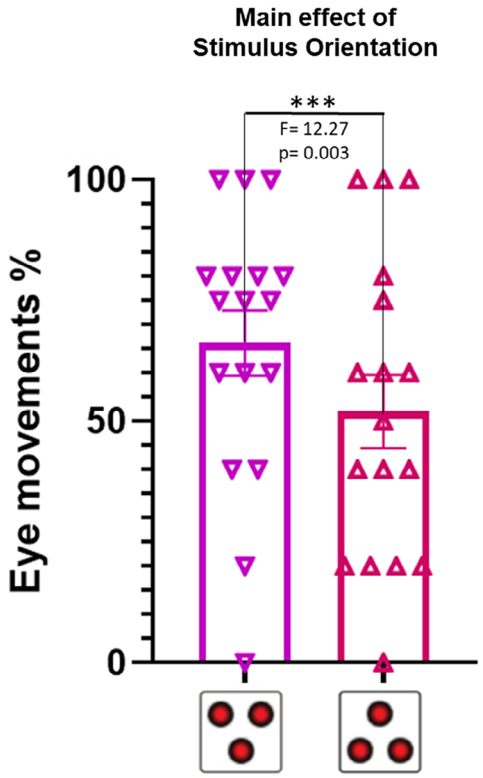

FIGURE 2 | Results Experiment 1a. Main effect of stimulus orientation: percentage of lens movements in response to FCs and ICs (see Supporting Information for further detail on statistical analyses). Notice the significantly greater number of movements in response to FCs. Error bars represent standard error of the mean (SEM). ***$p < 0.005$.

main effect of Stimulation condition ($F_{(1,7)} = 7{,}829$; $p = 0.027$; $\eta_p^2 = 0.53$), with significantly greater responses for the continuous light (68.4 ± 8.8%), rather than flashing light stimulation (46.2 ± 12.9%). No interaction between these two factors was found ($F_{(1,7)} = 0{,}007$; $p = 0.932$; $\eta_p^2 = 0.00$), indicating that irrespective of the stimulation condition, a greater number of fetal lens movements is observed for FCs (see Figure 3). However, even though the pattern of the results between stimulus orientation is similar for both light modalities, the main effect of Stimulation condition demonstrates that a significantly larger number of movements may be obtained with a continuous light stimulation. Such results are also supported by the non-Parametric Friedman ANOVA and Wilcoxon Matched Pairs Tests *T* described in the Supporting Information: *B. Experiment 1b*.

3.3 | Experiment 2: Pinpointing the Emergence of the Difference Between FCs and Ics

The repeated-measure ANOVA (factors: *Stimulus Orientation*: FC and IC; and *Gestational Age*: 26, 31, and 37 weeks) shows a significant main effect of gestational age ($F_{(2,23)} = 5.49$; $p = 0.01$; $\eta_p^2 = 0.3$). When exploring the main effect of gestational age, greater lens movements are observed at 37 weeks (62.2 ± 7.6%) as compared to 31 weeks (43.4 ± 5.6%; even though the difference is not significant at Bonferroni's post hoc comparison: $p = 0.429$) and 26 weeks (26.7 ± 5%; with a significant difference at Bonferroni's post hoc comparison: $p = 0.009$). See Figure 4A.

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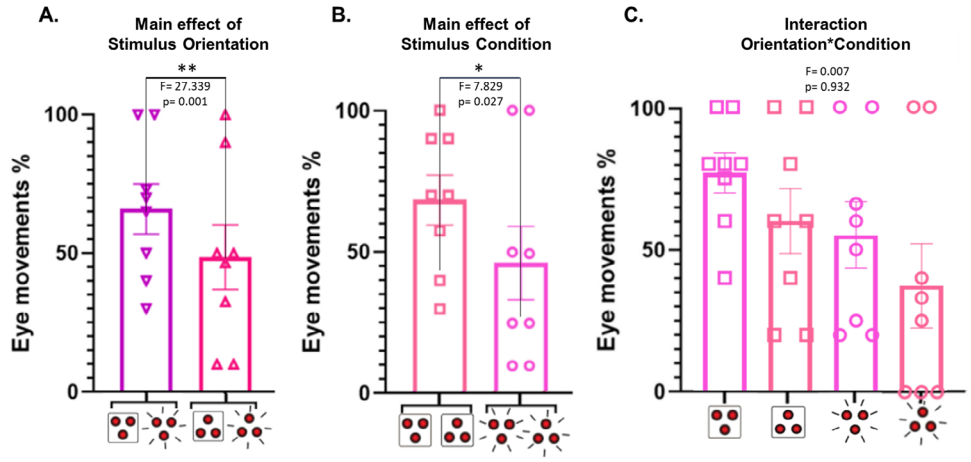

FIGURE 3 | Results Experiment 1b. (A [left panel]) Main effect of stimulus orientation: percentage of lens movements in response to FC and IC stimuli. Notice that movements in response to FCs are significantly greater than those in response to ICs. (B [central panel]) Main effect of stimulation condition: percentage of lens movements with continuous versus flashing light. A significantly greater number of movements is observed in response to continuous as opposed to flashing light (C [right panel]). Interaction: percentage of lens movements for FCs and ICs stimuli, with continuous versus flashing light stimulations. As depicted in the graph, no significant interaction between the two factors was found. Error bars represent the standard error of the mean (SEM). *$p < 0.05$; **$p < 0.01$.

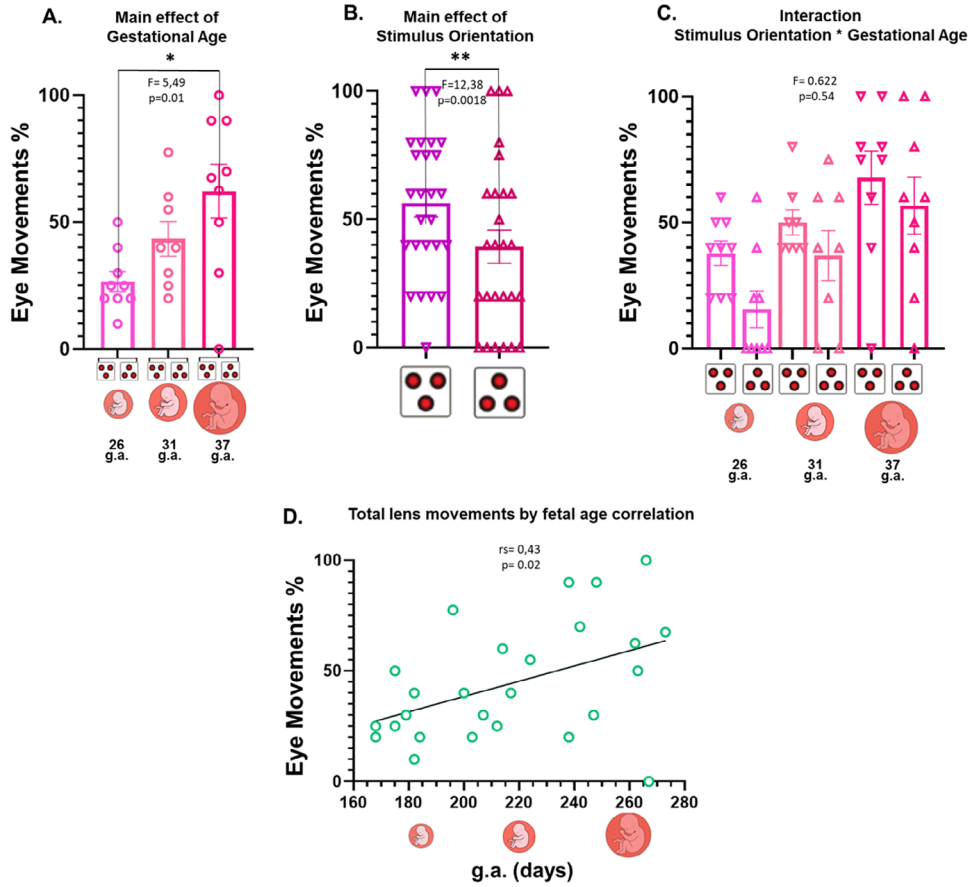

FIGURE 4 | Results Experiment 2. (A) Main effect of gestational age: percentage of lens movements observed at each time-point. (B) Main effect of stimulus orientation: percentage of lens movements for FCs and ICs. Notice the presence of a significant difference between the two conditions. (C) Nonsignificant interaction: Percentage of lens movements for FCs and ICs stimuli at each time-point. Results show significant difference between FCs and ICs at all time-points. Error bars represent the standard error of the mean (SEM). **$p < 0.01$. (D) Total lens movements by fetal age correlation: Significant positive correlation between the percentage of total lens movements and fetuses' g.a. in days.

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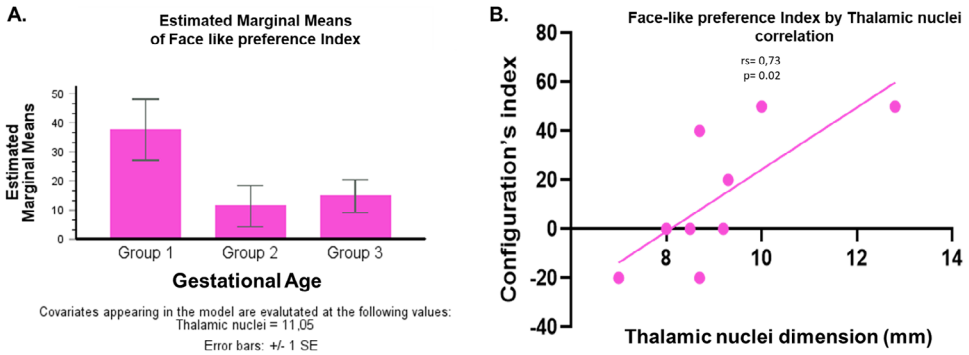

FIGURE 5 | Relation of face-like preference effect with the thalamic dimension. (A) Adjusted means of the Face-like preference Index in each time point. (B) Face-like preference Index by Thalamic nuclei correlation: Significant positive correlation between the Face-like preference Index (delta between movements in response to face-like and inverted configurations) and thalamic nuclei growth (mm) in 26-week-old fetuses.

Furthermore, we also find a significant main effect of stimulus orientation ($F_{(1,23)} = 12.38$; $p = 0.0018$; $\eta_p^2 = 0.3$), with overall greater lens movements in response to FCs (51.92 ± 4.8%) as compared to ICs (36.34 ± 6.3%). See Figure 4B. Importantly, no significant interaction was found ($F_{(2,23)} = 0.622$; $p = 0.54$; $\eta_p^2 = 0.05$), indicating that, beside the greater number of movements recorded at 37 weeks, a similar differential response between FCs and Ics is observed in all time-points (see Figure 4C). Such results are also supported by the non-parametric Wilcoxon Matched Pairs Tests *T* and Kruskal–Wallis tests described in the Supporting Information: *B. Experiment 2*.

To better explore the main effect of gestational age, we performed a correlation analysis to investigate the relationship between the percentage of observed lens movements (irrespective of the configuration type, either FCs or ICs) and the fetuses' age in days. Results show a positive correlation ($r_{s(26)} = 0.43$; $p = 0.02$) between the percentage of total lens movements and fetuses' age in days, as shown by the linear trend depicted in Figure 4D.

Furthermore, ANCOVA's results revealed a significant Gestational Age*Thalami interaction ($F_{(2,28)} = 4.67$; $p = 0.01$; $\eta_p^2 = 0.25$). Such results suggest that the differential responses to FCs and ICs may be predicted by the thalamic dimension. Moreover, such influence of the covariate on the outcome of the Face-like preference Index results effectively only at the first time-point (i.e., 26 weeks of gestation, see Figure 5A). Indeed, by comparing the adjusted means of the Face-like preference Index in each time-point, while controlling for the effect of the thalamic growth, a significantly greater Face-like preference Index was found in Group 1 (LS mean = 37.63), compared to Groups 2 and 3 (LS mean = 11.39 and 14.81, respectively).

To further explore the significant Gestational Age*Thalami interaction we run, at each time-point, a correlational analysis between the Face-like preference Index and the dimensions of thalamic nuclei.

Results show a significant positive correlation ($r_{(7)} = 0.73$; $p = 0.02$) with the thalami only in the group of 26-week fetuses (see Figure 5B). No significant correlation with any other time point was found.

4 | Discussion

In the present study, we had three main aims. First of all, we were interested in validating eye-lens movements as possible marker of fetal behavior. Then, we investigated the emergence of the preference for face-like configurations throughout the third trimester of gestation. Finally, we asked whether such preference is related to specific neurodevelopmental parameters. With a series of three experiments, we showed preliminary evidence that fetal preference for face-like configuration can be reliably detected by recorded lens movements, that this preference can be observed along the whole third trimester, and that, in the first time-point (i.e. 26 weeks g.a), this preference seems related to the thalamic nuclei growth. In the next paragraphs, we explored both methodological and neurophysiological aspects that might contribute to explain these results.

4.1 | Lens Movements as a Reliable Marker of Fetal Behavior

Through Experiments 1–2, having replicated the results of fetal responses to face-like stimuli previously shown by 4D ultrasound recording of head-turns, we showed that, in third trimester fetuses, it is possible to reliably track lens movements throughout 2D ultrasound. This result supports the view that, even before birth, eye movements are related to visual attention orienting and that they can be detected regardless of head movements. This finding is in agreement with a previous study that, by analyzing the 2D component of 4D ultrasound scans, demonstrated that fetal eye movements (observable in 2D) can be detected independently of head movements (observable in 4D) (Donovan et al. 2020). It is interesting to note that we recorded on average 3 (out of 5) lens movements in responses to FCs, whereas in the study by Reid et al. (2017), only 1.03 (out of 5) head turn directed toward the light stimulus was recorded. A first explanation of this numerical difference can be ascribed to the fact that, in the later stages of pregnancy, fetuses' head movements are often limited due to the constrained uterine environment (DiPietro, Costigan, and Voegtline 2015). However, it is also possible that eye-movements per se represent a more reliable measure of attention orienting to face-like configuration as compared to head

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turns. This idea has already been proposed by a previous post-natal study demonstrating a greater number of eye-movements as compared to head-turns while newborns were exposed to FCs stimuli (Johnson et al. 1991). Accordingly, another study failed in replicating the preference for face-like configurations in newborns when using head-turns, while succeeded when using eye-movements (Maurer and Young 1983). Similarly, results by Cassia et al. (2001) demonstrated that newborns oriented their gaze significantly more toward face-like configurations as compared to inverted ones (Cassia et al. 2001). After all, this greater reliability of eye-movements in post-natal studies is not surprising, considering that gaze behavior represents the gold standard for investigating implicit marker of attention orienting toward more salient stimuli, both in infancy and in adulthood. Eye movements are not only considered as a reliable outcome of attentional processing, but it has been proposed that attention allocation employs the same circuits used for oculomotor control (Rizzolatti et al. 1987) and that the ability to move our eyes is essential for the development of normal patterns of attention orienting (Smith, Rorden, and Jackson 2004). In our view, the present evidence endorses fetal eye movements as a reliable marker of attention orienting in utero. Moreover, such finding may open the way for future investigations of typical and atypical fetal behavior in response to environmental stimuli, even different than visual (e.g. auditory and /or vibrotactile). Together with theoretical and clinical implications for prenatal studies, this approach may also represent an optimal translational parameter, bridging the gap between research on pre- and post-natal life. Accordingly, tracking eye movements might allow the definition of an index of continuity and/or discontinuity in the behavioral patterns observed before and after birth. Nevertheless, we believe it is important to recognize that lens movements and head turns (as demonstrated by Reid et al. (2017)), both constitute distinct components of visual engagement with the stimulus. Exploring correlations between these measures in future studies would be very important for a comprehensive understanding of visual processing dynamics in human fetuses.

4.2 | Continuous Light as a More Effective Visual Stimulation in Triggering Fetal Lens Movements as Compared to Flashing Light

For what concerns the features of the visual stimulation, considering that in adults flickering stimuli seem to be very effective in capturing participants' attention (Stolte and Ansorge 2021), it could be argued that flashing rather than continuous stimuli, might be more suitable in attracting the fetal gaze. In previous studies (for a review please refer to Dunn et al. 2015), both single, continuous visual stimulations (Eswaran et al. 2002, Eswaran et al. 2004; Kiuchi et al. 2000; Reid et al. 2017), and set or trains of lights (Matuz et al. 2012; Sheridan et al. 2010) were employed to test the fetuses' visual responses. However, to the best of our knowledge, no study has systematically investigated which stimulation modalities is the best in eliciting fetuses' gaze orienting responses so far. When comparing the number of lens movements in response to a continuous versus flashing lights, even though both modalities were able to elicit gaze orienting behaviors, we demonstrated that the continuous light was significantly more effective in triggering lens movements (Experiment 1b). For this reason, we recommend to employ of a continuous visual stimulation in future studies.

With the data collected in this study, it is difficult to provide a conclusive interpretation as to why continuous stimuli are more effective than flashing ones in attracting the attention of fetuses, and it is important for future studies to further explore which factors specifically contribute to attentional engagement towards visual stimuli in fetuses. However, it has been previously suggested by Reid and Dunn (2021) that non-static stimulation may create greater alterations in the shape and in the perceived light intensity of the visual input as compared to static stimulation, due to the variation in tissue types across the maternal abdomen. Therefore, it is possible that continuous light stimulations represent the most effective way to induce a visual engagement with visual stimuli in human fetuses.

4.3 | Preferences for Face-Like Configurations is Already Present at the Beginning of the Third Trimester of Gestation and is Related to the Thalamic Nuclei Dimension

While findings of Experiment 1 confirm and extend to fetal lens movements the results by Reid et al. (2017), through the findings of Experiment 2, we showed that the preference for face-like configurations is similarly present at all time-points considered along the third trimester. Specifically, even though an overall greater number of lens movements is performed at 31 and 37 weeks g.a. as compared to 26 weeks g.a. (main effect of gestational age in Experiment 2), the preference for FCs is already present from 26 weeks g.a.

How to explain this early effect? FMRI or fetal-MEG studies (Fulford et al. 2003; Schöpf et al. 2014) often include later third trimester fetuses (typically after 33 weeks g.a.), therefore responses to visual stimuli in earlier time-points are rarely investigated. On the other hand, research on preterm newborns suggests that behavioral responses to visual stimulation (including horizontal tracking and the ability to fixate) are already present at 30 weeks g.a. (Ricci et al. 2010). Importantly, only a very limited number of preterm infants may be successfully assessed at such an early g.a., due to clinical instability (Ricci et al. 2010), and this may be considered one reason why there are only rare assessments of behavioral responses to visual stimulation before 31 weeks g.a. However, interestingly, other previous evidence suggests the presence of behavioral response to visual stimulation at 26 weeks g.a. (Polishuk, Laufer, and Sadovsky 1975). The early neurodevelopment of the fetal visual system might support the presence of such responses.

The formation of the visual system starts very early in uterus laying the foundation for postnatal visual experiences and further neural maturation (for an overview see Graven 2004 and Johnson 2020). Within the first trimester, the major retinal and eye structure morphogenesis begins (Quinn and Wijnholds, 2019; Glass 2002). Throughout the mid gestational period, the primary visual cortex and the critical connections between eyes and vision-related brain structures start to develop, with the accumulation of thalamocortical afferents in the superficial part of the subplate zone reflecting a transient endogenous pathway. Noteworthily,

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the lateral geniculate nucleus (LGN), a key thalamic relay station for visual information, starts receiving retinal inputs already at about 20 weeks g.a. (Hevner 2000). The third trimester is a critical period for the development of the fetal visual system, with the maturation and refinement of subcortical and cortical structures. At this stage, visual-evoked responses (VER) can be recorded with magnetoencephalography (Eswaran et al. 2004), suggesting that by this trimester we assist to the development of a functional visual system. Furthermore, at about 26 weeks g.a., the thalamocortical pathway is just established, a process that is almost simultaneous with eyelids opening (Donovan et al. 2020).

Accordingly, the results of the ANCOVA model performed in Experiment 2 suggest that the maturation of thalamic nuclei may be a key determinant in the early development of the preference for face-like configurations. Selectively for our first time-point (26 weeks g.a.), the thalamic nuclei dimension is significantly correlated with the Face-like preference Index (i.e., the amount of differential response between FCs and ICs). In other words, the larger the thalamic nuclei are at 26 weeks g.a., the greater is the differential effect between FCs and ICs (see Figure 5B). This finding, if confirmed by further investigations in larger sample, might be considered as a preliminary indication that, at the beginning of the third trimester—when the thalamocortical pathway has just established and the visual cortical layers are not fully developed—the thalamic nuclei, once sufficiently developed, may represent a key neural structure for face-like preference.

This evidence is in line with the dual route model of face processing, suggesting that faces are processed both through a subcortical route (involving the superior colliculus, pulvinar and amygdala) and a cortical route, involving several structures implicated in face identification (fusiform gyrus and inferior occipital gyrus), facial expression (amygdala, orbitofrontal cortex, and sensorimotor cortex), and eye gaze (superior temporal sulcus) (Johnson, Senju, and Tomalski 2015). In adults, the initial rapid activation of the subcortical route modulates the activity of cortical areas before or during the cortical processing of face-related visual input. In the early phase of human development, it has been proposed that the face-related preference is mainly supported by the subcortical route that not only detects the presence of faces and orients the newborn towards them, but might also activate face-related cortical regions and promote their development (Johnson 2005). Indeed, in newborns, structures on the subcortical route seem to be more developed as compared to those on the cortical visual route (Johnson 1990; Atkinson 2000; Born et al. 2002). Coherently, at birth, face-related preference was observed only when stimuli were presented in the temporal visual field that feeds into the subcortical route and not when they were presented in the nasal field that feeds into the cortical route (Simion et al. 1998). Interestingly for the purpose of the present study, within this subcortical route, the role of pulvinar in the thalamus seems to be implicated in attentional orienting of eye movements to "salient'" (biologically relevant) stimuli (Grieve, Acuña, and Cudeiro 2000), thus supporting our findings showing a correlation between the thalamic nuclei dimension and the preferential orienting of fetal eye movement to face-like configurations at 26 weeks (g.a.). However, this main involvement of the subcortical route does not exclude an early contribution of the cortical one, as it has been compelling demonstrated by an electrophysiological study showing that human newborns (at term) also rely on cortical areas for face-like processing (Buiatti et al. 2019). Coherently, our preliminary findings support the view that, at a very early stage (i.e., 26 weeks g.a.), a certain degree of thalamus development represents an essential prerequisite for the initial establishment of the preference for face-like configurations, while other, possibly cortical, structures may represent additional determinants of face processing in later stage of fetal development, as it is for newborns and adults (Buiatti et al. 2019; Toulmin et al. 2015).

4.4 | Possible Evolutionary Meanings of the Early Preference for Face-Like Configurations and Future Directions

If the preliminary results of Experiment 2 will be replicated by future studies, we should assume that already at the beginning of the third trimester of gestation, as soon as the thalamocortical connections are established (Kostović and Judas 2010; Frohlich et al. 2023), fetuses might be provided with a rudimental, but still effective, mechanism to process schematic visual stimuli and to discriminate FCs from other, less relevant, visual configurations. Such an early emergence of this discrimination within human ontogeny might appear surprising. However, from an evolutionary point of view, preferences for face-like configurations in newborns are widespread across species, being traced in mammals (e.g., human and monkeys; see e.g., Sugita 2008), birds (Di Giorgio et al. 2017), and even reptiles (Versace, Damini, and Stancher 2020). The presence of this phenomenon at lower phylogenetic steps seems to identify a primitive mechanism, mediated by ancestral structures of the nervous system (at least in reptiles), thus making the early-emergence of the preferences for FCs in human fetuses less unexpected. But *what* makes faces so important to explain such an early-emergence? In social species, such as mammals and birds, the early preferences for faces may be related to at least two different factors: (1) the necessity to support the interactions with conspecific, thus favoring postnatal care (*social hypothesis*); (2) the importance of paying attention to most informative body areas (*information hypothesis*—see also Versace, Damini, and Stancher 2020). We speculate that this early effect might be interpreted as the result of an innate reorienting of bottom-up attention toward face-like visual stimuli, expressed through fixation time and gaze orienting from a behavioral point of view (see the results in fetuses and infants; Dunn et al. 2015; Farroni et al. 2005), and through an enhanced neural entrainment from an electrophysiological point of views (as shown in newborns; Buiatti et al. 2019). Considering that the preference for face-like configurations was observed even in solitary species, such as tortoise (Versace, Damini, and Stancher 2020), it seems reasonable to hypothesize that this innate neural tuning for faces initially evolved as a basic, *non-social*, mechanism to support newborns' redirection of attention toward most informative body areas (*information hypothesis*). However, it is possible that in social species it was maintained also because it represents a clear advantage for the development of social interactions. To further explore the interaction between *information* and *social* factors in the development of this neural tuning in humans, future studies might be directed to test whether the presence versus absence of a conspecific (such as in singleton versus twin pregnancies)

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might influence the preference for face-like configurations in third-trimester fetuses.

5 | Limitations

While our study provides valuable insights on the differential fetal engagement with (more or less salient) visual stimuli, some limitations should be acknowledged. The final dataset used in our analyses was smaller than anticipated due to technical challenges in obtaining clear ultrasound images, as detailed Table 1. This reduction potentially compromised the statistical power of our analyses and limited the generalizability of our findings, particularly in Experiment 2, where the sample size was relatively small. To address this issue, we conducted nonparametric analyses (see sections B in Supporting Information). Despite the limited samples, however, both parametric and non-parametric analyses satisfy assumptions of homogeneity of variance and normality (see Supporting Information: *F* Test), and provide comparable results. Concerning the analyses exploring a relation between the Face-like preferential effect and the development of specific neural markers in Experiment 2, the effect size observed for the ANCOVA interaction was statistically significant but small ($\eta_p^2 = 0.25$), indicating that the thalamic growth explains the 25% of the variance of the Face-like preference effect only, thus highlighting an overall limited relevance of our predictor. Therefore, future studies are required to confirm our preliminary findings. Furthermore, given the logistical and technical challenges associated with prenatal investigations, future studies should anticipate high attrition rates and consider starting with larger sample sizes while maintaining rigorous data quality control procedures.

Author Contributions

Irene Ronga: conceptualization, methodology, investigation, data curation, writing–original draft, writing–review and editing; **Karol Poles**: data curation, formal analysis, writing–original draft, writing–review and editing; **Carlotta Pace**: investigation, formal analysis, writing–review and editing; **Marta Fantoni**: investigation, formal analysis, writing–review and editing; **Josephine Luppino**: investigation, writing–review and editing; **Pietro Gaglioti**: investigation, formal analysis, writing–review and editing; **Tullia Todros**: conceptualization, resources, formal analysis, writing–review and editing; **Francesca Garbarini**: conceptualization, project administration, writing–original draft, writing–review and editing, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Endnotes

[1]For the present paper, here a description of non-standard abbreviations: FCs, face-like configurations; ICs, inverted configurations; g.a., gestational age.

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