UNIVERSITÀ DEGLI STUDI DI PADOVA

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Dipartimento di Psicologia dello Sviluppo e della Socializzazione

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE PSICOLOGICHE

Psicologia dello Sviluppo e dei Processi della Socializzazione

XXV ciclo

FROM SOCIAL BRAIN TO ACTION AND PERCEPTION

Neural correlates of the early social abilities and their behavioural forerunners in typical and atypical development

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Riassunto

Il presente progetto di ricerca è rivolto allo studio dello sviluppo del "cervello sociale", una rete di regioni cerebrali specializzate per l'elaborazione degli stimoli sociali. Questo sistema è specializzato in senso evolutivo per l'interazione sociale e gioca un ruolo cruciale nello sviluppo sociale. Gli esseri umani sono "sintonizzati" socialmente, ma le origini di questo processi di specializzazione, ad oggi, sono in parte sconosciuti. Questa ricerca è focalizzata sulla comparsa dei precursori delle abilità sociali, e sull'elaborazione delle informazioni socialmente rilevanti nel primo anno di vita, nello sviluppo tipico quanto in quello atipico. In particolare sono state investigate le seguenti aree: i correlati neurali dell'elaborazione di azioni umane alla nascita; l'abilità di comprendere azioni dirette ad uno scopo nel primo anno di vita; gli endofenotipi neurocognitivi precoci del Disturbo dello Spettro Autistico (DSA) connessi ai precursori della cognizione sociale (es. pattern attentivi durante l'elaborazione dello sguardo e del volto; la percezione di azioni). Le popolazioni indagate sono infanti e neonati aventi diversi gradi di rischio familiare di sviluppare autismo. Gli scopi principale del progetto sono a) approfondire quando e come alcune abilità sociali vengono acquisite durante il corso dello sviluppo tipico; b) studiare - per mezzo di popolazioni a rischio- il ruolo di disarmonie precoci nella crescita sulle traiettorie evolutive atipiche; c) identificare possibili indicatori neurali e comportamentali precoci, i quali possano prevedere la futura comparsa di Disturbi dello Spetro Autistico (DSA).

Al fine di studiare questi aspetti, la tecnica di neuroimmagine funzionale è stata integrata con il quadro teorico e alle tecniche classiche delle neuroscienze cognitive in ambito evolutivo. Questi metodi complementari di ricerca permettono di studiare allo stesso tempo sia meccanismi comportamentali, sia le strutture funzionali del cervello sottostanti a tali sistemi. Quattro principali studi sono stati condotti con lo scopo di gettare luce sui meccanismi precocissimi dell'orientamento sociale e dell'elaborazione dell'azione e come si specializzano. Nel primo studio sono state adoperate procedure comportamentali neurocognitive assieme alla tecnica non invasiva di *neuroimaging* della spettroscopia del vicino infrarosso (fNirs) su un campione di neonati, al fine ti raccogliere dati diretti ed indiretti (comportamento visivo e cambiamenti di ossigenazione corticale) durante l'osservazione passiva di stimoli dinamici biologici, socialmente comunicativi (es. interazione faccia-a-faccia) o non comunicativi (azione *goal-directed*), in contrapposizione a stimoli dinamici non sociali e non biologici. Il secondo e terzo studio, rivolto a infanti (tra i 6 e i 9 mesi di vita) è centrato sul ruolo di indizi comportamentali (relativi agli stati mentali dell'agente) e ambientali (relativi al

contesto in cui o al modo in cui l'azione è eseguita) nei processi di attribuzione di scopo. Tre diversi paradigmi sono stati impiegati per valutare quali siano le caratteristiche più efficaci nel modulare la comprensione delle azioni altrui nel primo anno di vita. Il quarto studio consiste nel sottoporre a due paradigmi comportamentali consolidati nel campo dell'attenzione visiva un campione di bambini ad alto rischio di DSA (perché fratelli minori di un probando). Un compito aggiuntivo originale è stato proposto al campione a rischio, per valutare la preferenza spontanea per il movimento biologico su quello non biologico in scene realistiche di azioni dirette ad uno scopo. I dati dei fratelli di bambini affetti sono stati confrontati con le performance (agli stessi compiti) di un gruppo di pari con rischio di DSA non specificato (popolazione generale), per evidenziare la presenza di un ipotetico fenotipo attentivo peculiare nei parenti non affetti di bambini con autismo (fenotipo autistico diffuso)

The infant siblings' data were compared to the performances of peers (undergone to the same tasks) with unspecified risk for autism (i.e. general population) to highlight the presence of a hypothetical peculiar attentional phenotype in unaffected relatives of children with autism (Broader Autism Phenotype) .

Introduzione

Il **capitolo introduttivo** della tesi è volto a presentare la cornice teorica di riferimento dell'intero progetto, nello specifico tre approcci concettuali in neuroscienze cognitive dello sviluppo:

1 l'ipotesi del "cervello sociale" (Brothers, 1990) secondo cui vi è un complesso network di regioni cerebrali dedicate all'elaborazione di informazioni sociali ed evolutivamente specializzato per l'interazione sociale (es. elaborazione del volto, riconoscimento delle emozioni, discriminazione del movimento biologico).

2 L'ipotesi della Specializzazione Interattiva (Johnson, 2000) che teorizza la selettività della risposta di specifiche aree cerebrali a specifici stimoli ambientali. Tale fenomeno ha origine durante lo sviluppo post-natale attraverso l'interazione tra vincoli biologici e ambiente (meccanismi *"activity-dependent"*) portando alla creazione di aree funzionali localizzate e specializzate.

3 L'approccio Neurocostruttivista (Elman et al., 1996) interpreta lo sviluppo cognitivo come il prodotto di modificazioni bidirezionali tra i sistemi neurali (e le relative funzioni cognitive) e l'ambiente. All'interno di questa prospettiva è rilevante -ai fini della discussione- la posizione teorica relativa allo sviluppo atipico, secondo cui gli esiti fenotipici dello sviluppo vanno analizzati come risultati di un processo dinamico, pertanto lievi alterazioni precoci della traiettoria evolutiva possono avere effetti patologici in funzione dell'epoca in cui si sono manifestate (Karmiloff-Smith, 1998).

Il **secondo capitolo** ha come oggetto il background teorico relativo all'elaborazione precoce di stimoli sociali. Nello specifico verranno presentate le evidenze scientifiche principali riguardo elaborazione e orientamento automatico verso il volto; percezione e processi attentivi coinvolti nell'elaborazione dello sguardo e la discriminazione del movimento biologico. Questi processi cognitivi verranno analizzati anche alla luce delle anomalie presenti -nei suddetti processi- in individui con Disturbi dello Spettro Autistico e dei recenti dati presenti in letteratura.

Il **terzo capitolo** è dedicato al progetto sperimentale sull'elaborazione precoce delle azioni. In esso verrà presentato il Primo Studio, interamente indirizzato a neonati nei primi giorni di vita. Lo studio comprende due esperimenti comportamentali di preferenza visiva e due esperimenti di osservazione passiva con registrazione dell' attivazione corticale attraverso l'uso della tecnica di Spettrografia del vicino Infrarosso (fNIRs). La prima parte dello studio è costituita da due esperimenti:

4 Esp. 1.1: preferenza tra due azioni di prensione eseguite da un agente biologico (mano) e da uno non biologico (bacchetta con movimento meccanico);

5 Esp. 1.2: preferenza tra due azioni di prensione eseguite da un agente con aspetto umanoide e movimento non biologico (mano con movimento non biologico) e da un agente con aspetto non umano e movimento biologico (bacchetta con movimento biologico).

Dai dati di questo studio emerge una preferenza generale per l'elemento che esibisce una cinematica biologica, indipendentemente dal suo aspetto (mano + bacchetta biologica Vs bacchetta meccanica + mano meccanica). A livello di singoli esperimenti, invece non risultano esserci effetti principali. La seconda parte dello studio, basata sulla valutazione dei cambiamenti di ossigenazione corticale durante la presentazione di:
a) stimoli dinamici sociali Vs non sociali-meccanici (Esperimento 1.3)
b) stimoli dinamici biologici Vs non biologici-meccanici (Esperimento 1.4).
Dagli esperimenti con la fNIRS, l'analisi del segnale HbO_2 e dei valori beta, evidenzia la presenza di un'attivazione bilaterale della corteccia temporale posteriore per lo stimolo dinamico sociale (Esp.1.3: volto Vs oggetto), ma non per lo stimolo dinamico biologico (Esp.1.4: mano Vs bacchetta). L'attivazione cerebrale sembra dunque essere selettiva per uno stimolo biologico, ma solo se altamente comunicativo e socialmente rilevante come il volto. L'esperienza postnatale, seppur limitata, è sufficiente per la specializzazione delle aree cerebrali deputate all'elaborazione del volto, alla nascita non sembrerebbe esserci ancora lo stesso grado di specializzazione relativa all'elaborazione del movimento biologico. Dal confronto tra i dati di neuroimmagine e quelli comportamentali emerge una dissociazione in parte riscontrabile in letteratura.

Il **quarto capitolo** descrive lo sviluppo della capacità di comprendere le azioni altrui in prima infanzia. Verrà presentato lo stato dell'arte in letteratura riguardo al processo di attribuzione di scopo nel primo anno di vita; in particolare la rassegna delle principali evidenze scientifiche, i paradigmi sperimentali utilizzati dalla ricerca e le teorie di riferimento.

Il **quinto capitolo** presenta i primi due studi del progetto, quelli relativi alla comprensione precoce di azioni dirette ad uno scopo. Il secondo studio è composto da cinque esperimenti rivolti ad un campione di bambini di 6 mesi. La tecnica utilizzata è la familiarizzazione visiva. I primi due (Esperimenti 2.1 e 2.2) si avvalgono del paradigma classico Woodward (Woodward, 1998) e hanno dimostrato come i bambini di quest'età siano in grado di eseguire una attribuzione di scopo di azioni eseguite da un agente con cinematica biologica (indipendentemente dalla parvenza dello stesso: mano vs bacchetta) se prima familiarizzati ad un evento in cui l'agente effettua una scelta tra due opzioni (due possibili target). Gli

Esperimenti 2.3, 2.4 e 2.5 si avvalgono della versione *single paradigm*, perciò il target dell'azione (sia esso familiare o nuovo) è l'unico presente nella scena. In due dei tre esperimenti l'agente esibiva un movimento biologico (esp. 2.3: mano con movimento naturale; esp. 2.4: bacchetta con movimento biologico), nel terzo l'azione era eseguita da un oggetto privo di caratteristiche biologiche (bacchetta con movimento meccanico). Da questo secondo set di esperimenti emerge come l'assenza della scelta operata dall'agente (un solo oggetto presente nella scena) sia determinante per la capacità di attribuire uno scopo all'agente; questo elemento comportamentale è prioritario rispetto a caratteristiche fisiche secondarie (es. la "biologicità" del movimento e la parvenza dell'agente) che da sole non bastano a contribuire socialità all'agente. Tali caratteristiche fisiche vengono invece utilizzate ai fini dell'interpretazione dell'evento solo in assenza di altri elementi capaci di suggerire intenzionalità all'agente: nell'esperimento in cui ad agire è uno strumento con movimento non-biologico (apparenza non umanoide), paradossalmente, si verifica l'attribuzione di scopo, in parte in virtù della coerenza tra le caratteristiche fisiche (movimento e parvenza) e in parte perché un entità non-sociale (non biologica) può agire in modo finalizzato pur non possedendo intenzionalità. Il terzo studio è rivolto a bambini tra i 6 e i 9 mesi con lo scopo di indagare l'influenza dell'esperienza visiva di preferenza dell'agente (per uno tra due oggetti), sulle aspettative dei bambini relative al futuro agire dello stesso agente. Lo studio 3 è composto da due esperimenti. Si tratta di una familiarizzazione passiva all'evento di preferenza per uno di due possibili oggetti, seguito da una fase test in cui si mettono a confronto i tempi di fissazione per l'evento test in cui l'azione è rivolta all'oggetto familiare (presente in familiarizzazione) rispetto ai tempi di fissazione rivolti all'evento che mostra l'azione su un oggetto nuovo. Le condizioni sono due: una di controllo in cui l'oggetto familiare in fase test è lo stesso che veniva scelto in familiarizzazione (Esperimento 3.1) ed una sperimentale, in cui l'oggetto familiare della fase test è quello che non veniva scelto in familiarizzazione (Esperimento 3.2). I risultati dello studio suggeriscono due effetti: uno legato alla condizione (l'evento *scopo nuovo* viene preferito in situazione di controllo; l'evento *scopo familiare* viene preferito in situazione sperimentale) ed uno relativo al gruppo d'età (*trend* di risposta differenti nei bambini tra i 5.2 e 7.2 mesi rispetto al gruppo tra i 7.2 e i 9.2 mesi) che evidenzia come l'elaborazione dell'evento preferenza diventi più complesso con lo sviluppo.

Il **sesto capitolo** introduce un settore di ricerca molto recente: lo studio del fenotipo autistico diffuso nelle popolazioni a rischio di sviluppare Disturbo dello Spettro Autistico (DSA). Verranno presentate alcune delle popolazioni classificate come *high-risk* (HR) per fattori di rischio biologico (es. nati prematuri e/o sottopeso), genetico (es. fratelli di probandi) e

"ambientale" (es. individui i cui *caregiver* mostrano elevati tratti autistici). Lo studio del fenotipo autistico (BAP) in individui "non affetti" ha permesso, solo recentemente, di identificare alcuni *marker* comportamentali e neurofisiologici, indici precoci di rischio per il DSA. La valutazione di tratti autistici nella popolazione generale è una ulteriore strumento d'indagine. Questi approcci innovativi allo studio dello sviluppo atipico aprono nuove prospettive relative a *screening* neonatale, diagnosi precoce e intervento precoce, aspetti finora critici e determinanti per l'esito evolutivo e la qualità della vita degli individui con DSA e delle loro famiglie.

Il **settimo capitolo** presenta l'ultimo studio del progetto, il cui obiettivo è quello di identificare eventuali indici di rischio in una coorte di bambini italiani tra 0 e 3 anni. La prima parte dello studio è indirizzata ad un campione di neonati aventi un fratello o sorella con diagnosi DSA. Il protocollo sperimentale è costituito da tre esperimenti comportamentali:

1 Esp. 4.1: Preferenza visiva tra volto reale con sguardo dritto (contatto oculare con l'osservatore) e volto reale con sguardo orientato (assenza di contatto visivo).
2 Esp. 4.2: Paradigma *gap-overlap* con stimoli schematici sociali (volto dritto) e non sociali (volto invertito), per misurare i tempi di latenza saccadica e gli effetti della natura dello stimolo sui processi di facilitazione e disancoraggio attentivo.
3 Esp. 4.3: Preferenza visiva tra stimolo dinamico biologico e non biologico.

Il campione high-risk (HR) mostra dei pattern atipici nei comportamenti di *engagement* e *disengagement*, rispetto al gruppo low-risk (LR), durante i tre compiti sperimentali: a) stesso *trend* di risposta del gruppo LR per il compito di preferenza con volti reali, ma con un tempo medio di fissazione degli stimoli di molto inferiore; b) assenza del tipico *gap effect* e maggior RT di latenza nel caso dello stimoli sociale; c) assenza di effetto principale per la preferenza visiva di azioni biologiche (analogo al gruppo LR), con tempi medi di fissazione significativamente inferiori.

Nel **capitolo conclusivo** i risultati dei quattro studi verranno discussi alla luce della cornice teorica presentata in introduzione e delle emergenti posizioni concettuali. Aspetti critici metodologici del progetto verranno esposti in questa sezione, avanzando proposte per futuri studi volti a rispondere alle domande teoriche tutt'ora aperte.

Abstract

The present research project is addressed to the study of the development of the "social brain", a network of brain regions specialized for processing social stimuli.This system is evolutionary specialized to social interaction and it plays a crucial role in social development. Human beings are "socially tuned", but the origins of this specialization process are to date, in part unknown. This research is focused on the emergence of early precursors of social abilities, and on the processing of socially relevant information in the first year of life, in typical and atypical development. In particular the following areas have been investigated: the neural correlates of the human action processing at birth, the ability to understand goal-directed actions in the first year of life; the early neuro-cognitive endophenotypes of the Autism Spectrum Disorders (ASDs) related to the precursors of the social cognition (i.e. attentional pattern during face and gaze processing; the action processing). The populations studied are infants and newborns having different degrees of familial risk for autism emergence. The principal aims of the project are a) to investigate when and how few social abilities are achieved during the typical development; b) to study – by means of at risk populations- the role of early asynchrony in growth on atypical development trajectories; c) to identify some possible early behavioural and neurophysiological indicators which could predict the future emergence of Autism Spectrum Disorders (ASDs). In order to study these aspects, functional brain neuroimaging has been integrated with the theoretical framework and the classical techniques of developmental cognitive neuroscience. These complementary methods of investigation allow to study functional brain structures and behavioural systems at the same time. Four main studies have been conducted with the aim to shed light on the very early mechanisms of social orienting and action processing and how these specialized. The first study employed behavioral neurocognitive procedures and the non-invasive functional neuroimaging techniques (fNIRS - functional Near Infrared Spectroscopy) on a sample of newborn infants in order to collect direct and indirect data (looking behaviour and cortical oxygenation changes) during the passive observation of dynamic biological stimuli, socially communicative (i.e. face-to-face interaction) or not communicative (goal-directed action), compared to dynamic non social and non biological stimuli. The second and third study addressed to older infants (between 6 and 9 months old) is focused on the role of behavioural (i.e. related to the mental states of the agent) and environmental cues (i.e. related to the context where- or to the mode by which- the action is executed) in the goal attribution

processes. Three different paradigms have been utilized in order to evaluate which are the more efficient features which modulate the others' actions understanding in the first year of life. The fourth study consists in the administration of two well established behavioural paradigms in the field of visual attention to a sample of newborn infants at high risk for ASD (because later-born sibling of a proband). An additional original task has been proposed to the high-risk sample, to evaluate the spontaneous preference for biological over non-biological motion in realistic scenes of goal-directed actions. The infant siblings' data were compared to the performances of peers (undergone to the same tasks) with unspecified risk for autism (i.e. general population) to highlight the presence of a hypothetical peculiar attentional phenotype in unaffected relatives of children with autism (Broader Autism Phenotype) .

Chapter 1. STUDY 1: EARLY MOTION PROCESSING DURING HUMAN ACTION PERCEPTION

A complex network of brain regions seems to be preferentially dedicated to processing social information and evolutionary specialized to social interaction (social brain hypothesis, Brothers, 1990). These areas -including the superior temporal sulcus (STS), the fusiform "face area" (FFA), and orbitofrontal cortex- enable us to recognize other individuals and to evaluate their mental states. Social perception refers to the initial stages of evaluating the intentions and psychological dispositions of others by using their body movements, hand gestures, other biological-motion cues as gaze direction, and facial expressions (Allison, Puce, & McCarthy, 2000). The ability to selectively process information about conspecifics it is a kind of specialization but in literature is still open the debate about this ability: is it present from birth? It might be that the brain acquires expertise in processing social stimuli as a result of its being born into an intensely social environment. Several studies suggest an early specialization of a cortical network for the perception of biological motion, which becomes increasingly fine-tuned throughout development. Recent findings highlight that functional Near Infrared Spectroscopy (fNIRS) can reliably measure brain responses to biological motion and can detect social experience-dependent modulations of these brain responses. fNIRS is a noninvasive method for monitoring brain activity and involves measuring the absorption of near-infrared light passing through the skull. It enables evaluation of dynamic changes in the local microvascular concentration of oxygenated, reduced and total haemoglobin. In order to investigate the early emergence of the selective mechanism responsible for the human action processing, they have been observed the newborns' behavioral responses towards a human and a non human goal-directed actions (Experiment 1.1) and brain activity in the temporal region in response to biological and non biological events (Experiments 1.2 and 1.3). A visual preference task and a passive viewing paradigm have been used respectively in the first and in the second and third experiment. The fNIRS has been employed to evaluate the haemodynamic response in cortical areas of infants during the presentation of dynamic human or mechanical actions videos. The haemodynamic response (HR) to the experimental condition has been measured in relation to a longer control condition (to allow the HR initiated during the experimental condition to return to a baseline level); infants were shown mechanical movement in contrast to a biological movement. The brain activity was recorded in a region of the brain related to social perception (see Allison, Puce, & McCarthy, 2000; Lloyd-Fox et al., 2009).

1. Visual preference between biological and non biological goal directed actions in newborns

Infants are somewhat able to comprehend goal-directed action already during the first year of life (Tomasello & Haberl, 2003; Hamlin, Wynn & Bloom, 2007), even if they do not resort to mentalizing processes. The ability to perceive actions in terms of goals in a nonmentalistic way is still under debate in the scientific community, but two hypotheses are the most supported: according to the first already at birth by means of sensitivity to certain clues among which animacy and self propelled motion (termed: behavioural ostensive cues), infants are able to interpret goal-directed actions (Baron-Cohen, 1994; Leslie, 1994); the second approach is based on the link between first-person's experience and action understanding, thus infants can comprehend action only if is already in their motor repertoire or at least compatible with their motor skills (e.g. Sommerville & Woodward, 2005; Meltzoff, 2007). From the Mirror Neuron System field of literature has emerged clearly how the motor cortex is not only involved in the execution of actions but also in the cognitive understanding of motor acts. Perret and colleagues (1989) first demonstrated how – in monkeys, some cells in the analogous human STS area (Superior temporal Sulcus), respond specifically to hand movements and this responsiveness was grater when the actions were goal-directed, afterwards Grafton and collaborators (1996) confirmed this finding in human adults. Thus in the human brain there is a system devoted to hand action recognition, placed in left STS and left inferior Frontal Gyrus ([IFG]; Rizzolatti et al., 1996).

The action perception and action processing are closely linked on both outcomes level and inner activation level, in fact the Mirror Neuron System theorists support the idea of a special brain network (the premotor cortex, the supplementary motor area, the primary somatosensory cortex and the inferior parietal cortex) triggered as much by goal-directed action observation as much by goal-directed action execution (e.g. Iacoboni et al., 2005; Rizzolatti & Fabbri-Destro, 2008). Streltsova and colleagues (2010) have proposed two different functional aspects related to the activation of the mirror mechanism during movement observation in humans:

1. an automatic low-level ***motor resonance***, starting as soon as a movement or a goal-related motor act is observed. In humans motor resonance can be induced also

when a motor goal is not present in the observed behavior of others then irrespective of its goal-relatedness and social content.

2. The ***action understanding*** is the second functional aspect related to the mirror mechanism. Such aspect implies the activation of goal-related motor neurons in the brain of the observer matching the goal of the observed motor behaviour of others.

Moreover, the mentioned work -focused on hand motor acts and gestures- has shown that motor resonance can be strongly modulated in according to two different aspects of the observed hand behaviors: the presence/absence of a goal and its social relevance. There are others evidences showing that young infants shift their covert attention exclusively, or at least most effectively, during observation of human actions, and that supports the notion that early action comprehension is more specifically tuned to human actions (Daum et al., 2011). The study of human action processing is mandatory intersected with the study of biological motion (BM) processing. The most popular paradigm employed in this field is the point-light displays technique (PLDs), first introduced by Johansson (1973) as method for studying biological motion perception without the interference of the shape. Adults are able to recognize a configuration of moving dots (PLDs) as a walking person. Dynamic PLDs allow people to identify/discriminate BM from other kind of motion (i.e. scrambled or nonbiological). From this branch of study emerged the concept of the "life detector" (Troje & Westhoof, 2006) a system which seem to be innately tuned to makes human able to recognize conspecifics on the basis of local and configural features (i.e. speed, constancy). Vallortigara and Regolin (2006) enlarged this model to the animal kingdom as well: they found that displaying PLDs of a walking hen to naïve chicks they tended to align their body along the apparent direction of motion of an upright PL hen, but not to the inverted one. This result lead to a new hypothesis: an evolutional neural mechanism for detecting conspecifics, not limited to human beings. The latter findings suggest, so, that the presence of this life detector system might be experience-dependent. The unique and pioneering study in this direction is the experiment conducted by Simion, Regolin and Bulf (2007) on newborn infants using the PLDs technique and unfamiliar stimuli: participants were able to discriminate between BM and random motion and they manifest a spontaneous preference for the PLD of an upright walking hen. Indirect support to these findings comes from a study done on expectant women, the researchers did monitor the movements of a group of foeti revealing the presence, especially from the 22nd week of gestation, of kinematic

patterns of intentional actions (i.e. movement duration, peak velocity related to target); in other words the foetus' hand movements directed toward parts of his/her body were characterized by acceleration and deceleration phases apparently planned according to the size or delicacy of the target (Zoia et al., 2007). Despite this body of evidence coming from the use of the point-lights technique, not many others methods have been employed with this aim and even little is known about the processing of real every-day's life actions. Another very forerunner study, for age of participants, topic investigated and technique is the one published by Sarah Lloyd-Fox and colleagues in 2009. The study using functional near infrared spectroscopy (fNIRS),shown that five-month-old infants show bilateral activation of posterior temporal cortex sites in response to social video clips, a pattern that is not seen in response to static or dynamic non-social scenes. These data confirm the role of STS as biological motion detector as in infancy as, well largely reported, in adults and non-human primates. There are other converging lines of evidence about this topic and in particular there is a set of study that have been focused on the perception of biological versus non biological motion in adults comparing real (or computer generated) human actions to "robotic" actions. Several authors, using neuroimaging techniques, demonstrated the lack of premotor cortex activation during the passive observation of actions executed by non biological agents, supporting the concept that the human is strongly tuned on biological events and the absence of particular features in the action/agents caused a lack of cortical matching (absence of mirroring) and a subsequent lack of representation of the observed event as biological (Tai et al., 2004). Gazzola and collaborators (2007) in a similar study shown the lack of activation in the mirror neuron system (MNS) when participants were observing robotic action compared to the sight of human actions, reinforcing the hypothesis that MNS is sensitive to the type of movement exhibited by the effector/agent: different kinematics lead to different brain activity.

Shimada (2010) using NIRS technique and comparing human and robot agents showing either coherent kinematic patterns (human aspect- biological motion; robotic aspect- mechanical motion) and incoherent kinematic patterns (human aspect- mechanical motion; robotic aspect- biological motion), has shown that in healthy adults MNS activity is sensitive to congruency between appearance and kinematics of the agent (hereafter, "coherence hypothesis"): the mismatching between the two variable (aspect and motion) provoked a de-activation in the brain. The appearance information defines "who" is acting, but the kinematics information suggests how the agent performs the action. These two aspects – in my opinion- are closely interrelated: as the kinematics allow to human being

from birth to identify the agent's nature (see Simion, et al. 2008); on the other hand, the agent's nature should produce expectations in the observer about the motion features of the action's performer (e.g. studies on mentalizing in which moving geometric shapes are described as intentional; see Castelli et al., 2002; Zwickel, 2010). Whether the ability to infer kinematics from the visible aspect is available from birth, as the ability to recognize the nature of the agent from its kinematics, is to date unknown. The purpose of the first experiment is also to clarify this aspect.

1. Rational

Previous behavioural studies conducted on older infants (6 months old) by our research team (below presented, *see* Chapter 5) did highlight the role of motion in perceiving goal directed action and intentional agents. Considering these results it became urgent to conduct further studies on the early action perception/processing, using classical behavioural techniques, in order to deepen the role of motion features in perceiving agent from birth. Another aim of the present investigation was try to clarify if the salience of the perceptual-kinematic congruency showed both by adults (see Shimada, 2010) and infants (see 5 experiment contained in Study 2, Chapter 5) have their early roots since birth.

1.1.2 Method

The experiment is an infant controlled procedure, a visual preference between two simultaneously presented clips. There were two experimental conditions:

(a) appearance-kinematics coherence and

(b) appearance-kinematics incoherence.

The hypothesis of attended results was: a preference for the biological agent in the coherent condition and a strong violation of expectation in the incoherent condition. The expected preference for the biological agent in the first condition is motivated by the fact that the hand is partly familiar (see prenatal and postnatal experience); it displays biological perceptual features (appearance); and it displays attractive kinematic patterns (innate tuning on typical human motion properties). The result attended in the second condition could be due to the mismatch between aspect of the agent and type of motion

may be ambiguous: lack of preference of a preference for one of the two agents. In the second case the hypothesis was bidirectional: participants could prefer the hand is the external aspect is more salient than kinematics or they could prefer the tools if the motion is more salient.

1. Participants

Forthy-three newborns have been enrolled in this experiment; about who 7 were excluded for change of state; 1 for positional bias[1]; 2 for technical error; one for parental interference; and 2 because considered at risk according to biological parameters (i.e. born preterms or with low weight and/or with Apgar's index below or equal to 8). The final sample was composed by 22 healthy newborn infants (9 males and 13 females) with age ranging between 1 and 5 days-old, (mean: 58 hours; standard deviation: 24 hours). All the participants respond to the screening criteria of healthy delivery: a minimum Apgar score of 8 at 5 minutes after birth; a birth-weight ranging 2,600-4,000 grams (mean= 3397g); being a full term born and free of ocular and neurological defects.

2. Stimuli

The stimuli used were two short full-colored movies, presented at the same time, showing an agent (one one side the hand, and on the other side a tool) which enters the scene from above (upper external edge of the presentation screen), approaches the object-target already present in the middle of the stage (a red ball), than grasps it and reallocates it in the opposite side (in a symmetrical position on the stage respect to starting point). Respectively the pair of clips shown in the coherent condition (*see*

Figure 1):

[1] When infants look more than 85 % of the total fixation time (both experimental phases) in one direction than the other, regardless to the stimuli's position, they are excluded from final sample to avoid the presence of unreliable fixation times in the dataset, due to postural biases rather than spontaneous preferences.

1. as *biological coherent stimulus* a clip in which an hand grasps the target on the surface with its natural fluid motion;

2. as *non-biological coherent stimulus* a clip in which a rod grasps the target on the surface moving in a rigid/mechanical way.

In the incoherent condition the stimuli were (*see*

Figure 1):

1. as *biological incoherent stimulus* a clip in which an hand grasps a red ball on the surface showing a rigid/mechanical movement;

2. as *non-biological incoherent stimulus* a clip in which a rod grasps a red ball on the surface moving in a fluid way as shifted by an unseen person.

The movie's duration was of 2 minutes, the whole action (approach-grasping-release) lasted 10 seconds, and it is presented in loop (with an interval of 1.7s from end of one cycle to the beginning of the next one) until the end of the clip. To manipulate the kinematics of the two agents, the original videos were corrected by means of two editing softwares (Adobe Premiere and Adobe Photoshop). The editing procedure had the aim to equalize the length, luminance and contrast of the pair of clips and to synchronize the different phases of the action by manipulating the speed of the presentation. Moreover in order to obtain the biological hand stimulus and the biological tool the clips were kept as were filmed in terms of number and sequence of frames; on the contrary to get the mechanical ones several frames were cut, duplicate and placed in a different order to confer the typical features of the nonbiological motion (arrhythmic, rigid, constant); doing that the agent was moving on with the same speed (one move every 10 frames) and for the same amount of space. On the other hand, the biological agents exhibited the natural human motion's features, among which a fluidity and reduced speed in reaching related to the proximity of the target (classical arm peak velocity at the beginning of the motor act and changes in kinematics in respect to object's size and distance). The tool's kinematics,

in the incoherent condition was perfectly comparable with the natural hand's motion, given that the tool was moved by an unseen person; during the editing phase from each frame of the video the model's arm has been removed. The two films were presented together, the initial frame of each clip contained the target on the stage (starting point) and the final frame for every clip shown the agent placing the object on the opposite side of the stage (end state). The parallel presentation of the couple of clips was always specular (action from the center to the side of the screen) in order to make the whole scene symmetric. This escamotage was taken to comply with the newborn's visual characteristics, is easier for the neonate to follow an action occurring from the center to the periphery due to the immaturity of the cortical regions responsible for the central ("foveal") sight (Fantz, 1963). The procedure of the classical visual preference provides for the presentation of two phases, the second stage usually consists in the presentation of the same elements (stimuli seen in phase 1) in reverse position, to control any bias related to the position of the stimuli. In this case, to maintain the mirroring and characteristics above described, not only have been swapped the positions of the two agents in the second trials, but also presented the clips were mirrored, to restore the previous schema (target-object in the center and action which develops outwards).

Figure 1 ***Stimulus used in Experiment 1.1.*** *On the right side the stimuli of coherent condition: the arm moves in a natural way, the rod moves in a mechanical way; on the left the stimuli of incoherent condition: the tool is being moved by an unseen person displaying a biological kinematics the arm (by digital editing) shows mechanical motion. The position of the two agent is switched and mirrored among the first and the second trial (phases), the target of the action is always in the centre of the screen, whereas the agent are reversed. The order of presentation of the two phases is between participants (within condition).*

The choice of the stimuli, goal-directed actions is motivated by several findings in literature. First of all the human brain is specialized for the perception of grasping movements, in particular there is a system devoted to the hand action recognition located in the left STS and in the caudal part of the IFG (Rizzolatti et al., 1996). The action selected in finalized to the reallocation of an object in another position on the stage, we know that the activation in the brain is greater when there is a clear goal in the motor act (Gazzola et al., 2007) and that the pattern of activation differed according to whether the observed action is meaningful (ventral visual pathway) or meaningless (dorsal pathway) (Decety et. al., 2007). Moreover Craighero and colleagues in 2011 demonstrated that even newborns are more sensitive to the presence of a goal in the action and they display a preference for the grasping actions only when are directed to a perceivable target and the movement develops from the body of the agent to the goal-object and they do not reliably longer the stimulus when the action occurs in the other way around (from the object toward the agent's body). Finally hand actions seem to be very salient early in the development: the visual preference appears in the first months of life and the motor development of manual actions is achieved within the first 6 months of age (Del Giudice et al., 2009).

1. Setting and Apparatus

The infants were tested in the S. Polo Hospital (Monfalcone, Go, Italy) in the neonatal lab coordinated by Dr. Teresa Farroni. Testing was carried out in a darkened and quite room. During the experimental sessions the infants sat on the experimenter's lap at 30 cm distance from a monitor screen of 60 cm x 34 cm (in 4:3 presentation each peripheral stimulus could subtend about 21° -horizontally -and 35° -vertically- of visual angle). The newborns' eye level was aligned to the centre of the screen. A video camera, placed above the monitor was focused on the infants' face in order to shoot and record baby's eye movement. Filming the participant's face allows the experimenter to monitor newborn's movements, position and possible change of state, on a second screen, out of the baby's sight; in addition the recordings of newborns' visual fixations are employed for the coding procedure. During the experimental session caregivers stayed in the room, but were told to

not interact with the infant, in order to avoid any kind of interference on the testing procedure.

2. Procedure

Once the newborn was awake and quiet, was placed in front of the presentation monitor, held by the experimenter and the experimental session started. The presentation is composed by two trials: in the first presentation the infants are shown the human agent on one side and the mechanical tool on the other. The presentation of the first phase lasts a maximum of 2 minutes, according to the infant-controlled method the presentation is interrupted as soon as the infant makes a disengage (to look away from the screen or to close his/her eyes) longer than 10 seconds, after a cumulative fixation time of at least 20 seconds (time needed to see the action twice) during which infant must have seen both the agents (left and right), otherwise the presentation continue to permit the newborns to explore both the side of the scene. In absence of a spontaneous disengage of 10 seconds the presentation of the first trial automatically stopped after a cumulative fixation time of 60 seconds, to allow the infants to observe also the second scene before became fuss. The second trial has a maximum length of 2 minutes too, the presentation goes on until the baby makes a 10 seconds disengage, or the video stops (because maximum fixations' criterion is reached) or he/she get tired.

3. Variables/Coding

The frame by frame videocoding of newborns' eye movements, provided two measures for evaluate the visual preference: the fixation times toward the two agent and the number of orientations in the two trials. There were two instructed coders, blind to the hypotheses, the inter-agreement rate between them has been calculated on the 20% of the total experimental group (both conditions). The recording of five infants, taken randomly from the whole sample, were re-coded by two judges: they used a code range of 3 seconds to obtain quantitative data of fixations and orientations from the first 90 seconds of each selected experimental session. The data obtained from the recoding shown an high Pearson correlation on the raw data (r= 85%); a Cohen's K equal to 0,72 (using an error's threshold of 500 ms), and a good agreement among judges equal to 87%.

1. Analysis and Results

Analysing the distribution of the total fixation times one participant has been excluded from the final sample because out of the range +/- 2,5 standard deviations (outiler). In the table below is illustrated the distribution of the sample along the experimental conditions.

Participants in the experimental conditions		order		Total
		A	B	
conditions	coherent	4	7	11
	incoherent	7	3	10
Total		11	10	21

Table 1 *Participants' distribution across conditions and order of presentation (phase1 and phase 2).*

The first analysis on the data has been conducted on the singular conditions as independent experiments, to verify the presence of principal effects of the stimuli. Descriptive statistics of the two experimental groups are illustrate in Table 1. A t-test on matched means has been done on the first and second conditions separately, leading to no significant results for fixation time in millisecond (coherent condition: t_{10}= 1.843, p > .092; incoherent condition: t_9= 1.082, p > .307), number of orientations and fixation rate.

		HAND		TOOL		total	
condition	measure	*ms*	*orient.*	*ms*	*orient.*	*ms*	*orient.*
Coherent	mean	58520	21,08	39413	19,17	97933	40,25
	st.dev.	26287	5,25	18468	8,32	27838	12,45
Incoherent	mean	44436	20,90	53318	23,40	97754	46,80
	st.dev.	25853	13,85	22073	11,82	40468	23,65

Table 2 *Means and Standard Deviations of fixation time and orientations towards the two stimuli and during the whole presentation.*

A t-test on independent means has been performed on the variables fixation time, fixation rate (percentage of stimulus' fixation on total fixation time), and number of orientations towards both the stimuli (hand and tool) using the belonging condition as pooling factor (coherent Vs incoherent). The result shown a reliable difference in the fixation time of the tool agent between conditions (t_{19}= 2.420, p = 0.026), thus infant newborns looked more at

the non-biological agent when it displayed human kinematics (means: 53318 ms Vs 35076 ms). The rate of fixation time (stimulus' fixation in ms / total fixation in ms) resulted significant as well (t_{19}= 2.410, p = 0.026). The difference between the fixation times towards the agent with human aspect (hand) was not significant (t_{19}= 1.349, p = 0.193). The number of orientation responses in respect the two stimuli did not reveal any reliable result.

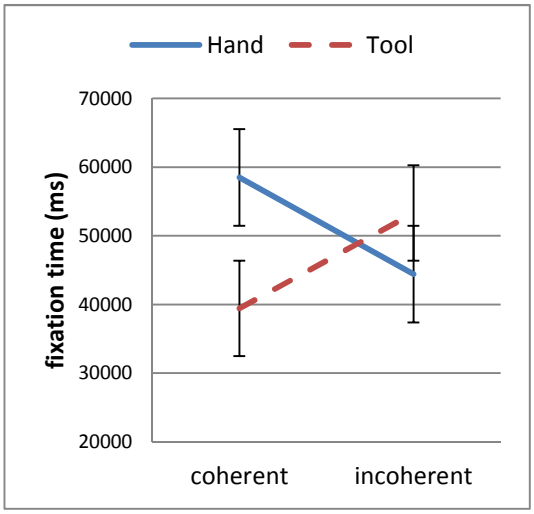


Figure 2

These results were confirmed by a non-parametric Mann-Whitney test, according to, the mean fixation time towards the tool was higher in the incoherent condition, when it displays human motion properties (Z= 2.605, p= 0.008), also the rate of fixation times resulted greater in the second condition (Z= 2.008, p= 0.043). Finally only the responses respect the aspect of agents, across conditions, has been compared with a t-test, which result not significant (t_{21}= 0.876; p = 0.391)

Figure 3 average of *total fixation time towards the stimuli divided by aspect and by kinematics.*

At the same way the kinematics per se has been compared showing a reliable differences (t_{21}= 2.154; p = 0.043): infant newborns exhibited greater fixation time towards the agent who was moving in a biological way, regardless to its aspect (hand or tool). In summary even if the raw data show a greater fixation time towards the hand in the coherent condition and towards the tool in the incoherent condition, these differences are not significant, thus there is not a specific effect of the stimulus per se. In general in terms of aspect there is no preference for one agent over the other one, but considering the kinematics per se, emerged a preference of newborn infants to orient their attention toward the element with humanoid motion features.

Figure 4 *Average fixation times (in milliseconds) towards the two kind of motion, divided by experimental conditions, and in the whole sample*

2. Conclusions

The first experiment shown a lack of difference in the infant newbors' behavioural responses towards the biological and non biological agent, both the conditions, that is when they exhibit a coherent pattern of kinematics (human appearance and biological motion Vs nonhuman aspect with mechanical motion) or when there is a mismatch between the external features and the motion properties. Nevertheless newborns tend to look reliably longer to the stimulus in the couple which manifest characteristics typical of the human action. Taken together the results confirm the previous result about the spontaneous preference for the biological motion regardeless to the configuration (aspect)

and the familiarity of the stimulus reported by Simion and colleagues (2007). The absence of principal effect of the condition may also suggests that the use of echological stimuli evaluated with the visual preference paradigm is a method not enough sensitive to record a possible different processing of the biological motion than biological motion.

The critical issues of this experiment are the use of realistic events rather than the stylized ones employed before in the studies with the point-light-dysplays technique. The real clips used as stimuli in the present study are composed by very complex images, so the presence of several confounding variables might prevent the baby to isolate the crucial features, rather than the presentation of point-light videos in which the original shape is seriously degraded and stylized. Another aspect to take in account is the presentation of part of the body (in the human agent stimulus), which could have compromised the process of detection of biological features: previous studies use the whole body (human beings or animals) walking. Providing partial information about the human agent could be a crucial aspect in determine the lack of evidence, infants are better in configure processing, thus present only local detail of the agent might prevent the detection of the shape otherwise recognisable as biological. The foot motion (see Troje & Westhoff, 2006; Chang & Troje, 2009) seem to be very important for the activation of the "life detector" system.

1.2 Cortical oxygenation changes in newborns during passive viewing of biological and non biological goal directed actions

1. Rational

Given the ambiguous results of the previous experiment, another technique have been proposed to investigate the presence of differential processing system for biological and non biological motion. In literature Lloyd-Fox (2009) has recently demonstrated the sensitivity of the Near Infrared Spectroscopy technique in highlight cortical activation in response to stimuli observation in infancy. For this reasons the behavioural study has been re-run, using the same stimuli, to check if the newborns' visual patterns of responses were linked with the neurophysiological data. The hemodinamic response to neural activation has been measured by the NIRS (InfraRed Spectroscopy), a non-invasive optical technique which detect changes in cortical oxygenation between oxyhemoglobin (HbO2) and

deoxyhemoglobin (HHb). This device returns the representation of neuronal activation in monitored brain regions in response to the task (visual stimulation paradigm).

The study had, thus, a double purposes. The first aim is to examine the activation of the newborn's brain motor areas, in particular of inferior frontal and temporal regions which have been demonstrated to be involved in the processing of goal-directed action in adults. These region partly overlap with an area that seem to be already specialized at 5 months in the social perception (STS), thus to investigate the activation of these area in response to social relevant stimuli, as the human action ,could be possible to evaluate the functioning of the social brain network at birth, in other words try to understand the role of postnatal experience on these complex neural circuits. The second reason which aimed this experiment is the possibility to assess potential early differences in processing between biological motion inserted in goal directed-action as opposite to biological motion per se. In order to investigate the early emergence of these mechanisms we have observed the newborns' brain activity in the temporal region in response to a social and communicative stimulus involving biological motion in comparison with non-biological stimulus using the NIRS technique.

Starting from the evidence provided by Lloyd-Fox in 2009, who compared dynamic social and dynamic non-social stimuli, using the same technique on 5 month-old infants, the following hypotheses have been elaborated: 1) if infants are engaging the same neural mechanisms for processing the dynamic social stimulus face as to process human goal directed action then the activation between the two stimulus categories (biological action vs non biological action) will be different and the brain areas involved in the biological (Bio) stimulus might be the same or sharing region of activation found in the previous study (Lloyd-Fox, et al. 2009); 2) if the posterior STS region is not yet specialized for dynamic complex biological stimuli -as the hand goal directed action- then no difference in activation between the two experimental conditions will be observed.

2. Method

The procedure implied the passive observation of two video clips representing a grasping action made by an hand (biological stimulus) and by a tool (non-biological stimulus), which were the experimental stimuli. The presentation of the stimuli was spaced out with sequences of static images, used as a baseline, which has been compared, in terms of brain activation, with the patterns elicited by the dynamic stimuli.

1. Participants

The study was conducted at the Pediatric Unit of the Hospital of Monfalcone. Twelve full-term newborns (postnatal age 24 – 120 hours; mean 72 hours) were recruited for the study through the Pediatric Unit of the Hospital of Monfalcone. All infants had normal birth weight (>2,400g) and Apgar index scores (between 8 and 10 at the fifth minute after birth).

2. Stimuli

The stimuli were extrapolated from the behavioural experiment described above (1.3). The clips from the couples used in the coherent condition of the previous experiment were presented individually. The experimental stimuli, thus, were:

3. A dynamic biological stimulus (an arm grasping a toy and moving it in another location on the stage), and

4. a dynamic non-biological stimulus (a tool performing the same action, but showing mechanical motion features)

The visual stimulation paradigm was adopted from a previous fNIRS study with five-month-old infants (Lloyd-Fox et al., 2009).

The baseline condition consisted in naturalistic static non-social stimuli: full-colour images of different type of transports (cars or helicopters) presented randomly to permit babies to remain awake and oriented to the screen (see Figure 5).

Figure 5 ***Stimuli and procedure of Experiment 1.2.*** *Adapted and modified from Lloyd-Fox et al., 2009*

The baseline sequence was presented randomly for 10 seconds with each image presented for a pseudo-random duration (1 – 3 seconds). These images were selected to be colourful,

complex and interesting, and ensured that newborns remained attentive to the screen. The overall surface area of the displayed experimental stimuli and baseline stimuli were equivalent. Note that fNIRS studies with adults use a blank screen as the baseline but this is not possible when working with infants, therefore the static images act as the baseline for the activated experimental period containing the human action video clips.

Figure 6 ***Examples of images presented in the Baseline (B) condition.*** *Adapted from Lloyd-Fox et al., 2009*

- **Setting and Instrumentation**

The setting is the same described in the relative section of Experiment 1.1, with the addition of the NIRS headgear. The infants were sat on experimenter's lap at 30 cm from the screen (26 inch), their eye movement and alert status were monitored and recorded by a camera placed above the main screen. The device employed is a commercial frequency-domain oximeter (Imagent, ISS Inc.) with 16 sources (32 laser sources, 16 at 690nm, 16 at 830nm) and 4 photomultiplier tube detectors and a sample rate of 16 Hz. The optical source fiber and detection bundles were arranged in two flexible rubber probes, fixed through a custom-built helmet. There were 10 channels (couple source-detector) for each hemisphere. Each probe was placed on the temporal region, with the midpoint at a fixed distance of 6cm from the center of the forehead, aligned approximately with T3/T4 of the 10-20 system on the average newborn head (17 infants; median cranial circumference: 34.5 ± 1.4cm). The posterior half of each probe lies approximately over the scalp locations T5/T6. The separation between channels was 1.8cm, which corresponds of a mean depth sensitivity of 1cm.

- **Procedure**

Before the infants' began the study, measurements of their head circumference, and distance between glabella, ears and inion were taken and the location of the channels and probes relative to these anatomical landmarks were recorded. At the beginning of each experimental session, when the newborn was quiet and in an alert state the experiment held

him/her seating in front of the screen and in the meanwhile the NIRS headgear was placed on the infant's head aligned to scalp and anatomical landmarks (10-20 electrode system). The stimulation paradigm started and the presentation of the biological action (BM) and mechanical action (MM) continued on a cyclical loop with the baseline sequence (B): the order was B-BM-B-MM (10 seconds for each condition, 40 seconds for each trial) until the infants became tired or fussy (the average length of the presentation was three minutes). The loop-style presentation of the stimuli and the short time duration of each condition were adopted for balancing possible loose of subjects' attention along the procedure. The attentive response should therefore result averaged over the stimuli, thus reducing its significance. The average time-duration of the presentation ensured that the subjects were exposed to a sufficient number of cycles for assessing the response to the block-task paradigm.

Figure 7 ***Probe location and channels disposition.***

- **Data acquisition and processing**

The measurements adopted for the experiment were changes in HbO2 and HHb concentration (μmol) relative to baseline. The haemodynamic indicators of neural activity were computed only for valid trials, actually attended by newborn infants. Fast Fourier Transform of the signal was performed to compute the AC component (amplitude), DC component (average Continuous Wave "CW" intensity) and phase shift of the waveform

signal from the 20 channels. The concentration changes were assessed using the modified Lambert Beer law with an estimated Differential Pathlength Factor (DPF) .The data were band-pass filtered (0.04-0.5 Hz, ***FIR*** digital ***filter***) to attenuate slow drifts and high frequency noise, mainly caused by physiological noise such as breathing and heart pulse.

Figure 8 Graphic representation of the channels disposition on the hemispheres.

Movement artefacts, were identified on and removed from the time course of each channel' signal by means of a semi-automated procedure and linear interpolation. In addition typical video-coding of the visual fixation responses was performed to be able to exclude invalid trials (not observed by newborns) from the compute of the beta-values, to execute an extra behavioural analysis to verify visual preference for the stimuli employed.

1. Analysis and Results

We first controlled the behavioural pattern of responses in terms of fixation time. As demonstrated by the previous experiment newborns were equally attentive to both types of stimuli: the looking time difference was not significant (mean of 8.4 sec for the BM stimuli and 8.2 sec for the MM stimuli); the number of trials per condition that were rejected on the basis of inattentiveness were again similar in both conditions (15 for the BM stimuli and 17 for the MM stimuli).

The analysis of change in blood oxygenation in the sample have been executed by the Infrared Imaging Lab (ITAB) of the Institute of Advanced Biomedical Technologies (Department of Neuroscience and Imaging of Chieti).

The analyses consisted in an Independent Component Analysis (ICA). The first step was to eliminate the components characterized by a positive correlation between oxy-haemoglobin (HbO_2) and deoxy-haemoglobin (Hb); than motor artefact were removed and finally the stimuli onsets were reconstructed. Using the same procedure adopted in fMRI studies a general linear model was performed in order to convolute the stimuli with the Hemodynamic Response Function (HRF) for the compute of β parameters necessary to fit with a model the experimental data of HbO_2 . The adult HRF has been used because of lack in literature of studies on such young children and the substantially invariance of the size of statistical results -reported on older children- changing the HRF. A t-test has been run on the participants' β values to evaluate if there was a statistical reliable activation in the brain region of interest (ROI)[2] in response to the passive viewing of the stimuli. The comparison did not revealed any remarkable different pattern of activation in the channels (t_9=0.9961, $p > 0.05$). Finally a general linear model considering as factors ROI and hemisphere (HEM) has been performed. From the results of the Anova results is confirmed the lack of effect of the two variable taken individually and the absence of an interaction (for details see Table 3). It is important to note that there was not a significant correlation between the age (in hours) of our newborns, and the strength of their activation in response to the Biological stimulus.

variables	Sum of Squares	d.f.	Estimated variance	F	P-value
ROI	0.119	1	0.11867	0.04	**0.8509**
HEM	0.021	1	0.02083	0.01	**0.9372**
ROI*HEM	1.765	1	1.76541	0.53	**0.4701**

Table 3 ***Results from the Anova performed on beta-values adopting hemisphere and regions of interest as factors.***

In summary the blood oxygenation signals and beta values did not emerge a particular increase of HbO_2 concentration in the lateral temporal cortex in response to the biological motion. The results suggest that the STS area is not activated in the newborn infants of our sample by the realistic stimulus representing a goal-directed action which involves biological motion (BM).

[2] Left and Right hemispheres (2) * anterior and posterior channels (10).

Paired t-test	Hand biological motion (BM)	Tool mechanical motion (MM)
Hand (BM)	T=0 p=1	T=1.1 P=0.27
Tool (MM)	T=-1.1 P=0.27	T=0, p=1

Table 4 Paired t-tests mean HbO_2 (GLM t-scores).

1.3 Cortical oxygenation changes in newborns during perception of social and non social dynamic stimuli

1. Rational

The results of the first Nirs experiment (1.2) were in line with the behavioural data: there was not any cortical activation for the biological stimulus (BM) than the non biological one (MM). In light of these lack of evidence, but also for the previous finding which have shown that young infants exhibit a bilateral activation of posterior temporal cortex in response to social video clips, but not in response to static or dynamic non-social scenes, it became important to replicate these finding. The most important reason is to verify if these pattern of activation to social and communicative events is not present at birth because the underlying neural circuits are not yet specialized or simply because the stimuli utilized in Experiment 1.2 involve human action, but are not enough salient, communicative and/or meaningful for the neonate to elicit the social brain network. The findings in five month-olds correspond well with functional MRI studies in adults of the activation of the posterior superior temporal sulcus, suggesting precocial functional specialization of some parts of the social brain network. While these previous results indicate rapid development of parts of the social brain, they cannot resolve the long-standing issue of whether or not these

regions are functional prior to relevant sensory experience, since by five months of age most babies have engaged in hundreds of hours of face-to-face contact with other humans.

1.3.2 Method

The procedure implied for the third experiment is analogous the one seen in experiment 1.2: a passive observation of two video clips, procedure adopted by the original work of Sarah Lloyd-Fox (2009). The stimuli shown to newborn infants are exactly the same created by the author, who permitted us to utilize them, thanks to the long-lasting collaboration between our and hers research team. The clips represent even in this case human actions (biological stimulus) compared to non human event (non-biological stimulus), but are more complex and, above all, in the first case they convey social and communicative contents. The presentation of the stimuli was alternated with the baseline, described before. The expected results were the following:

1. if infants are engaging the same neural mechanisms for processing social stimuli as adults then activation in the current study would be localized to the posterior-temporal region of the probes for the dynamic social stimuli, and not for the dynamic non-social mechanical stimuli.

2. However, if the posterior STS region is not yet specialized for dynamic social stimuli in newborns, then no difference in activation between the two experimental conditions will be observed.

- **Participants**

The study was conducted at the Pediatric Unit of the Hospital of Monfalcone. Fifteen newborns (postnatal age 24 – 120 hours; mean 56 hours; 11 spontaneous labour and 4 caesarean section) were recruited for the study through the Pediatric Unit of the Hospital of Monfalcone. A further two infants were excluded due to technical problems with data collection. All infants had normal birth weight (>2,400g) and Apgar index scores (between 8 and 10 at the fifth minute after birth).

- **Stimuli**

The stimuli for the replication of Lloyd-Fox's study consisted in full-colour, life-size social video clips of an actress either videoclips of objects in movement. The experimental conditions consisted of dynamic social video clips (S) of a women who either moved their eyes left or right, their mouth in silent vowel movements, or performed hand games; 'Peek-a-boo' and 'Incy Wincy Spider', and dynamic non-social video clips (M) of machine cogs, pistons and moving mechanical toys (see Figure 9).

Figure 9 Procedure and dynamic stimuli in Experiment 1.3. *Adapted from Lloyd-Fox et al., 2009*

These stimuli were selected because they involved complex interacting curvilinear motion patterns that served as a good control for complex manual and facial motion. The baseline as in the previous experiment were sequences of images of transports presented randomly and occurring between each experimental trial. The experimental trials, setting, apparatus, the procedure and baseline condition were identical to Experiment 1.2.

- **Procedure**

The infants sat on an experimenter's lap and were encouraged to watch the stimuli displayed on a 26-inch screen with a viewing distance of approximately 30cm. The visual stimulation paradigm was adopted from a previous fNIRS study with five-month-old infants (Lloyd-Fox et al., 2009): following blocks of B-S-B-M presentation (10 seconds for each condition, 40 seconds for each trial) as long the infant can tolerate before a change of state. All the requisite of the Spectrometry device were identical to ones described on the relative section of experiment 1.2. The variables measured were the same (HbO_2 and Hb), and the processing of the data acquired was made following an identical procedure.

- **Data acquisition and processing**

Changes in oxy-haemoglobin (HbO_2) and deoxy-haemoglobin (Hb) concentration (μmol) were calculated relative to baseline and used as haemodynamic indicators of neural activity only for valid trials. A total of 22 trials out of 75trials were corrected for movement artifacts. For each infant we included data from 4 to 7 (mean 5) full responses to each B-S-B-M sequence. The neural activity in response to the S and M stimuli respect to B stimulus was modeled as a square-wave function lasting 10 seconds. This covariate was convolved with the adult hemodynamic response function (HRF) and compared to the filtered HbO_2 concentration changes to yield appropriate predictors (beta values). This was done in a generalized linear model (GLM) and in analogy with fMRI analysis.

The mean square deviation between the model and filtered HbO_2 concentration changes was evaluated. Trials with channels that showed deviation within the population ≥ 99% percentile were disregarded. The rejection rate for stimuli was 7% on average, with a maximum of two disregarded responses of the same stimulus in one newborn.

1. Analysis and Results

Also in this case the looking time behaviors were controlled in newborns: they were equally attentive to both stimuli (8.9 sec for the S stimuli and 8.5 sec for the M stimuli), with no significant differences in respect to the time spent looking one stimulus over the other one. The number of rejected trials per condition because infants did not attend them were again alike in both conditions (10 for the S stimuli and 12 for the M stimuli).

Statistical analysis was performed on beta values of HbO_2 concentration changes. We evaluated the average response for each channel weighted on the number of received stimulus trials for each infant. A t-test analysis evaluated statistical significance of the average activation/deactivation within each channel (activation/deactivation defined as a significant increase/decrease in HbO_2). The standard deviation of the maximum displacement (half head circumference) among newborns, measured from the centre of the forehead assumed as reference point, was estimated as low as 0.7 cm, in comparison with a source-detector distance (that is the order of magnitude of spatial sensitivity) set to 1.8 cm. The group analysis of activation/deactivation within each channel among different children

should be considered significant when the inter-fibre distance is larger than the standard deviation of cranial circumferences. Therefore, given the actual experimental settings, data from the same channel among different children can be considered as coming from similar cortical areas. Two Regions of Interest (ROIs) were identified for each hemisphere: fronto-temporal (FT) and temporo-occipital (TO). Three-way (stimulus: S vs. M; hemisphere: right vs. left; region: FT vs. TO) multivariate ANOVA was performed on beta values dataset for each channel response. Statistical significance p-value was set to 0.05. A two dimensional topographic image (**Figure 10**) was reconstructed, considering the spatial sensitivity profile in reflectance geometry.

Figure 10 *Two dimensional topographic reconstructed image of the HbO_2 concentration changes. From representation appears clear the bilateral posterior activation only during the observation of the social sequence stimulus.*

The statistical analysis of HbO_2 signals and beta values using uncorrected t-tests showed relevant activation to the S stimulus in channel 4 (t=2.74, df=14, p=0.016, uncorrected) and channel 18 (t=3.75, df=14, p=0.002, uncorrected). Applying Bonferroni correction only channel 18 was statistically relevant (p=0.04, corrected). No channels showed significant activation/deactivation to the M stimulus (**Figure 11**).

Multivariate ANOVA indicated significant effects of Stimulus (M stimulus vs S stimulus) (F=11.33, p=0.0005), the Region-of-Interest ROI (fronto-temporal and temporo-occipital) (F=4.52, p=0.034) and the interaction between ROI and Stimulus (F=4.02, p=0.045), but not of hemisphere (F=2.92, p>0.05). No significant HHb changes were found. For the averaged haemodynamic time courses for the change in concentration of HbO_2 and HHb in

response to the S and M stimuli for all channels see Figure 11 and 12. In line with previous studies, the increase in HbO_2 concentration during S reached a maximum value towards the end of each trial, at 7 to 8 seconds post stimulus onset. The reconstructed topographic image of the HbO_2 concentration changes, suggested bilateral posterior activation for the S stimulus (Figure 10). T-test outcomes (uncorrected and corrected) for the HbO_2 signal on channel 4 and 18 showed that the activation in the right posterior area was greater than in the left posterior area.

Figure 11 *Averaged haemodynamic time courses for the change in concentration of HbO_2 and HHb in response to the Social stimulus for all the channels. As put in evidence, there are relevant activation in* ***channel 4*** *and* ***channel 18****.*

Secondary analysis comparisons were made for each experimental condition to investigate the effect of postnatal age within the group. This analysis was conducted on channel 4 and 18, given that the significant response in these channels is in a similar region to that found in the five month old infants. Linear regression of normalized beta-values of HbO_2 changes within channel 4 and 18 was conducted as a function of age (hours from the time of birth). The regression shows that there was a significant effect of age (ch4: $p=0.0147$ negative related, ch18: $p=0.037$ positive related) on the degree of activation (see Figure 13) in response to the S stimulus. There were no significant regression effects within these

channels for the M stimulus. the effect was not evident when gestational age was considered, indicating that very early postnatal experience is an important factor.

Figure 12 *Averaged haemodynamic time courses for the change in concentration of HbO_2 and HHb in response to the Mechanical stimulus for all the channels. None channel showed significant activation/deactivation.*

Figure 13 ***Linear regression of normalized β-values of HbO_2 changes as a function of age in channels 4 and 18 . The regression shows a negative relation of channel 4 with and a positive relation in channel 18 on the amount of activation in response to the S stimulus.***

In conclusion an analysis of variance (GLM) has been executed on the data obtained from the two studies in order to compare the brain activation in responses to the different type of event shown to the newborn infants. From the t-tests (as illustrated in Table 5 Paired *t-tests mean of HbO2, GLM t-scores comparing different stimulations (Experiment 1.2 and Experiment 1.3)*appears clearly that the social dynamic stimulus (which contains the actress' face) elicits a brain pattern of activation in the selected regions (fronto-temporal occipito-temporal) which significantly differs from the cortical activity produced by the other stimulation: hand action (BM); mechanical action (MM); mechanical object (M).

Paired t-test **T=tscore**	**Face (S)** **vs**	**Hand (BM)** **vs**	**Object (M)** **vs**	**Tool (MM)** **vs**
Face (S)	T=0, p=1	**T=-3.2** **P=0.002**	**T=-3** **P=0.002**	**T=-2.25** **P=0.02**
Hand (BM)	**T=3.2** **P=0.002**	T=0 p=1	T=1.6 P=0.06	T=1.1 P=0.27
Object (M)	**T=3** **P=0.002**	T=-1.6 P=0.06	T=0 p=1	T=-1 P=0.32
Tool (MM)	**T=2.25** **P=0.02**	T=-1.1 P=0.27	T=1 P=0.32	T=0, p=1

Table 5 ***Paired t-tests mean of HbO_2, GLM t-scores comparing different stimulations (Experiment 1.2 and Experiment 1.3)***

2. Conclusions (experiment 1.2 and 1.3)

The second experiment on newborns compared biological and non biological goal directed actions- showing only the effector (arm vs tool). The analysis of the cortical oxygenation changes during passive viewing of human and non human actions has revealed a lack of selective activation for the social stimulus in newborns. With regard to the dynamic social and non social stimuli (persons Vs object) compared in the third experiment, it emerged an activation selective to the dynamic social stimulus over bilateral posterior temporal cortex and an early localization correlated with age in hours over the first days of life. These data are concurring with the evidences coming from literature on later development (see Lloyd-Fox et al., 2009 – study on 5-month-olds).

3. Discussion

The first experiment, the behavioural one did not produced remarkable results, suggesting and absence of spontaneous preference for one of the two agent. In the two neuroimaging experiments a different pattern of activation has been found. From experiment 1.2, the equivalent of the visual preference executed with the functional Nirs the results were analogous: the activation brain pattern did not highlight any reliable difference between the two experimental stimuli. The results of the third experiment (1.3) are –instead- consistent with the very early activation of the brain network responsible for social perception. The trend between the hours of life and the oxygenation change in the brain during the observation of dynamic social stimulus, is negative in one site and positive in another cortical site. This opposite tendency could indicate the specialization process of the brain regions: assuming that, for example, is possible to think that one area of the cortex could be more susceptible to environmental cues (like the face and the biological motion) in the first hours of life an less in the following hours/day because of a constant re-organization of functions and system in the neonate's brain (which develops very quickly in the postnatal period). An alternative account is that a cortical functionality –ready to social

perception- is present from the prenatal stages, but the impact with the experience triggers the emergence of different processing systems. Thus, even very limited experience of face-to-face interaction with other humans may be sufficient to cause stimulus-specific activation of relevant cortical regions.

The presence of the face - a very special category of stimuli- seems to have affected the results of the study showing activation of the STS, likely for three reasons: a) in the previous experiment the comparison between biological and non biological actions was based on the presentation of a limited perceptual view of the motor act: only the effector (arm vs tool); b) the eye gaze is an essential communicative cue for early social development (Farroni, et al., 2002), therefore its presence in the social clips was very salient for newborns. c) The third possible interpretation for the lack of evidence in the second experiment is related to the multimodal nature of the STS (Wright, Pelphrey, et al., 2003; Schultz et al.,2012). The social condition in Experiment 1.2 shows a silent actress that moves their mouth, according to the hypothesis of *learned audio visual association* (i.e. visual experience of seeing moving mouth accompanied by sounds) there is the possibility that even with a short postnatal experience infants may have associated seeing moving lips with the human voice sound; for this reason the social dynamic stimulus may elicit temporal cortex activation independent by the social meaning of the scene, but by virtue of this audio-visual matching.

The analysis of the cortical oxygenation changes during passive viewing of human and non human actions has revealed a lack of selective activation in newborns; in contrast a subsequent study run by other colleagues in the research team (not included in the current dissertation) found a clear localization and differential activation for the social stimulus over the non-communicative one (using the same clips of Experiment 1.2) in a 5 month olds sample. According to the Interactive Specialization model of functional brain development, whereas some cortical areas may be also initially responsive, but less finely tuned to specific visual stimuli. All together these data suggest the presence of a strong specialization process for the encoding of socially-informative stimuli in the first months of life.

Chapter 5

Chapter 2. EXPERIMENTAL PROJECT ON GOAL ATTRIBUTION IN INFANCY (STUDIES 2 and 3)

The ability to interpret others' acts, like intentional grounded, is an inescapable stronghold for the child development and for everyone's adaptation to the environment. The prerequisite of this skill resides in the understanding of goal directed actions. This ability appears already in the first year of life (e.g. Aschersleben et al., 2008; Csibra, 2008; Sommerville & Crane, 2009), it's fundamental for the social and cognitive growth, and represents one of the Theory of Mind's forerunners (e.g. Meltzoff & Brooks, 2008). In the last decade the interest for this topic in the scientific community is strongly enhanced in the field of social cognition, in part supported by the new related findings coming from the neuroscience. In particular has been somewhat revolutionary the discovery of a class of neurons (that we share with nonhuman primates) triggered by the perception -as well as the execution- of others' motor acts. Functionality of the so-called mirror neurons and neural network involved in the human action processing have been largely investigated in adults by the Mirror Neuron System theorist (MNS: Iacoboni et al., 2005; Rizzolatti & Fabbri-Destro, 2008). Even though is still unclear how works the action understanding mechanism in the infant brain, numerous behavioural evidences from developmental psychology testify the presence of the prerequisites and few early manifestations of others' action understanding, yet in the two years of life. There are many speculative approaches for goal's attribution in infancy: *experience-based* theory (e.g. Woodward et al. 1999;1998; 2009; Meltzoff, 2002; Sommerville et al., 2005; 2009); *cue-based* theory (e.g. Premack, 1990; Csibra & Gergely, 1998; Kirali et al., 2003; Biro & Leslie, 2007) and *teleological stance* (Gergely & Csibra, 2003) which partly overlap each other, creating a prosperous theoretical concerning debate. To comprehend the very early understanding of goal-directed actions in humans two behavioural studies have been conducted on infants in the first year of life (5 experiment on 6 months old; 2 experiment of infant ranging between 6 and 9 months-old).The methods used were: behavioural techniques (habituation infant

controlled and visual familiarization) with violation of expectation task in classical and modified Woodward's paradigm and in a novel paradigm created on purpose.

STUDY 2.a Woodward paradigm: how motion makes infants see social agents (Experiments 2.1 and 2.2)

Do exist special cues that enable infant to make goal attribution? One on the aim of the present and the followings experiments described in this chapter is to comprehend whether there are different categories of cues. The context's cues could have a different role if they are related to the agent or to the goal directedness of the action. Several studies have demonstrated that there are almost two categories of cues that work in the process of action's understanding: behavioural cues (i.e. related to who is acting) and environmental cues (i.e. related to the context in which the action is executed). Until now these cues have been used alternatively, according to the chosen paradigm. In example the most popular techniques, the Woodward's paradigm and Csibra's paradigm are conceived to elicit goal's attribution by convey information about the agent's intention; on the contrary the paradigm employed by Luo and Baillargeon is based on the presence of physical constraints or agent's feature which drive the process of goal's attribution. These different set of cues seem to imply two levels of cognitive processes. The information about the agent (as the preference between two possible visible goals) suggest an high-level of interpretation and make possible inferences about other's mental states and intentions. On the other hand the cues about the action (i.e. the outcome, the efficacy, the possible environmental constrains) can be elaborate at low-level, because they are connected with physical knowledge earlier available in the development (see Spelke). *Are these two categories of cues independent? Do they elicit different interpretation or goal attribution?* Shifting this debate on the early development, the intention is to investigate the role of these two families of cues in the first months of life, to figure out whether one category of information more salient and effective in built the social ability to understand others' actions and less dependent on experience and maturation. In literature there are several experimental approaches to study infants' goal-attributions, in most of which infants repeatedly witness a goal-directed action and later their reaction are assessed to similar actions performed under modified environmental circumstances (e.g. Woodward, 1998). The Woodward's paradigm is based on the presentation of the preference information (i.e. a second possible goal) during the familiarization phase, according to the assumption that

infants' encoding of the goal of actions witnessed during familiarization can guide their representations of goals and actions during the following test phase. In the single-target versions (Luo & Baillargeon, 2005; 2007; 2009) of the Woodward's paradigm, indeed, no preference information is provided, there is only one at a time visible object (old or new); that expedient is enough to prevent the generation of expectations, in the observer, about the agent will. The availability of various cues related to goal-directedness could compensate for the lack of preference information. Efficacy also termed "means selection" it is one of them: the outcome of an action, given the physical constraints, it depends on the way in which the goal is achieved (Southgate & Csibra, 2009; Hernik & Southgate*, under review*). The presence of environmental obstacles accompanied with aequifinal variations in the agent's behavior (i.e. change the route/modality to reach the previous goal) makes infants able to attribute intentions even to non human elements. This category of cues has been often contrast with the agent's preference for one -among more possible- target ("outcome selection"); thought to be even a crucial cue as well in the "Woodward scenario" in generating expectations about the future behavior of the actor. The present research is focused on the role of kinematic cues and on the effect of the previous outcome selection in modulate infant's anticipation of an action and goal's attribution evaluated by means of violation of expectation tasks (VoE).

4. Rational

The focus of this broad project was to verify the role of special categories of cues on the ability to understand goal-directed actions in infancy. Six months old infants took part in a visual familiarization procedure, paradigm adopted from Woodward (1998). They saw an agent perform and hand action on a target rather than another (two possible visible outcomes) during an initial phase, then a couple of similar events was shown them: the agent acting alternately a) on the previously un-chosen target (*new goal*) and b) on the *familiar goal*. The visual behaviour of infants in respect to the two test events was measured. Two experiments, using this paradigm, have been run; the variables manipulated to get the different experimental situations were:

1. the *pathway* of the action (from above or from the side);
2. the *action's goal* (i.e. *cube* or *ball*);
3. the *agent's aspect:*

Exp.5.1: a human arm (biological appearance)

Exp.5.2: a tool (non biological appearance).

The hypothesis underlying this part of the study was to verify whether infants (at 6 months) are able to discriminate goal directed actions and whether they respond in a different way on the basis of the appearance of the agent (biological or non-biological). In other words, by replicating the Woodward's original study would have been possible to proof the power of our stimuli and procedure in generalizing the previous results (Woodward, 1998; Biro & Leslie, 1997). This step was mandatory in order to goes on in the research project further slightly manipulating the variables considered and especially modifying the original paradigm to test new hypotheses, next described. In operational terms the attended result is a preference -based on looking time behaviour- for the test situation showing the *new goal* event (Woodward, 1998), regardless to the nature of the effector (human aspect vs non-human aspect). Infants at that age should be able to interpret actions as goal-directed in a "human-shape-independent" way (Biro & Leslie, 1997), on the condition that specific behavioural cues are provided (i.e. self-propelled motion, see description in Chapter 4, *for a review* Scholl & Tremoulet, 2000).

2.1.2 Method

1. Participants

The whole sample recruited for the experiment was composed by 42 six-month-old infants (age range in days: 178-192). The participants who successfully completed the experimental session and were include in the final sample are respectively 16 (10 males and 6 females; mean age: 183,2 days) for the human agent condition (experiment 5.1) and 13 (7 males and 6 females; mean age: 179 days) for the non-human condition (experiment 5.2). 13 infants were excluded from the sample for technical problems (4), fussiness (3), because they did not attend the stimulation (2), for parental interference (2).Two additional infants were omitted in the analysis phase because outliers (in respect to the average) on the base of the total fixation time.

2. Stimuli

The stimuli presented during the experiment were three full colour video clips. Each of them the agent came on the stage, reached the target closer to its entrance's point (left or right), and grasped it. The whole action lasts 10 seconds and is presented in loop throughout the movies interspaced, every time, by a blank screen of 1.7 seconds for a maximum of 2 minutes in the familiarization phase and for a maximum of 1 minute in the test events phase. The target event was presented for first during the familiarization phase, afterwards in the following test phase other two clips were presented responding to the following criteria: the same agent perform a similar action either on the familiar object (the action target in familiarization) and on the *new goal* (i.e. the object not grasped during familiarization), the presentation of the two test events is sequential. In the *new goal* scene the pathway of the effector (arm or tool) is identical to the one seen before; in the *familiar goal* scene the agent reached the target through a different pathways (i.e. from above vs from the side). The agents were life-size (the tool was built with similar size of the arm) and the targets occupied the same space on the stage (cube: 14 cm for each side; ball: 14 cm of diameter).

The targets present on the stage are the same from familiarization phase to the end of the experimental session: always a cube and a ball, always one red and one blue (*see*

Figure 14). The combination shape-colour of the target was counterbalanced, in the same way the target chosen at the beginning, the order of test events' presentation and the action's pathway (new or familiar) were randomized between participants.

The video employed as stimuli were obtained starting from recorded real scenes. A digital editing process (using Adobe premiere and Adobe Photoshop) permitted to improve the luminance and contrast and to equilize the length of the videoclips. The non-human agent stimulus was obtained by paper rode put in motion by an actor (and subsequently made invisible in the final clip).

Figure 14 *Stimuli of Experiment 1 and 2.*

3. Apparatus and setting

All the infants have been tested in the DCNL babylab (Developmental Cognitive Neuroscience Laboratory) coordinated by Dr. Teresa Farroni, at the Developmental Psychology Department[3]. The equipment consists in a car seat facing a 23'' screen monitor (distance between babies eyes and the monitor was between 60 and 70 cm), where above is located a camera to record infants' eye movements. Behind the screen there is a curtain to divide the participant from the experimenters area. Out of baby's sight, indeed, there is the computer which manage the experiment's phases a second screen to monitor the infant's behaviour and a mixer to record baby's gaze and stimuli's presentation at the same time. The session is started by an experimenter via computer (the experimental script is run with the software E-prime 2.0) and while a second experiment is coding on-line –by pressing joysticks- the visual behaviour of the participant (through the curtains).

[3] University of Padova

Figure 15 ***Experimental Setting for study 2*** **.**

4. Procedure

Once the infant is placed on the car seat looking at the screen, with eyes aligned to the centre of the screen the experimenter started the familiarization phase. The presentation of the movie goes on until the infant reach the criterion of a cumulative fixation time of 80000 milliseconds or the sequence stops automatically after 2 minutes. When one of these criteria is met the test events presentation begins. The two test events are presented sequentially in a randomized order between subjects. The criteria for interruption of the test event movies is a cumulative fixation time of 40000ms or the end of the video (after 1 minute). A shared parameter between familiarization and test events' presentation is the obligatory experience of the whole scene, which means that infants must have seen the all scene before the experimenter skip to the next one. Even if the measured variable in the total fixation time towards the events (new goal Vs. familiar goal) the on-line coding permit to verify immediately in the infant is observing only part on the scene (left or right). In order to avoid bias in the data, the experimenter was allowed to drawn the baby's attention on the unattended part of the familiarization scene (by sounds) whenever the infant had not seen the whole scene after the first 10 seconds of the familiarization. Nevertheless the on-line coder was blind to the stimuli on the screen (visible only by a second experimenter), thus is coding was only referred to the side where the infants were looking at.

5. Variables/Coding

Recordings of the infants' eye movements during familiarization and test phases were later coded also off-line, frame by frame, by a second and a third coders unaware of the study's hypotheses.

The coders recorded, separately for each stimulus, the number of orienting responses and the total fixation time. A comparison between on-line and off-line coding was performed to check for technical error, change of state and positional bias. Moreover the inter-agreement reliability between the off-line judges was analyzed on raw data (Pearson correlation) and by Cohen's Kappa (using data of the first minute of the sessions, recoded in wider intervals to create the contingency tables of agreement).

The mean estimated reliability between on-line and off-line coding for 10% of the sample was of 96% for the fixation time and 91% for the number of orientations (Cohen's K) .

Using looking time and number of orientation responses of each participant the group's averages of both measures for both condition (hand Vs tool) was computed for each stimulus.

5.2.3 Analysis and Results

To verify the presence of a preference for one of the two test events (new goal Vs. familiar goal) a t-test on paired-sample two-tailed was carried out on the infants' average looking fixation time toward the two stimuli. For the arm condition (Experiment 1) did not result a significant difference ($t_{(15)} = .958$, p= 353) in terms of orientations between the new goal (average: 8.15) and the familiar goal events (average: 7.37). On the other hand, from the looking time behavior emerged a reliable difference ($t_{(15)}= 2.372$, p= .032), between the new goal (mean = 34585 ms, St.dev.= 5188 ms) and the familiar goal (mean = 27422 ms, St.dev.= 11773 ms). For the tool condition (Experiment 2) did not result a significant difference in terms of orientations ($t_{(12)}= 1.104$, p= .291) between the new goal (average: 6.84) and the familiar goal events (average: 6.0). The analysis of fixation times revealed a reliable difference ($t_{(12)}= 2.508$, p= .027), between the new goal (mean = 33435 ms, St.dev.= 10397 ms) and the familiar goal (mean = 29568 ms, St.dev.= 12432 ms).

The examination of data compared by target object, color and pathway did not revealed any spontaneous preference or effect on the fixation times towards the stimuli.

Figure 16 *Looking time's average towards the two test events in both experimental conditions.*

5.2.4 Conclusions

In summary six months old infants looked significantly longer to the new goal test event than at the familiar goal (new pathway) in both conditions (human agent Vs. non-human agent) indicating that infants interpreted the action as directed to the original target and are able to attribute goal to human as well as to non-human agents confirming previous evidence (Woodward, 1998; Bíró & Leslie, 1997).

1. STUDY 2.b Single paradigm: when lack of preference is lack of intention (Experiments 3, 4, 5)

1. Rational

In the classical Woodward's paradigm – employed in the previous experiments (1st and 2nd)- there are always two objects present in the scene of stimulation (in familiarization/habituation phase as like as in the test phases), in other word two possible targets of the action, that expedient makes visible to the observer the choice of the agent, the preference between object A and object B. The so called 'Woodward effect' (preference for the *new goal* stimulus rather than *new pathway* stimulus) could be forced by the presence of that cue, suggesting the will of who are acting (e.g. grasp the blue cube in the place of the red ball). The method used in our next experiments (from 3st to 5th) is instead a single-target version of the paradigm, in other word one-goal version of the task. Recently has been demonstrated that infants do not form the expectation that an agent will continue to approach the same previously approached goal target if this object was initially the only potential target available to the agent (Luo & Baillargeon, 2005).

To test the effect of preference information ("outcome selection") on goal's attribution 3 additional experiments have been run, degrading the amount of information in the familiarization phase: only one target is present on the stage. The following test events are provided of a unique object too. The procedure and stimuli were identical to the first two experiments, except for the presence -either in familiarization phase and test phases- of the second object (the un-chosen one in the familiarization of the classical Woodward scenario). The variables manipulated were:

1. the *pathway* of the action (from above or from the side);
2. the *action's goal* (i.e. *cube* or *ball*);
3. the *agent's aspect* (human: arm; non-human: tool);
4. *agent's motion features* (*biological* or *not-biological*).

The match between the agent's aspect and agent's kinematics lead to 3 experimental situations:

1. experiment 3: an arm which moves displaying its natural motion pattern;
2. experiment 4: a tool with *biomechanical-motion* (fluid, arrhythmic);
3. experiment 5: a tool with *mechanical motion* (rigid and rhythmic);

The speculative question which aimed this set of experiment is: Does the presence of a choice between two objects is necessary for the goal attribution mechanism in six-month-old infants?

The following experiments were conceived to answer this question about the influence of the amount of information incidental to the action shown during the familiarization; the additional manipulation of kinematics element is related to a secondary aim: evaluate whether in absence of a clear perceivable evidence of the agent's preference (i.e. intention), infants could employ physical elements as the kind of motion to confer

intentionality to an entity. According to these theoretical questions the hypotheses and attended results are the following:

1. if infants at 6 months are able to attribute goal to an agent even when the action is not result of a selection (one target means forced-choice), the other's action understanding at this age is not yet modulated by precursors of the TOM, which means that not implies mental states attribution and is founded on more low-level cues.

2. The goal's attribution ability seem to be extended also to non-human agents according to previous findings; if the ability to attribute intention to entities with no-human aspect is compromised when an extra perceptual clue -as the kind of motion- is in contrast with the human features, therefore this skill -in 6 months old- in more based on environmental/physical cues than on behavioral cues.

1. Method

3. Participants

The whole sample recruited for the study was composed by 93 six-month-old infants, tested between five months and three weeks to six months and one week of life (postnatal age range in days: 178-192). The participants who successfully completed the experimental session were 66 distributed in the 3 experiments as below:

1. experiment 3 (arm): 23 infants (12 males and 11 females) with a mean age of 184 days.
2. experiment 4 (bio-mechanical tool): 20 infants (13 males and 7 females) with a mean age of 180 days.
3. experiment 5 (mechanical tool): 23 infants (14 males and 9 females) with mean age equal to 185,28 days.

27 additional infants were excluded from the sample for technical problems (8), fussiness (8), because they did not attend the stimulation (4), for parental interference (1) or because outliers (6) in respect to the average of the total fixation time.

4. Stimuli

The stimuli employed in the three experiments were real colour video clips, identical to the ones used in experiment 1 and 2:

experiment 3: an arm which moves displaying its natural motion pattern (as in Exp-1);

experiment 4: a tool with *biomechanical-motion* (as in Exp.-2).

For the third condition a new stimuli was created by manipulating the video of the tool previously used:

experiment 5: a tool with *mechanical motion* (rigid and rhythmic).

In order to get the mechanical kinematics several frames were cut, duplicate and placed in a different order to confer the typical features of the nonbiological motion (rhythmic, rigid, constant). In this way the natural human features of the movement were eliminated conferring to the agent a "robotic" traits.

The only characteristic which differentiate the video used for these experiment from the ones employed before is the absence of the second target in both familiarization phase and test phase. The stimuli are illustrated in Figure 17.

Figure 17 Stimuli employed in experiments 3, 4 and 5

Apparatus, setting and procedure are identical to the ones engaged for the previous two experiments.

5. Variables/Coding

The variables measured on-line and off-line by the coders were again looking times towards each test events (new goal Vs. familiar goal). The mean reliability between on-line and off-line coding for 10% of of each sample of participants was analyzed by Cohen's Kappa.

Exp.1 : for fixation time was found a 91% of concordance, for the number of orientations was found an agreement rate of 84% between the two coders.

Exp.2: for fixation time was found a 96% of concordance, for the number of orientations was found an agreement rate of 95% between the two coders.

Exp.3: for fixation time was found a 96% of concordance, for the number of orientations was found an agreement rate of 91% between the two coders.

1. Analysis and Results

For evaluate the presence of a preference for one test event over the second one also in the three condition of the single target version paradigm, a series of t-test on paired-samples were performed on the infants' average looking fixation time and number of orientations towards the two stimuli. For the arm condition (Experiment 3) did not result a significant difference ($t_{(22)}$ = .117, p= .908) in terms of orientations between the new goal (average: 7.17) and the familiar goal events (average: 7.07). Likewise the looking time behavior did not differ ($t_{(22)}$= -685, p= .50), between the new goal (mean= 32914.78 ms, St.dev.= 11655.15 ms) and the familiar goal (mean = 34786.09 ms, St.dev.= 11632.20 ms). For the bio-mechanical tool condition (Experiment 4) did not result a significant difference in terms of orientations ($t_{(19)}$ = -.815, p= .426) between the new goal (average: 6.89) and the familiar goal events (average: 7.42). The analysis of fixation times revealed a lack of difference ($t_{(19)}$= -197, p= .846) between the new goal (mean = 36622.30 ms, St.dev.=

9220.89 ms) and the familiar goal (mean = 36088 ms, St.dev.= 9487.42 ms). For the last condition, the mechanical agent (Experiment 5) did not result a significant difference ($t_{(22)}$= .339, p= .738) in terms of orientations between the new goal (average: 8.31) and the familiar goal events (average: 7.86). A reliable difference in looking times ($t_{(22)}$= 2.628, p= .015), emerges, instead between the new goal (mean= 37031.30 ms, St.dev.= 9723.52 ms) and the familiar goal (mean = 30820 ms, St.dev.= 11821.04 ms). The examination of data compared by target object, color and pathway did not revealed any spontaneous preference or effect on the fixation times towards the stimuli. The results of the three experiment are illustrate in Figure 18.

Figure 18 ***Results of experiments 3, 4 and 5 of Study 2.***

Finally the three experimental conditions have been merged to execute a series of general linear models (repeated measures analysis of variance) on the three experimental samples, with the aim to check the presence of possible overall interactions or main effects. First of all the one Anova has been run considering the belonging condition as between-subjects factor and the fixation time towards the two test event (in milliseconds) as within-subject factor. The result denied the presence of a principal effect of the test event type (new goal Vs. familiar goal) along the conditions ($F_{(1,63)}$ = 1.162, p = .285) and also a lack of interaction *condition X event type* ($F_{(2,63)}$ = 2.641, p = .079). A second Anova was run to ascertain the influence of the external features of the agent (aspect) on the infants' pattern of responses. Data from the 4th and 5th experiments (agent= tool) were put together and

compared to the data of 3rd experiment (agent= arm). The analysis do not support the role of the aspect: assuming the event type as within factor and the appearance of the agent as between factor a no significant effect of the event type emerged ($F_{(1,64)} = .284$, $p = .596$) and the interaction *condition X event type* was not reliable ($F_{(1,64)} = 2.914$, $p = .093$) too. This data suggest a low salience of the aspect's features of the effector in infancy; responses to the test events -in term of fixation time- in six-month old infants do not seem to be highly influenced by the presence or not of humanoid perceivable external features of the agent. A last critical analysis has been execute to verify the role of the kinematics on infants' visual behaviour in responses to the two test events. The data of experiment 3 and 4 have been dropped together to compare biological motion to non biological motion (experiment 5) Assuming the event type as within factor and the kind of motion displayed (biological vs mechanical) as between factors a significant interaction *condition X test event* ($F_{(1,64)} = 4.913$, $p = .030$), supporting the previously exposed hypothesis of a strong influence of the kinematic patterns of the effector on the goal attribution process in 6 month olds.

Figure 19 *Average fixation time (msec) spent by the infants looking at the two test events comparing the biological kinematics to the mechanical kinematics).*

2. Conclusion

The second set of experiments (3-4-5) revealed a lack of evidence for the 2 biological condition (arm and tool) and a reliable effect only in the third experiment the one in which

the agent is a tool displaying mechanical motion properties. In the first two experiments infants looked equally at the two test events, while in the mechanical condition infant shown a novelty preference for the new goal.

3. Discussion (2nd Study)

In general the first part of the Study replicated the finding in literature about the ability of infants at around 6 months of age to interpret goal-directed action as intentionally based regardless the aspect of the agent per se. the presence of particular cues makes possible the attribution even on inanimate or non human agents. The results of the second part of study 2, all together demonstrated that infants at 6-months old regardless to the aspect of the agent are not able to read the action shown as intentional because of the absence of optional target. Indeed in both the first conditions (arm and biomechanical tool) infants looked equally the two test event, showing a lack of preference, the preference found in experiment 1 and 2, and due to the violation of expectation caused by the change of the agent's goal.

The lack of a cue about the agent's preference (outcome selection in familiarization) prevent the infant to build a representation useful to anticipate the future behavior of the agent. Actually the absence of a choice between two potential targets do not provide the infant of enough evidence to interpret the entity as an "agent" that is someone (or something) able to act under intentions on the environment, producing effects. Without initial choice infant could not generate reliable expectations and both the test events became "equiprobable". The results of experiment 2 (tool with human motion) could be also explained on the bases of a mismatch between appearance of the agent (non human) and its movement (biological, because driven by an unseen person). In support of this interpretation ("coherence hypothesis") Shimada (2010) using NIRS technique has shown that in healthy adults MNS activity is sensitive to congruency between appearance and kinematics of the agent. It is possible that infants resort to other perceptual cues -like the coherence- when they cannot explain the event using theirs inner repertory of experiences; so in that case they might have used perceptible and physical elements to understand the seen action. This hypothesis is in accordance with the *cue-based theory* and with the evidence shown by Biro and Leslie (2007) which displays how infants need more cues -for the goal attribution- only when the agent is non biological. Only in the condition in with aspect and motion properties are matched (experiment 3: mechanical tool) infants looked

longer at the new goal event than the familiar goal event. This critical result may be interpret in different way: the more direct interpretation is that infants recognize the mechanical tool as an agent and given that they attribute it goals and intention. A second interpretation in connected with the use of a critical paradigm: the violation of expectation method (VoE). This paradigm is not sensible in discriminate between the absence of goal attribution and the inability to generate an expectation. This argument could be applied on the lack of evidence emerge from experiment 3 and 4; but it makes sense also to explain the unattended result in the mechanical condition. Indeed if no human cues are provided, as in the third experiment, in which aspect an kinematics are somewhat coherent but unfamiliar for the infants. That may be sufficient to categorize the entity as non-intentional and in light of that representation the ability of a non-human "intentions-free creature" of execute an act with a novel end state (new goal) might me largely more surprising than seeing a the same effector (as mechanical device) slightly changes the modality of execution of the same act. These data, in accordance with the literature, uphold that the ability to understand other's intentions at six months old, maybe due to experience (Woodward, 1998), might be carried out by the presence of special context's cues (Biro, et al., 2007; Sommerville & Crane, 2009) or by particular agent's features (i.e. the kind of motion). On the basis of these assumptions it is important to consider not only the appearance of the agent in the study of infants' goal attribution and comprehension, but also the motion's features of the action and other possible cue (as the presence of the 2nd object) which contribute to the interpretation of the agent as biological or not and more or less "intentional".

2. STUDY 3: Expectations on goal directed actions based on a preference event

In light of the findings emerged by the previous study (2nd Study) the role of the preference cue in the goal attribution mechanism seems to be crucial in generating expectations about the future behaviour of an agent. The focus of the body of researches in this field has always been on the "positive expectations" generated by a previous experience, but in the context of goal-directed action in the typical Woodward scenario every active choice is accompanied by an antagonist passive choice, that is whenever the agent acts on one of two possible target is also omitting to perform an action on the second

possible target. Therefore when infants are processing events like these they may potentially build a bidirectional representation composed by a positive action (the choice) and a negative/or lack of action (the missing choice of the second target). It's well known that infants from 6 months old are able to represent an action as goal directed and to interpret events containing certain information (agent-action-goal) like intentional. In the literature, by the way, there are no studies focused on the interpretation of the avoidance, in terms of anticipation (see Higgins et al., 1997- Social-cognitive motivational system for self-regulation of goal pursuit- promotion vs prevention) or lack of action (omission). In the classical Woodward scenario all the investigations have been about the selection of one of two possible target and the expectation that this situation generate in the infants; using this paradigm is possible to evaluate how the babies perceive a novel event –test phase- in the same scenario: a new goal (the agent act in the same way but on the other target) or a new pathway (the agent act in a different way to get the same goal/target). What we know about how infants perceive a negative goal?

Starting from these consideration the aim of this part of the project is to verify the influence of the information modulated by the outcome selection presented in familiarization on the generation not only of positive expectations (i.e. the object more likely to be choose is the same on which the agent acted previously), but also of negative expectations (i.e. the object less likely to be chosen in future because ignored by the agent before).

1. Rational

Infants begin to interpret others' actions that they see as goal-directed around 6 months old. This skill is one of the forerunners of a more high level ability: to understand others' intention, desires, thoughts. This achievement is crucial in the social development, and that's the reason why is important to investigate how babies get these information from the very beginning. It's well known from the literature that infants at this age perceive an event in which an agent perform an action on one of two possible targets as an expression of the agent's will and preference across them. The way in which the researchers have investigated this early ability is using the looking times technique: in this kind of paradigm an event is shown (the agent's preference for one of the targets) several times in order to familiarize the infants to it and make them able to build a representation about the goal directed action; this representation allows them to have expectations about the future

agent's behavior (i.e. its goal). After the familiarization two test events are shown to the infant; these scenes differ each other's in terms of outcome: the familiar goal (the action's target is the same seen before) vs. a new goal (the action's target is new compared to the familiarization). From about 6 months old infants show surprise (increased looking times) when the agent displays a new goal, that suggests that their expectations about the agent's goal have been violated, the new goal event is in conflict with the representation which the infant has created according to his previous experience (familiarization). The unexpected scene brings infants' attention explaining why they look longer in this case.

These kind of everyday events in which there is a goal-directed action in a multiple choice scenario- always involves an explicit content and implicit content: the visible content is the "active" deed and its consistent positive outcome (i.e. the agent acts on the target A); on the other hand there is also a "passive" or omitted act and its lack of outcome that is a sort of "negative side of the goal" (i.e. the agent doesn't act on the target B). Using a modified Woodward's paradigm we want to investigate how they perceive the systematic avoidance of a target despite another one. The expectations that this event creates in the infant are only related to the effective goal ("chosen object") or are they able to represent also the negative persistence of the behavior? In other words we want to evaluate whether in infancy the goal's concept may be related also to negative tendencies (i.e. "the agent has selected this object because it doesn't want /like the other one"). In a goal-directed action which imply to choose a possible target there is also a "non-goal directed event", the representation of the "non-chosen target". Removing from the initial scenario the selected target, about which the infant built his/her expectation about the agent's future actions ("selectivity cue") and introducing a new possible target in the scene we have the chance to check how infants interpret the negative goal, that is, the target which has been ignored/avoided during the familiarization phase. The aim of the study is to understand if the infants' representation of goal directed actions does contain elements related to the passive/negative side of the preference (i.e. the unchosen object), if the infants are attending also the not preferred target in this kind of scenario. If infants are able to build a complex representation of this instance, they should generate not only positive expectations about the future agent's goal (most likely target: the familiar one) but also negative expectations (most unlikely target: the ignored one). Comparing the infants' responses towards a *completely new object* (that doesn't involve any goal's expectations) versus the responses towards a familiar but -according to their previous experience- not preferred object (negative goal's expectations).

2. Method

A modified version of the Woodward paradigm has been created in order to test the theoretical hypotheses described above. The revised version of the original scenario (Woodward, 1998) is inspired by a similar method employed in a study on the representation of negative events (omission) in 7 month-old infants (unpublished data). The study has been presented as a poster by Feiman, Cushman, and Carey (2011)[4]. The procedure consists of a familiarization phase in which the same act is performed by the agent on one of two possible targets over and over again (outcome selection) to allow the infant to create a representation of the agent's preference for one target over the other one. After the familiarization an orientation trial is presented: two target on the stage one is familiar (already seen in familiarization) and the second one is novel. Than the test phase begins and two events are shown to the infants: the agent acts on the familiar object either on the novel object. The present experiment consists of two conditions:

Experiment 5.1 : evaluation of infants' visual responses to a familiar expected (likely to occur) event compared to a neutral (no expectations) novel event.

experiment 5.2: evaluation of infants' visual responses to a novel unexpected (unlikely to occur) event compared to a neutral (no expectations) novel event.

- **Participants**

The whole sample recruited for the experiment was composed by 74 infants (42 males and 32 females; mean age: 230.04 days; st. dev.: 30.74), tested between five months and two weeks to nine months and two weeks of life (age range in days: 167-290). The participants who successfully completed the experimental session and met all the inclusion criteria are 41: 18 participants in control condition (experiment 3.1) and 21 in the experimental condition (experiment 3.2). 35 infants were excluded from the sample, respectively: for

[4] Feiman, R., Cushman, F.A., & Carey, S.E. (2011). Infants fail to represent a negative goal, but not a negative event. Poster presented at the Society for Research in Child Development Meeting, Montreal, Canada.

technical problems (10), change of state (9), because they did not attend the enough stimulation (14), or because consistent in outliers (in respect to the average) on the base of the total fixation time (2).

- **Stimuli**

The stimuli presented during the experiment were full colour video clips representing a person's arm which grasps one object, of the two present on the stage. The clips were life-size. In familiarization the agent grasps always the same object, even if the position of the target is switched in every trial. During the following orientation trial the new object is always presented with one of the familiar object, respectively the chosen one in control condition, and the un-chosen one in experimental condition. In the test phase the infants were shown the agent grasp the new object or the familiar object present in the orientation trial, the two event were sequential (the presentation order was randomized between subjects). Thus participants could see two alternative pair of test events according to the condition they belong to:

1. in control condition infants were presented with the actor grasping the previously chosen object (familiar goal- familiar object) and with the actor grasping the new object (new goal- new object);

2. in the experimental condition infants were presented with the actor grasping the previously un-chosen object (new goal- familiar object) and with the actor grasping the new object (new goal- new object).

Three object target were used: a puppet a cup and a ball, very similar in size, but easy to discriminate as belonging to different categories. Spaepen and Spelke (2007) provided evidence that infants are predisposed to represent reaching actions as directed to categories of objects rather than to specific object when target with similar features were employed. The ball was always the new object, the target introduced on the stage after the familiarization in place of one of the two familiar object. The puppet and the cup, instead, could be the chosen or the un-chosen object of familiarization (variable randomized between subjects). Each video has the same length and structure: at the beginning only the two objects are visible (2 seconds), than the agent comes on the scene from above, approached the target, and lift it up (3 seconds), the action is interrupted at the point of its

end state and the outcome phase begins: the last frame of the action keep to stay on the screen for a maximum of 30 seconds.

- **Apparatus and Setting**

All the infants have been tested in the DCNL babylab (Developmental Cognitive Neuroscience Laboratory) coordinated by Dr. Teresa Farroni, at the Developmental Psychology Department[5]. The equipment consists in a car seat facing a 32'' screen monitor (distance between babies eyes and the monitor was between 60 and 70 cm), where above is located a camera to record infants' eye movements. Behind the screen there is a curtain to divide the participant from the experimenter's area. Out of baby's sight, there is the computer which manage the experiment's phases, a second screen to monitor the infant's behaviour and a mixer to record baby's gaze and stimuli's presentation at the same time. The session is started by the experimenter via computer (the experimental script is run with the software E-prime 2.0).

[5] University of Padova

- **Procedure**

Once the infant is placed on the car seat looking at the screen, with eyes aligned to the centre of the screen the experimenter started the familiarization phase. The presentation of the two movie (showing the agent taking the same object, but in different location) is repeated twice. The maximum duration of the video is 35 seconds. The presentation of each video continues until its end unless the infant meet the following criteria: to make a disengage (look away) of more than 2 seconds, but only after have seen at least the 66 % of the action (2 s out of 3s) and at least 1 second of outcome phase (still frames). The orientation phase only require the infant to look at both the objects, the presentation goes on until the end of the clips (35 s) or the occurrence of a disengage longer than 2 seconds. The presentation on the two test events is repeated twice in a sequential order.

- **Variables/Coding**

The variable measured for the current study are the looking time behavior expresses in number of orientations and fixation time (in milliseconds) addressed to the test events (4 clips, 2 types of event) only during the outcome phase. The coding of the variable has been executed by two coders blind to the hypotheses of the study. The mean reliability between off-line codings for 10% of the whole sample of participants was analyzed by Cohen's Kappa: for fixation time 97% of concordance, for the number of orientations 95% of agreement. The coders computed the fixation time and number of orientation toward the two test events globally (the whole scenes) and partially (related to subcategories of the stimuli). The looking behavior has been computed also for a) time sent looking the new object compared to the familiar object; b) time spent looking the at the dynamic portion of the scene (action) compared to the static portion of the scene (omission); c) time spent looking at the chosen object compared to the unchosen when the object is new or when is old.

1. Analysis and Results

The analyses of data for the current study are subdivided in three analytic levels:

1. event: the infants' looking behavior oriented to the whole test scene (new goal vs familiar goal);

2. matched portions: the infants' looking behaviour adressed to categories of stimuli across the test events (new object Vs familiar object; action Vs omission). To obtain these data the fixation time recorded for each part of the scene (left or right) was added up with the portions of the other scene. In example the omission is the total amount of time spent by the infant in looking the portion of the scenes in which the action is not occurring (omission's fixation time in new goal event + omission's fixation time in familiar goal event).

3. Single portions: infants' fixation time toward the single portions of each test scene, taken individually (i.e. chosen-new-object, unchosen-new-object

condition		Media	Deviazione std.
control	ms_grNEW_event	13460,00	7374,541
	ms_grOLD_event	11780,00	4826,305
	vc_grNEW_event	11,33	6,928
	vc_grOLD_event	10,56	4,355
	rate-fix_grN_event	,5183	,12944
	rate-fix_grO_event	,4817	,12944
experim.	ms_grNEW_event	16117,14	9142,871
	ms_grOLD_event	15395,71	10695,892
	vc_grNEW_event	12,67	8,656
	vc_grOLD_event	14,10	10,963
	rate-fix_grN_event	,5243	,15449
	rate-fix_grO_event	,4757	,15449

To verify the presence of a preference for one of the two test events (new goal Vs. familiar goal) a series of paired-t-tests on were carried out on the infants' mean looking fixation time toward the two stimuli. First of all the data have been examined under the event level (responses to the whole scene). A t-test on the entire sample split by conditions revealed the lack of significance in average fixation time (ms), number of orientation (visits count, VC) and rate of fixation (fix-rate) between the two test events in both the conditions .

Table 6

Given the wide range of age tested, the absence of differences might had been due to opposite trend in infants' responses between younger and older babies. For this reason the sample was divided in two range of age (Table 7).

Crosstabulation: condition * age_ranges				
condition		age_ranges		Total
		5.3 to 7.2 (168-225 days)	7.2 to 9.2 (226-283 days)	
	control	9	9	18
	experimental	10	11	21
Total		19	20	39

Table 7

Performing the a paired t-test on the dependent variables of EVENT (ms, Vc, and fix-rate), this time split by age ranges, emerged a significative opposite trend between younger and older infants: the younger looked more at the *new goal event* (t_{18}= 2.374, p= .029), whereas the older looked more at the *old goal event* (t_{19}= 2.374, p= .024). A paired t-test on rate of fixation time toward the test events, grouped by age and condition shown a difference in younger infant responses (between the two events) to the experimental condition: younger infants look longer (t_8, p= .033) the new event (neutral event) while the older infants look longer (t_{10}, p= .032) the grasping action on the familiar object (i.e. unexpected event).

Two additional t-test on event's variables, split by order and split by chosen object in familiarization confirmed that there are no reliable differences in the infants' responses related to these variables. Finally a repeated measures GLM has been conducted. Events' measures were the within factors, and ages ranges and condition the between factors revealed a within subjects effect event X age range ($F_{3,33}$= 4.788, p= .007). About the matched portions of the test event scenes, there is a general preference (considering the whole sample, all the ages, all the conditions) to look longer the new object than the old one (t38, p= .005) and the active portion of the scene rather than the side in which nothing is happening (t38, p= .0007). Checking for this aspect in different condition and age group, appears clear that the fixation time oriented to ACTION is greater than the fixation time toward OMISSION in both the age groups but only in experimental condition, while the object NEW is preferred to the OLD object only for younger infants.

Descriptive Statistics

condition		N	Mean	Std. Deviation
control	ms_grNEW_event	18	13460,00	7374,541
	ms_grOLD_event	18	11780,00	4826,305
	vc_grNEW_event	18	11,33	6,928
	vc_grOLD_event	18	10,56	4,355

experim.	ms_grNEW_event	21	16117,14	9142,871
	ms_grOLD_event	21	15395,71	10695,892
	vc_grNEW_event	21	12,67	8,656
	vc_grOLD_event	21	14,10	10,963

1. Discussion

The results of the experiments 3.1 and 3.2 shown a different pattern of responses between the two range of age investigated in the fixation times toward the two compared event. In both conditions infants saw as new goal the action performed on the just introduced novel object in respect to, none expectations could have been created yet. In the control condition the neutral event is compared with an expected event according to the infant's previous experience indeed, the target and the action have been already seen by the infant (familiarization). The data shown that infants look equally at the two test events in control condition, indicating that the two event are considered with the same probability to occur. The absence of violation of expectations is consistent in both the age group; this results is in line with evidence coming from the single target paradigm. Even if our stimuli contain two targets, these targets are not the two presented in familiarization, so the pattern of responses elicited is similar to the one obtained when a second possible target is introduced on the stage only after familiarization (see Hernik and Southgate, 2012) . On the other hand in the experimental condition we compared the neutral event with an unexpected event: the agent grasp a perceptually familiar object, but not experienced before as agent's goal. In this condition both the age group shown a novelty preference for one of the events over the other, but the trend in opposite among younger and older infants. As expected older infants find more surprising the unexpected event, while younger infants looked longer at the neutral one. The results in infants over 7 months old suggest that at this age

they are able to build positive and negative expectations on the base of the previous experience: the familiarization convey several behavioral cues about the will of the agent. On the contrary before 7 months of age the interpretation of children in information-deprived context -such the one employed- is strictly founded on low-level cues: the neutral event perceptually is the more salient because contain a major amount of perceivable novelty (new object plus new goal) compared with the unexpected event that contains a familiar object.

Chapter 3. EARLY BIOMARKERS IN INDIVIDUALS AT HIGH-RISK OF AUTISM SPECTRUM DISORDERS

Introduction

The focus of this project is the development of Social brain, therefore in order to deepen the knowledge about the social cognition, it is important to study the developmental

disorders which may affect that area. The autism fit into this group of disorders. Autism disorder until now has been defined as characterized by social deficits, communication deficits, and restricted, repetitive, and stereotyped patterns of behaviour (American Psychiatric Association, 2000). A revision into a single diagnostic category – the *Autism Spectrum Disorder* (ASD[6])- will be proposed in the next *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, under review, due on May 2013), which will be soon released; the manual will adopt a dimensional approach to diagnosing disorders that fall underneath the autism spectrum umbrella[7]. The innovative categorizing includes collapsing social and communication deficits into one domain, so the ASD diagnosis will be described in terms of severity of symptoms in the following domains (see ***Errore. L'origine riferimento non è stata trovata.***):

1. social communication (SC),
2. fixated/repetitive or restricted behaviors or interests (RRBs)

These two deficits spheres were vaguely roughed out already one century ago (1912), when the term "autism" [8] was conied by the psychiatrist Paul Eugen Bleuler, who described it as a schizophrenic symptom. Long before the autism was named, however, several cases displaying this set of symptoms have been documented, among which the most popular is likely the "Wild Boy of Aveyron"[9]. About three decades later the term creation, Bleuler's terminology was adopted by Hans Asperger, his research was focused on a particular condition observed in a group of children: they were showing the autistic behaviour, but the linguistic deficits (Asperger Syndrome). Meanwhile, working separately, another psychiatrist, Leo Kanner, was studying the same general disorder, and only in 1943 he began using this word referring to its modern meaning, as we know it today (Kanner, 1968)[10]. Despite the long history of the autism, nowadays still very little is known about its causes and functioning, but in the last two decades many progresses have been done in this direction.

Moreover researchers are capitalizing on autism investigation, with the intent to increase the scientific knowledge on both typical and atypical development. Assuming the social

[6] This acronym will be used in place of "Autistic Spectrum Disorder/s" in the rest of the dissertation.
[7] Set of neurodevelopmental disorders included in ASDs definition: autistic disorder, pervasive developmental disorder-not otherwise specified [PDD-NOS] and Asperger syndrome.
[8] from the Greek *"autos"* which means "self" and *"ismos"* which means state of being: state of being absorbed by one's self.
[9] Documented by Jean Itard in *Mémoire* et *Rapport sur Victor de l'Aveyron* (1801, 1806)

nature of core impairments in ASDs, the condition could be a model syndrome through which investigate social perception, cognition and behavior. Schultz (2005) argues that the social deficit is the only one that diagnostically differentiates ASDs from other neurodevelopmental disorders. For this reason may be crucial to shed light on the neural basis of social dysfunction to identifying autism's causes. Its early onset and familial pattern strongly suggest a biological basis, and, in fact, there are now substantial data implicating brain based as well as genetic mechanisms. One critical issue is the diagnosis timing due to the lack of evident early signs. In example the absence of language, typical in a group of ASDs, is not observable in the early months of life, does not make possible a diagnosis before 2-3 years old. For these reasons it seems to be necessary to detect the earliest behavioural and neurophysiological manifestations of ASD. The search for the precursors of the disorder (also named *biomarkers*) is motivated by theoretical and practical benefits: a) the understanding of the nature ASDs, and the neurodevelopmental processes underlying it; b) an effective screening leads to earlier diagnosis which translates into a timely intervention and therefore into a better prognosis for the individual. ASD is a subclass of the recently defined *Broader Autism Phenotype* (BAP), which describes individuals having autistic-like traits (or an higher biological risk to show ASD), but who might not have autism. To study individuals from this wider group (BAP) might be the new direction to comprehend the origins of this developmental disorder and to trace the earliest brain and cognitive signs of autism. In the following paragraphs the topics above mentioned will be described in more details.

Proposed DSM-5 criteria for autism spectrum disorders
An individual must meet criteria A, B, C and D:

[10] Original paper published on *Nerv Child* in 1943, later reprinted in *Acta Paedopsychiatr.* in 1968.

A. Persistent deficits in social communication and social interaction across contexts, not accounted for by general developmental delays, and manifest by all 3 of the following:
1. Deficits in social-emotional reciprocity; ranging from abnormal social approach and failure of normal back and forth conversation through reduced sharing of interests, emotions, and affect and response to total lack of initiation of social interaction.
2. Deficits in nonverbal communicative behaviors used for social interaction; ranging from poorly integrated- verbal and nonverbal communication, through abnormalities in eye contact and body-language, or deficits in understanding and use of nonverbal communication, to total lack of facial expression or gestures.
3. Deficits in developing and maintaining relationships, appropriate to developmental level (beyond those with caregivers); ranging from difficulties adjusting behavior to suit different social contexts through difficulties in sharing imaginative play and in making friends to an apparent absence of interest in people
B. Restricted, repetitive patterns of behavior, interests, or activities as manifested by at least two of the following:
1. Stereotyped or repetitive speech, motor movements, or use of objects; (such as simple motor stereotypies, echolalia, repetitive use of objects, or idiosyncratic phrases).
2. Excessive adherence to routines, ritualized patterns of verbal or nonverbal behavior, or excessive resistance to change; (such as motoric rituals, insistence on same route or food, repetitive questioning or extreme distress at small changes).
3. Highly restricted, fixated interests that are abnormal in intensity or focus; (such as strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interests).
4. Hyper-or hypo-reactivity to sensory input or unusual interest in sensory aspects of environment; (such as apparent indifference to pain/heat/cold, adverse response to specific sounds or textures, excessive smelling or touching of objects, fascination with lights or spinning objects).
C. Symptoms must be present in early childhood (but may not become fully manifest until social demands exceed limited capacities)
D. Symptoms together limit and impair everyday functioning

Table 8 New criteria for autism spectrum disorders according to the APA(under review)[11].

1. Recurrence and causes of ASDs

Epidemiology

The prevalence[12] for unspecified Pervasive Developmental Disorders (PDDs) has been declared of about 30/10,000, including: autistic disorder (AD), Asperger disorder (AS), PDD not otherwise specified, and CDC, the childhood disintegrative disorder (Charman,

[11] American Psychiatric Association, due on May 2013.

2002; Fombonne, 2009). Another statistic survey executed by the Centers for Disease Control and Prevention report an estimated prevalence of 1 to 500 for a strict diagnosis and 1 in 150 using broader diagnostic criteria (CDC, 2007). With regards to incidence[13], instead, recent studies using updated diagnostic criteria and differing methods from multiple countries have identified rates ranging from 2.0 to 12.0 per 1,000 children with "best estimate" rates ranging from 2.0 to 6.0 per 1,000 children. Autistic disorder has increased in recent surveys and the current estimated prevalence is around 20/10,000; probably the reason for that increase are: the broadening of the concept, the expansion of diagnostic criteria, the development of services, and an improved awareness of the condition, but others unknown factors might have also contributed to that trend (Fombonne, 2009; Rutter, 2005). Although autism was once considered to be relatively rare, striking new prevalence above-mentioned have prompted the CDC and the World Health Organization to declare autism a public health crisis. ASDs are reported to occur in all racial, ethnic, and socioeconomic groups, but the gender ratio is highly skewed, with about 80% of affected individuals being male[14]. A substantial influence on estimation of ASD recurrence is also represented by the "stoppage", the phenomena consisting in the tendency of families with an affected child to decide not to have any more children. The main effect of the reproductive stoppage is an underestimation of the proper rate of neurodevelopmental risk in the later born siblings (Jones & Szatmari, 1988; Ozonoff et al., 2011) . For all these reasons comes the urgency to shed light on this set of disorders in order to determine the still unclear etiology to date.

Etiology

The origins of ASDs are mainly unknown, for long time it has been hypothesized to exist a common cause on a genetic, neurologic and cognitive level, but today researchers are always more convinced that to an heterogeneous set of impairments corresponds a series of causes (*Happé & Ronald, 2008).* Autism is likely the result of a variety of causes among which familial, infectious, neurologic, metabolic, immunologic, and environmental. The etiopathogenesis of autism is complex and still not fully understood, but there is a rich corpus of research supporting –by indirect evidence- a strong genetic contribution in the condition's emergence. Studies on monozygotic twins show a concordance of about 60%

[12] Prevalence refers to the number of existing cases in a defined group of people during a specific time period.
[13] Incidence refers to the rate of new cases of the disorder within a period of time (usually a year).
[14] ASDs are almost 5 times more common among boys (1 in 54) than among girls (1 in 252).

with an estimation of 90 % of heritability (Steffenburg et al., 1989; Folstein & Rutter, 1977; Bailey et al., 1995; Le Couteur et al., 1996; Szatmari et al., 1998 [15]) .

Figure 20 *Different models for how single or multiple genetic and/or environmental risk factors in infancy lead to ASD behavioural outcomes in childhood. From Elsabbagh & Johnson, 2010.*

Although studies on genetic etiology of ASD have proved a very hight rate of heritability (even over 90%) the phenotypical expression of the condition might be determinated by aggregate biological (pre-determined) and non-biological constraints, whose interaction makes possible reaching the threshold (ASD manifestation). The compounded effect of genetic and environmental risk factors -over the development- can determine the resulting phenotype, the multi level interaction (biological X non-biological) lead to variable developmental trajectories, not otherwise predictable (*see* **Errore. L'origine riferimento non è stata trovata.**).

2. Diagnosis of ASDs

Clinical assessment

In order to evaluate a case of autism the specialists have to review the anamnesis, development, cognitive and communicative degree according to the cronological age and integrate these information with the observation of possible symptoms. The diagnosis of Autism Spectrum Disorder is mainly based on the detection of behavioral atypicalities manifestation, given that as to date it has not yet been found a reliable biomarker. The behavioral assessment is made by mean of standardized diagnostic instruments built on the

[15] as cited in Losh, 2008, p. 829.

inclusion criteria presented in the two main diagnostic manuals (DSM-IV16 and ICD-1017). There are numerous assesment tools used for the clinical evaluation of autism (for a review see Lord & Corsello, 2005) , the ones used by the majority of the specialists are presented below (see

Table **9**). Despite the large number of instruments it has recently established a strong consensus among academics on the use of two instruments for scientific aims: the Autism Diagnostic Observation Schedule (ADOS; Lord et al., 2000) and the Autism Diagnostic Interview-Revised (ADI-R: Lord et al., 1994), respectively, a structured observation protocol of the individual and a semi-structured interview adressed to the caregivers of the individual suspected to have an ASD.

Critical Diagnosis' timing/Autism's Diagnosis Issues

ASD is usually recognized in the preschool stage, but the knowledge achieved nowadays, should allow experts to make a diagnosis by age two.. Many studies show that usually the age in which individuals receive the diagnosis is four years old or later (ADDMNS[18], 2007; Shattuck et al., 2009; Yergin-Allsopp et al., 2003)[19]. Regardless the heterogeneity of primary symptoms, autism condition is distinguishable from other developmental disorders by virtue of the core deficits in social functioning.

Instruments' category	Assessment tool	Coding method
SCALES MEASURING CORE DEFICITS	**Social Responsiveness Scale** [SRS] (Constantino,2002)	Partents/Caregivers/ teachers questionnaire
	Pervasive Developmental Disorders Rating Scale [PDDRS] (Eaves, 1990)	partents/caregivers/ teachers questionnaire
	Children's Social Behavior Questionnaire [CSBQ] (Luteijn, et al., 2000)	Partents/Caregivers questionnaire

[16] APA (1994). Diagnostic and statistical manual of mental disorders (DSM-IV).

[17] WHO (1992). International statistical classification of disease and related health (ICD-10).

[18] Autism and Developmental Disabilities Monitoring Network Surveillance Year 2002

[19] As cited in (Barton, Orinstein, Troyb, & Fein, 2013) .

RATING SCALES	**Childhood Autism Rating Scale** **The Childhood Autism Rating Scale** [CARS] (Schopler, Reichler, & Renner, 1986)	Rating scale based on history, oservation and report (therapists and clinicians)
	Revised Behavior **Summarized Evaluation**[BSE-R] (Barthelemy et al., 1997)	Rating scale based on behavioral observation (therapists and clinicians)
	Autism Behavior Checklist [ABC] (*part of* ASIEP; Krug et al.,1980)	Caregiver rating
	Gilliam Autism Rating Scale [GARS] (Gilliam, 1995)	Parents questionnaire
DIAGNOSTIC INTERVIEWS	**Autism Diagnostic Interview-Revised [ADI-R]** (ADI: Le Couteur et al., 1989; ADI-R: Lord et al., 1994).	semi-structured, investigator based interview for caregivers.
	Diagnostic Interview for Social and Communication Disorders [DISCO] (Wing et al.,2002)	semi-structured interview
DIRECT OBSERVATION SCALES	**Autism Diagnostic Observation Schedule**[ADOS] (Lord, Rutter, DiLavore, & Risi, 1999; Lord, Risi, et al., 2000).	structured protocol for the observation of social and communicative behavior.
	Psychoeducational Profile-Revised [PEP-R] (PEP: Schopler & Reichler, 1979; PEP-R: Schopler, Reichler, Bashford, Lansing, & Marcus, 1990),	Structured protocol for the observation of 7 developmental areas.
DIAGNOSTIC & BEHAVIORAL ASSESSMENT	**Communication and Symbolic Behavior Scales, Developmental Profile** [CSBS DP] (Wetherby & Prizant, 2002).	Structured observation and parental reports (6 to 24 mos.)
	Children's Communication Checklist [CCC] (Bishop; 1998)	Checklist adressed to parents/ teachers/ therapists/ clinicians

Table 9 *Most used assessment instrument for ASDs diagnosis. Built according to* (Lord & Corsello, 2005) .

The symptoms expression is very often readily detectable during a face-to-face interactions with children and adults; conversely, to date, in early infancy there are not effective and reliable indicators -for clinicians- to establish the presence (or the later emergence) of ASD in individuals. Numerous symptoms which are present from birth may go undetected for may months. The condition is currently detected on the base of the presence of peculiar behavioural features which may take very different form in infancy, moreover the most popular assessment tools for the screening of ASD have been validated for children

starting from around 18 months, the field addressed to the building of new instruments for the detection of ASD in early infancy is still constantly evolving. reports suggest that the diagnostic strategies that are effective in 3-year-olds might not be as effective for those under 30 months of age (Turner & Stone, 2007). One of the challenge in detecting social deficit in ASDs is the need to spot a lack of behaviors (i.e. minor frequency) rather than the presence of atypical activities, easier to observe. Often these deficits are masked by parental support. The importance of a systematic screening for ASD lie on the idea that an early identification may permit to begin sooner treatments and interventions which really could make the difference in terms of developmental trajectories (brain plasticity), altering the atypical pathway and preventing the emergence of the full syndrome, that means more positive short-term and long-term outcomes (Dawson, 2008; Rogers, 1996; Rogers & Vismara, 2008).

3. Biomarkers of ASDs

During the last decade a new enhancing field of research has developed: the seek for biological warning indicators suitable to being used as marker of Autism Spectrum Disorders. In 2012, Guinchat and colleagues have published a complete review on the very early risk factors for autism. The topic is developed classifying the biomarkers in term of period of appearance: before, during or after birth. The analysis took in account 85 previous selected work published since 2007 to 2011 responding to the following search criteria: 'autism' in combination with 'prenatal' or 'perinatal' or 'pregnancy' or 'neonatal' [20] (see

Table 11). From the meta-analysis emerged that the principal prenatal risk factors closely associated with the ASDs are familial or related to the pregnancy; among which advanced parental age; and health problems or stress during the pregnancy. With regard to the perinatal risk factors the strongest associations reported through the survey are the gestational age and problems during the delivery. At last, the major postnatal risk factors documented are related to situations of adverse birth conditions as low Apgar index, low birth weight and/or atypical growth and brain disease such as encephalopathy (for a summary see **Table 10**).

[20] Guinchat et al. (2012), p.289, Figure.1.

Risk factors	Studies reporting most robust associations between autism and the following pre peri- and natal risk factors
FAMILIAL FACTORS	Parental age; parity; mother born abroad.
MATERNAL PREGNANCY FACTORS	Bleeding; pre-eclampsia
DELIVERY FACTORS	Breech presentation; Scheduled caesarean; Small for gestational age
BIRTH WITH ADVERSE CONDITIONS	Prematurity; low Apgar; Hyperbilirubinemia; Low birthweight/slow growth; Encephalopathy; Birth defects

Table 10 *Major risk factors for autism reported in the period 2007-2011.*
Table adapted from Guinchat et al., 2012.

Nevertheless, several papers have stressed the hypothesis that only few biomarkers (between the many detected) could have an impressive impact in the genesis of the disease: anatomical and functional brain abnormalities in early infancy. Courchesne and Pierce (2005) in a review described in detail, as more reliable risk factors of autism, the following biological signals:

1. reduced Head Circumference (HC) at birth;
2. abnormally accelerated brain growth in the first months of life;
3. premature cessation of further brain growth.

Head circumference and patterns of growth

Many authors have investigated the growth parameters (e.g. Courchesne et al., 2003; Dawson et al., 2007; Muratori et al., 2012; Mraz et al., 2007; Webb et al., 2007; Whitehouse et al., 2011; Rommelse et al., 2011) proving an high rate of macrocephaly in ASDs than in the general population. The reason of this interest is, above all, because standardized value across the general population already exist (i.e. WHO Child Growth Standards), and secondly because to record and monitoring these values is relatively simple and non-invasive for children. The meaning of this field of research is the attempt to detect precocious warning biomarkers and link them to future brain abnormalities. The first study in this direction has been conducted by Courchesne and colleagues (2003): they

found two phases of brain growth abnormality quite efficient (present in the 60% of ASD sample)to consider the clinical onset of autism. From analysis came to light a reliable difference in birth HC (smaller) of infants with ASD compared with the normative data, then a faster and excessive growth of head size between 1 and 2 months and between 6 and 14 months.

Although the biological cause of the brain volume increase remains unknown there are several possible interpretations according to the author (2003):

excessive numbers or rates of growth of neurons and/or glial cells, excessive number of minicolumns, excessive and premature expansion of dendritic and axonal connections, and/or premature myelination. The causes also remain identified and could reflect an abnormal acceleration of postnatal growth processes or a failure of late prenatal and early postnatal regressive processes. The brain volume increase could also reflect either aberrant compensatory responses to adverse prenatal conditions or deviant biological mechanisms that are first expressed in early postnatal life. (p.341-342)

Other growth parameters, such as height and weight, have been examined in infancy and early childhood, supporting the idea that children with ASD, compared to infants with typical development, are significantly longer and/or heavier, but this evidence are still debatable (e.g. Mraz et al., 2007; Muratori et al, 2012).

Early Brain Abnormalities

A very supported hypothesis argues that few anatomical structures and disrupted neural circuits are involved in the ASD emergence, among which, frontal lobe; cerebellum; and limbic system (especially amygdala and hippocampus).These early brain anomalies could be really injuring whether they take place during a developmental critical period. Brain atypical functioning might be closely connected with the rapid and atypical brain growth, explained before. Variation in head growth during infancy leads to both functional and anatomical effects, which could explain the variability in trajectories of symptoms (Lewis et al., 200821). Moreover there are controversial evidences of both over- and under-connectivity in adults with ASD (e.g. Murias et al., 2007 21; Belmonte et al., 2003; 2004), phenomena which might be interpret as a consequence of an unexpectedly chaotic and rapid brain development which creates too many non-adaptive connections (Courchesne et al., 2003) and/or too less functional neural network.

[21] As cited in Elsabbagh et al., 2010.

Prenatal Risk Factors	
FAMILIAL FACTORS:	Advanced maternal age ; Advanced paternal age; Mother born abroad; Parity;
PREGNANCY FACTORS:	Diabetes; Bleeding; Psychotropic drugs; Pre-eclampsia

2. Perinatal risk factors	
GESTATIONAL AGE	Preterm birth; Post-term birth
DELIVERY-RELATED RISK FACTORS	Breech presentation; Induced labor; Precipitous labor; Prolonged labor; Cesarean section, Scheduled caesarean, Meconium; Fetal distress.

Post-Natal risk factors	
BIRTHWEIGHT & GROWTH	Low birthweight; Lower birthweight; Small for gestational age; Head circonference; Apgar;
MARKERS OF HYPOXIA	Lack of first cry, breath or oxygen; blue baby; Respiratory distress syndrome or assisted ventilation or asphyxia.
OTHER SPECIFIC CONDITIONS AT BIRTH	Hyperbilirubinemia; Neonatal encephalopathy; Birth defects; Neonatal or congenital infections.

Table 11 *Risk factors associated with autism [a)prenatal; b) peri-natal; c)post-natal] . Variable took in account for the meta-analysis (period 2007-2011) reported in Guinchat et al., 2012.*

One reason for the presence of opposite interpretation is the multi-level meaning of the word "connectivity" (Belmonte et al., 2004). There is a local connectivity, that is within neural region, and a "long-connectivity, between functional brain areas but it also be classified as physical (synapses) and computational (information transfer). In the "autistic brain", likely due to perturbations in the synaptogenesis/pruning processes, different category of network seem to develop together, but with an opposite trend: long-range under-connectivity and local overconnectivity; moreover there is an excessive physical at the expense of computational connectivity which might be responsible for the difficulties in discriminating noise from signals (see Figure 21).

Figure 21 Selected from Belmonte et al., 2004. *Potential effects of network connectivity patterns on brain activation. on the left (normal brain): strong local connectivity and selective long-range connectivity, this computational structure guarantees an efficient information transmission and a functional activation during a visual task (red spot: activated regions).In the network on the right ("autistic brain"): strong connection between subregions is not differentiated, and computationally meaningful long-range connections fail to develop leading to an excessive and localized activity.*

About anatomical differences, have been documented also an enlarged cerebral white and gray matter (Courchesne et al., 2001; Hazlett et al., 2005; 2011; Schumann et al., 2010) in infants with ASD, especially in the frontal lobes (Carper et al., 2002), with atypical patterns of development in the first years of life and the amygdala appear larger in size as well (Schumann et al., 2010).

All together, these neurological discoveries may indicate an atypical brain development in children with ASD, but these biomarkers taken individually are not enough "reliable, sensitive or consistent" across the autistic population to be used as screening method (Elsabbagh et al., 2010).

4. Neurobiology Of Autism

In order to understand which brain substrates are involved in the social skills and how do they work in early stages of development, is necessary to investigate the neural correlates of the social brain both in adults and in clinical populations. Indeed, the study of atypical development can tell us more about the typical development.

Since the proliferating in literature of evidence about abnormalities in the brain of individual with ASD, different theoretical models has been introduced. According to Schultz (2005) the neurobiology of ASDs it can be summarized through two hypotheses on the origin of the disease (damage against single Vs multiple neural circuits):

an initial injury "attacks" numerous independent brain systems and provokes various impairments which get to the autistic phenotype expression;

an early damage affects a unique or a couple of neural systems, altering, through the development of the system, the typical trajectory and giving rise to several symptoms.

Even though the full manifestation of the disease in later childhood and in adulthood implies deficits related to multiple systems, the original state is still unclear. Whether the first stage is a limited insult or a multiple systemic brain disturbances the full syndrome manifestation is reached through an evolving process which negatively modifies the experiences responsible for "modeling" neural basis and behavior (theory called "*Big Bang Model"* , Schultz, 2005). An indirect proof of the role of early brain damage on the etiopathogenesis of autism is brought about by Badawi (2006) who has underlined a strong association between neonatal encephalopathy (NE) and the development of ASD : infants "hit" by NE are about six times more likely manifest autism than control. One of the first neurobiological interpretation of the Autism Spectrum Disorder is the Amygdala theory, introduced by Baron-Cohen (2000), based on the set of data supporting the abnormal size and functioning of the amygdala region (evidence from: post-mortem; animal model; similarity with patients following amygdalotomy; comorbidity with tuberous sclerosis; structural and functional neuroimaging). Mark Johnson (2005) has postulated another model which fits for typical development as well as for neuro-atypical prospective of autism. According to this model ("subcortical route") the autistic disorder generates from abnormalities in cortical and subcortical regions associated with the 'social brain'. One of the possible consequences is a visual processing less tuned to the low spatial frequencies (LSFs), that means a minor activity in subcortical pathway. The resulting lack of specialization of the cortical circuits for high spatial frequencies (HSFs) that causes a bias towards featural rather than configural face processing, documented in the individuals with ASD (for more details on face processing in autism see Chapter 7).

5. Broad Autism Phenotype

The clinical studies on families including at least one ASD proband have documented among relatives a phenotype similar in quality to the defining traits of autism, but much milder in expression. This set of features occurs more frequently among unaffected relatives of autistic individuals than controls and reflects autism symptom areas (*see*

Table 12). As Molly Losh presents in a recent review, in order to better grasp the genetic and neurobiological etiology of ASDs it has been proposed a novel methodological approach: the investigation of the subclinical markers of the disease.

LANGUAGE IMPAIRMENT	1.	Delayed acquisition
	2.	Narrative and/or pragmatic language deficits
SOCIAL FUNCTIONING	1.	Delayed social behavioural development
	2.	Socially reticent, undemonstrative personality, few reciprocal friendships
RESTRICTED INTERESTS AND BEHAVIORS	1.	Rigid/perfectionistic personality
NEUROCOGNITION	2.	Social-cognitive impairment
	3.	Executive control deficits
	4.	Featural processing bias

Table 12 *Summary of Broad Phenotypes Identified in Relatives (from Losh, 2008)*

These markers are also named "endophenotypes", they include indicators on a behavioural, psychological, and neuropsychological level but not only. The underlying hypothesis is that clinical outcomes of ASD might be deeply comprehend through the study of these indicators, which are present among both affected and unaffected individuals (e.g. Losh, M., 2008; Dawson et al., 2002; Pickles et al., 2000). Evidence of an intermediate phenotype in autism was first reported in 1977 in a work by Folstein and Rutter, in which the examined language and cognitive abilities in twin pairs (monozygotic [MZ] and dizygotic [DZ]) wherein only one of them was diagnosed as autistic. The authors found similar cognitive and language pattern in the unaffected individuals. The presence of a broadly defined phenotype in the unaffected twin was remarkable higher in both MZ and DZ sibling groups (MZ= 82%; DZ= 10%). Family studies as the one described above (see Bailey *et al.*, 1996 for a review) have shown that the genetic liability for ASDs makes the close relatives of the proband at higher risk (than individual without familial history of ASDs) of developmental disorders (especially PDD) as well as for indirect associations of deficits: in social communication or in interests and behaviors. Sub clinical behavioral features of the autistic syndrome have been observed in the unaffected relatives of children with autism (Piven et al., 1997; Szatmari et al., 2000; Sung et al., 2005; Constantino et al., 2006), as have event-related potential (ERP) abnormalities (Dawson et al., 2005), functional magnetic resonance imaging (fMRI) variations (Dalton et al., 2007), and a variety of psychological and personality traits that are similar in nature to the phenotypic

characteristics of autism (Murphy et al., 2000; Losh et al., 2008). The expression `Broader Autism Phenotype' (BAP) defines this range of "pseudo-deficit" exhibited by individuals who do not meet the diagnostic criteria (Fombonne et al. 1997; Piven et al., 1997). The genetic susceptibility might be expressed in unaffected relatives of people with ASD , who exhibit phenotypic features of psychological functioning milder but qualitatively similar to defining features of autism (Folstein & Rutter, 1977; see Piven, 2001 for a review). Studies of such subclinical phenotypes among relatives have confirmed that BAP's features parallel the core symptom domains in autism, but subtle in expression and not usually associated with any functional impairment (Piven J.,2002). Whereas autism involves impairment across the three classical symptom domains (social interaction, communication and behavioural patterns); evidence suggest that these features may decouple and segregate independently in relatives without autism (Losh, et al., 2007; Piven et al., 1997); in fact they appear to be uncorrelated in neurotypical populations (Happé et al., 2006). In literature many studies show that performance patterns in unaffected relatives are similar to the patterns of impairments in ASDs in all the core symptom domains (Baron-Cohen et al., 2001; Happé et al., 2001). Losh and colleagues (2009) show that social cognition most robustly differentiate performance of individuals with autism and parents with the social BAP from controls without ASD and BAP. Parents with the social BAP are not impaired clinically, but show neurocognitive patterns similar to those observed in autism.

1. Research on Early signs of ASDs

The search for the earliest neural indicators of autism is justified by three reasons: methodological , speculative and pragmatic. The former is the latest progress in neuroimaging methods and the enhanced competence developed by specialists in the use of these techniques, which allow a deeper analysis of the neurophysiological features also in atypical development. The last two reasons are connected with the increasing need to understand the origins and the functioning of the most severe and prevailing neurodevelopmental disorder, having a two-fold purpose: to lower the diagnostic age (practical) and shed light on the mechanisms by which the developmental pathway is adversely modified (theoretical). Until now the ways research has choosen/adopted, to investigate this topic, are retrospective and prospective studies on ASDs.

Retrospective Reports

In a recent review Elsabbagh and Johnson (2010) highlight that infants who go on to a later diagnosis of ASD, do not show – in the first year- many overt behavioral indicators which allow the clinicians to make an early diagnosis. Only starting from the second year of life the early signs of atypicality emerge as measurable by clinical tools (for details, see *Table 13*).

Characteristics of ASD emerging between 12 and 24 months	
1.	Deficits and delays in emerging joint attention [Yoder *et al.,* 2009, Sullivan *et al.,* 2007]
2.	Decreased response to name [Nadig *et al.,* 2007]
3.	Decreased imitation [Bryson *et al.,* 2007]
4.	Delays in verbal and non-verbal communication [Mitchell *et al.,* 2006]
5.	Motor delay [Yoder *et al.,* 2009]
6.	Elevated frequency of repetitive behaviours, e.g. hand waving [Loh *et al.,* 2007]
7.	Atypical visuo-motor exploration of objects [Ozonoff *et al.,* 2008]
8.	Extremes of temperament [Garon *et al.,* 2009]
9.	Decreased flexibility in disengaging visual attention [Bryson *et al.,* 2007]

Table 13 *Early signs of autism: initially variable and low predictive, their value increase with the age of children. From Elsabbagh & Johnson, 2010*

This concept summarize the necessity to examine the first year of life in children later included in the ASDs group, in order to sift out the behavioural patterns looking for potential clinical markers in early infancy. This demand has been embraced by the scientists involved in the field, here is why, in the last 30 years[22], family home movies have become of high clinical interest. Retrospective studies[23] based on homemade videotapes, have allowed specialists to spot several consistent signals of autism onset, many months (at times years), before the official expression of the diagnosis (conventional made around 3-4 years of life). In 2010 Catherine Saint-Georges and collaborators have reviewed 18 previous works (out of the original 41 documented) conducted on family home movies of infants who were later diagnosed with ASD. From the analysis turned out that the most warning signals -in the first two years of life- of future emergence of the condition are: spending less time looking at people; less visual interaction with others a poorer quality of eye contact; less response to their own name; less positive facial expression and joint attention. All together these data suggest a general poor interest for social stimuli and a wider focus on nonsocial entities (e.g. Swettenham et al., 1998).This methodological approach, therefore, has brought to detection of reliable and observable

[22] The first study reported in this field is dated 1975.

[23] A retrospective study looks backwards and examines exposures to suspected risk or protection factors in relation to an outcome that is established at the start of the study.

indicators in toddlers, laying the foundations for an early identification (between 18 and 24 months) of infants "at high risk".

Prospective Studies

Another method to investigate early clinical markers of ASD is to detect the relationship between an aspect shared by some members of a group and the future emergence of the disorder . This approach is named "prospective study[24]" and should be adopted in the investigation of atypical development, but the requirement is to observe a very large sample of individuals taken out randomly from the population, a practice really too expensive rather than the benefits. The key is to sample the experimental group from a limited cohort in which there is an higher probability of emergence of the disorder investigated (greater than in the general population). These pre-selected groups are named high risk populations, and on them, the research on ASD's biomarkers has been focused in the last ten years. The literature on the topic has shown numerous evidence across the long-life path, identifying disorder-associated factors in infants (from 4 months) as well as in adults[25]. The prospective studies on ASD, in the modern meaning , have been introduced recently with many experimental researches addressed to later-born infant siblings of diagnosed children (Baron-Cohen et al., 2002; Elsabbagh and Johnson, 2010; Yirmiya and Charman, 2010; Zwaigenbaum et al., 2007).

1. High risk populations

The introduction of the Broader Autism Phenotype concept and the importance of prospective studies on ASD lead to the consideration of the groups which, according to the disorder's biomarkers already founded, are classified as at high-risk (HR). In this section three HR populations will be described, putting them in order from the less investigate to the most contemplated in literature: babies whose parents show high autistic traits; preterm infants and infants siblings of children with autism.

[24] A prospective study watches for outcomes, such as the development of a disease, during the study period and relates this to other factors such as suspected risk or protection factor(s). The study usually involves taking a cohort of subjects and watching them over a long period.

[25] see paragraph about infant siblings and parents of ASD individuals.

Individuals with high rate of autistic traits

Being an infant whose parents show high Quantitative Autistic Traits (QAT) or clinical evidence of the Broader Autism Phenotype (BAP) it might be an important risk factor. The Broad Autism Phenotype it has been defined "the expression of the genetic liability to autism, in non-autistic relatives of autistic individuals" (Hurley, 2007); therefore the Sub clinical markers of the autistic syndrome may be traced also in the parents. Previous studies shown that parents with the social BAP are not impaired clinically, but they show neurocognitive patterns similar to those observed in autism (Losh et al., 2008; Abrahams & Geschwind, 2008) on the core symptom domains, not combined with any functional or clinical impairment (Baron-Cohen et al., 2001; Happe´ et al., 2001). Literature proves that unaffected relatives show parallel performance patterns rather than autistic individuals (Losh et al., 2007; Piven et al. 1997). The social domain most differentiates performance of individuals with autism and parents with the BAP from controls without ASD and BAP. Moreover the relatives of individuals with autism have an increased risk for affective disorders (Delong & Dwyer, 1988; Piven et al. 1991; Smalley et al. 1995). Another way to investigate the autism's intermediate phenotypes in unaffected adults is the evaluation of the presence of autistic traits. There are many instrument to evaluate quantitative autistic traits (e.g. QAT, Virkud, 2009). Here it will be briefly presented only two of them, which is being use in a part of the research still ongoing: the Autism Questionnaire (AQ) created by Baron-Cohen (2001), and the BAP-Q (Piven et al., 1997). Both the instrument are self-report questionnaire; the former contains five subscales: social skills, attention switching, attention to detail, communication and imagination. Several administrations of the questionnaire to parents of ASD proband have shown that the social skills and communication subscales distinguished parents of autistic individuals from parents of normal controls (Bishop et al. ,2004). The second questionnaire the other hand the BAPQ was designed to measure particular "personality and language characteristics that we have previously postulated as defining features of the BAP, including social personality, rigid personality and pragmatic language deficits in non-autistic parents of autistic individuals" (Hurley, 2007). The BAP-Q is composed by 3 subscales: aloof personality; rigid personality; pragmatic language problems. The use of autistic trait evaluation might be used in the opposite direction, not only to verify the presence of BAP in parents of already diagnosed children, but also to temporary classify infants whose parents showing QAT (clinical as well as subclinical, if higher than the general population) as at high-risk an monitor them, looking for early sign of ASD or the presence of the BAP. The previous

approach is never been used before in the study of intermediate phenotype and is part of the present research project, but given that is still in progress the data are not going to be presented in the dissertation.

Preterms

It has been observed a significant association of autism-spectrum disorders with very low gestational age (Johnson & Marlow, 2009; Johnson et al., 2010; Moster et al., 2009). Extremely preterm children are at high risk for neurodevelopmental disability, behaviour problems and social difficulties, and impairment in executive functions, all of which are also impaired in children with ASD (Johnson et al, 2010). The prevalence of autism is approximately 65-times higher than average, and the prevalence of ASD are 4-to 12- times higher in preterm children. A couple of studies (see Johnson et al., 2010 for a review) have reported positive results on screening tests for autistic features in about 21-25 % of very preterm infants; and also individuals extremely/very/ low birth weight (ELBW <1000 g; VLBW< 1500g; LBW< 2500g) have a 1 to 4 % prevalence of diagnosis of ASD (positive screening at school-age). Limperopoulos and colleagues (2008) have identified several factors that increase the risk for a positive screening, in addition to lower birth weight and gestational age: male gender, prenatal infection, greater illness acuity, and abnormal MRI studies.

Siblings

The importance to include unaffected close relatives (parents and siblings) in studying ASD became evident when previous studies realized that there is an enhanced prevalence for autism among siblings of autistic probands. The recurrence estimated, indeed, is from 3% to 8 % in families with at least one affected child (Micali et al., 2004). According to Smalley and colleagues, in 1988 the rate of ASD was about 75±100 times than in the general population[26], and nowadays sibling recurrence risk in autism has been estimated to be approximately 10% (Murphy et al., 2000; Pickles et al., 2000) . Given that several evidence suggesting that genetic factor plays a critical role in vulnerability to ASD (Losh, Sullivan, Trembath, & Piven, 2008), studies that investigate sub-syndromal autistic impairments among siblings of probands with pervasive developmental disorders have multiplied in recent years, confirming that the genetic susceptibility factors is responsible for common, sub-clinical social impairments in this population follow called “at high risk”

[26] Smalley, Asarnow, & Spence, 1988.

(e.g. (RW.ERROR - Unable to find reference:837). Later born infant siblings might share some characteristics with probands, given the genetic lability, even if they do not themselves go on to receive a diagnosis (Elsabbagh & Johnson, 2010). The aim of a subset of studies on unaffected relatives is to compare the infant siblings of children diagnosed with ASD (ASD-Sibs) and infants siblings of typically developing children (TD-Sibs), with no family history of autism. Sigman and Yirmiya conducted the first pioneering sibling study (2006), glimpsing the remarkable high-potential of this new methodological approach and the importance to focus scientific efforts on risk populations to lower the diagnosis age. Cognitive neuroscience methods might answer the questions that the overt behavioral markers of ASD may not, because they are hardly noticeable in the first year. Direct measurements of cognition and brain function might reveal atypical trajectories of development before the emerging in the infant of the overt abnormal social behaviours. Differently from classical studies, the proposed approach adopted from the field of complex disorders (e.g. schizophrenia) and focused on the study of intermediate phenotypes, has already documented group differences between infant siblings (at high risk, HR) and control (low risk, LR), yet during the first years of life. The discrepancies between the two groups (ASD-Sibs vs. TD-Sibs) are more consistent by 12-14 month of life (Gamliel et al.,2007; Zwaigenbaum et al., 2005), but many authors report other effective factors of discrimination yet in the first year (Elsabbagh et al., 2009; Landry & Bryson, 2004; McCleery, Allman, Carver, & Dobkins, 2007; McCleery, Akshoomoff, Dobkins, & Carver, 2009). The domains in which have been demonstrated a very early gap in the performances, between HR group than LR group, are visual attention (McCleery et al., 2007), eye contact (Elsabbagh et al., 2009a)., face processing, motor skills, precursors of language, flexibility of the attention switch process (Elsabbagh et al., 2009b) and inhibitory control (Holmboe et al., 2010). The gap between the pattern of evidence got measuring brain function by developmental cognitive neuroscience techniques and the results coming from the assessment of explicit social comportments is matter of debate. Elsabbagh and Johnson (2010) have proposed three possible accounts for this discrepancy: methodological issues; a general early BAP; the role of brain plasticity. The former explanation is based on the methodology used: cognitive behavioural techniques seem to be more sensitive at indicating ASD risk in infants earlier in the development. The second proposal justify the inconsistencies by virtue of the presence of an infant BAP[27]: this

[27] Broader Autism Phenotype (Piven, Palmer, Jacobi, Childress, & Arndt, 1997)

pattern causes the variability (in the early stages of development) in the expression of risk outcomes. The last account is built on the brain plasticity concept: even if in a subgroup of individuals few initial subtle differences become associated and lead into an atypical trajectory of development; the most of the infants are able to restore the typical development pathway by the intervention of brain adaptation processes (*see* canalization, afterwards presented).

This new perspective in the study of neurodevelopmental disorders, could be helpful also in the theoretical debate about development and functioning of the "social brain" (Adolphs, 2009; Brothers, 1996; Dunbar, 2003; Grossmann & Johnson, 2007; Johnson, 2009).

Figure 22 *Hypothetical variable trajectories in at-risk infants compared to typical group. Findings support the variability in the onset of behavioral symptoms.*
From Elsabbagh & Johnson, 2010 .

According to the scientific area of developmental cognitive neuroscience, studying unaffected relatives of ASD probands it is another way to better understand the straightforward mechanisms underlying the appearance of social and cognitive skills (and their neural correlates), by comparing typical versus atypical developmental trajectories. The emergence timing of the primary warning signals of ASD, and the heterogeneity of symptoms and phenotypical expression has brought scientists to assume the existence of different developmental trajectories of autism (*see* ***Errore. L'origine riferimento non è stata trovata.***). Some children show a regressive path with loss of abilities in the second year of life; in others symptoms appear sooner and the severity of the condition gradually increase in time. One of the principal aim of studying the ASD-Sibs as high-risk population, is to determine the different effects of early perturbations on developmental trajectories. An early alteration of the evolving route could reset a developmental trajectory

or, in other cases, the typical developmental trajectory can be resilient through canalisation[28], a process by which brain adaptation and plasticity maintains or restores the typical trajectory. Therefore, that being so, it is required to study prospectively from infancy the cognitive development in the siblings of older children with autism (Elsabbagh & Johnson, 2010).

Chapter 4. STUDY 4 : Risk-markers of Autism Spectrum Disorder in a cohort of Italian infants

As introduced in the previous chapter dedicated to the theoretical background (see Chapter 6), in literature there are always more evidences about early atypical responses to social tasks in infant siblings of a child with autism (ASD-Sibs). Recent publications have shown behavioral differences from 6 to 9 months between siblings of older children with ASD (high risk-HR) and siblings of children with typical development (low risk-LR) (see Elsabbagh et al. 2009, 2012; Ozonoff et al., 2008 for review). These data suggest the presence of uncommon cognitive mechanisms and related underlying brain correlates in infants at familial risk for autism; which seem to share much with their affected relative. As introduced previously in the dissertation, the face is one of the most salient perceptual input for human beings, the stimulus par excellence for the investigation of social perception (on behavioural and neural level). For this reason, in order to understand social impairments in ASD, face processing should be one of the future research's object. Infants with autism, in the first two years of life do not spent too much time looking at other persons, they instead seem to be more attracted by things. In other words, in ASD is common a less defined classification of what is social meaningful and what is not, affected children fail to orient to both social and nonsocial stimuli, but they show worse performances in the social domain. Many other behavioural and electrophysiological findings (listed below) indicate impairments in social processing, in attentional mechanisms and in visuo-spatial processing (e.g. during human movement perception) in both individuals diagnosed as autistic and "at risk" relatives (siblings and parents of probands).

[28] "*process through which brain adaptation and plasticity maintains or restores the typical trajectory*" (Elsabbagh & Johnson, 2010) .

Since current findings support the idea that discrepancies in the behavioural phenotype between LR and HR infants may be observed starting from about 9 months of age (Elsabbagh et al. 2009) or as early as in the first semester of life (Ibanez et al., 2008; McCleery et al. 2007; Merin et al., 2007), to be able to evaluate the role of experience in the genesis of broader autism phenotype, it is dramatically crucial to study ASD-sibs at birth. The last three decades of studies on newborn infants in the field of developmental cognitive neuroscience have shown that typical infants respond preferentially to face-to-face interaction with conspecifics from the very beginning (in most of the cases, within the first hours of life). These rudimentary responses are believed to be the basis of social development (Johnson 2005). Many authors have speculated that the lack of responses or the disruption of these neonatal mechanisms, already proved in the typical development, might contribute in the later emergence of ASD (Dawson et al., 2004; Johnson 2005; Schultz, 2005); or alternatively, newborns showing these atypicalities (commonly referred to as an 'endophenotype') could be part of the BAP, that means having an higher risk for autism than general population.

Could be possible detect specific behavioral phenotypes that may be the prodromes of autism before the beginning of the full syndrome manifestation? Are there particular signs evident from birth?

In order to answer this questions and to shed light on some of the critical autism's domains from the first days of life, were chosen three tasks to administer to a cohort of at risk newborn infant siblings. The selection criteria for the tasks were: being linked to atypical cognitive mechanisms and/or brain processes already investigated in the "affected" population; being suitable to be run with newborn infants; being based on a well-proven technique with typical newborns. Two of the designated tasks are similar to those already existing in literature (about BAP), and the third one is a novel task in this field; nevertheless none of which had never been used with newborn infants at-risk for ASD. The whole experimental protocol consists in two classical paradigms: "visual preference" and the "gap-overlap task". none of which had never been used with newborn infants at-risk for ASD;

The basic aim of the research project which is going to be presented here, is to identify very early group differences -based on behavioural responses to perceptual and attentional tasks- between newborn infants at low-risk (LR) and at high-risk (HR) for autism. By

testing newborns might be possible detect the earliest developmental atypicalities – where present- that may be associated with autism or the broader autism phenotype (BAP).

This is a pseudo-longitudinal study: the experiments have been run on a sample of newborn infants, in the future –where possible- further studies will be conducted on the same sample to track the developmental trajectory of the cognitive abilities already investigated. Birth information have been recorded as: being full-term or preterm; the head circumference, delivery method (natural or cesarean) and the Apgar index; these data, especially the morphological measures, will be used to further prospective analysis as well. The high-risk group was composed of healthy newborn infant ASD-siblings, pointed out by clinical services; the control low-risk group, instead was composed of healthy newborn infants without familial history of autism, recruited directly in the maternity ward of the hospital that house the neonatal lab of the research team.

There are many methodological issues inherent in studying high risk groups (e.g. Zwaigenbaum et al, 2006; Yirmiya & Ozonoff, 2007), therefore also the present study has several challenging aspects, among which, the small size of the sample, due to the very costly recruitment, and partly dependent problem of limitations in statistical analysis. In terms of recruitment has involved four regions of Italy: Veneto, Friuli Venezia Giulia, Toscana and Lazio. The clinical services and research teams operating in the territory (among which the two major Italian centre for diagnosis in neurodevelopment disorders[29]), provided the contacts of families of affected children, whose mothers were expecting a child. About the recruitment procedure, two were the principal obstacles: a) the ASD-infant siblings sample in the general population is relative small, connected with the reproductive stoppage frequency; b) the experimental sessions must have taken place within the first 5 days of life of the newborn, in the event that the mother was still hospitalized, not always has been possible to execute the observation in the maternity ward.

Given that the methodological approach is based on individual differences, it is not easy to find great variability in the data (Elsabaggh & Johnson, 2010), since for the data analysis of the experiments described below, it has not been possible resort to classical ANOVA or

[29] The research centre "IRCSS Stella Maris" (Calambrone-Pisa) and the Pediatric Hospital "Bambin Gesù" (Rome).

non-parametric methods, likely has been adopted some strategies proper of clinical case literature and qualitative analysis techniques.

Low-risk and high-risk infant newborns have been compared by using behavioral marker tasks designed to assess attention to social and non-social stimuli offering some support to previous behavioral studies with older individuals with ASD, and suggesting the presence of social attention processing abnormalities very early in the development.

The present study is in line with the new trend of research in this field: the exploration of *gene-development-environment* interactions in longitudinal perspective; the aim is to outline the interplay between attentive biases and experience in developmental disorders (Scerif, 2010).

1. Eye contact effect in newborn infants with genetic risk of ASD (Experiment 4.1)

The social deficits in autism have been for long time considered only as a lack of adaptive communicative and interactive behaviours, easily observable in the affected individuals. Years of remarkable studies in this field, revealed how the evident social impairments were only "the tip of the iceberg"; the key, to shed light on these difficulties, is the study of processes underlying them. In a very enlightening work Swettenham and collaborators (1998) compared typically developing, developmentally delayed and autistic infants in their second year of life about spontaneous shift of attention. The number of shifts made by participants between the following pairs of stimuli was observed: a) between an object and another object, b) between an object and a person, and c) between a person and another person. From results emerged group differences which demonstrate that infants with autism yet at 20 months of ages show abnormalities in attention switching than the other two control groups. The pattern of responses in the ASD group displayed shorter fixation time towards people than objects, and fewer shifts of attention between an object and a person, and between person and person. This study is one of the first supporting the presence of impairments in ASD not only in the social communication but also in social perception and social orienting.

Previous works, in line with these first evidence, have demonstrated that children with autism have deficits in attentional (dis)-engagement mechanisms (gap effect, Van der

Geest et al., 2001), and in processing of social information, particularly faces (e.g. Dawson, 2005; Golarai et al., 2006; Sasson, 2006). Impairments in face processing (on several levels) in children with ASD, have been largely documented in the last forty years; the fundamental finding are following summarized:

1. children with ASD have an opposite attentional bias than controls: they focus on the lower part of the face (Langdell, 1978);

2. they process faces as healthy children process objects (e.g. Joseph & Tanaka, 2003; Deurelle et al., 2004; Davies et al., 1994, for neuroimaging data see Schultz et al., 2000): paying more attention to parts and details (local/featural analysis), rather than to the global picture (holistic/configural processing);

3. better discrimination in identity recognition tasks, based on mouth than eyes; the opposite trend has been found in typical children (ASD: Langdell, 1978; TD: e.g. Tanaka & Farah, 1993);

4. intact mouth processing in children with autism (Joseph & Tanaka, 2003) despite impairment in processing information from the eyes (Swettenham et al., 2001).

5. A greater salience of mouth over gaze. Eye tracking studies confirmed that children with autism look longer the mouth region rather than eyes area in other's faces if compared with developmental delayed or typically developing peers (e.g. Klin et al., 2002, Jones et al, 2008; Neumann et al, 2006). This phenomenon could be also explained according to an affectively-based reason: the well-known avoidance for eye contact (e.g. Davidson & Irwin, 1999; Trepagnier, 1996, 1998).

1. lack of face-inversion effect (Langdell, 1978; Tantam, Monaghan, Nicholson, & Stirling, 1989;Carey & Diamond, 1994);

2. impaired processing of gaze direction (e.g. Courchesne, 1997; Spezio et al., 2007). Young children with autism can differentially process direct and averted gaze when viewing faces (Grice et al., 2006).

3. abnormal activation of the social brain network during face perception and eye processing (e.g. Hadjikhani et al., 2007; Spezio et al., 2007; Ashwin et al., 2007)

On the other hand in population at high risk for ASD there are, likewise, atypical profiles in the social perception domain, especially in face processing. Behavioural,

electrophysiological and neuroimaging studies have documented atypical responses (i.e. visual scanning and attentional mechanisms), and neural correlates of face and gaze processing in individuals at risk for autism. For example Dawson and colleagues (2005) found a significant decrement in face recognition ability in parents of affected children; from the ERP study emerged an abnormal brain responses pattern to faces –compared to nonface- in the close relatives, which reflects the kind of brain activity recorded in individuals with ASD. Moreover a smaller activation of Fusiform area in adult sibling of probands, has been found to be correlated with diminished gaze fixation (Dalton et al., 2007). Electrophysiological recordings on infant siblings of a proband (ASD-Sibs) show an atypical latency for the P400 and atypical gamma band activity when processing direct eye-gaze (e.g. Elsabbagh, et al. 2009a; Elsabbagh, et al. 2009b).

On the basis of this set of consistent data, Elsabbagh and collaborators hypothesized that atypical sensitivity to eye gaze may be a reliable early biomarker , in other words a precursor of the distinctive communicative and social deficits of ASD and a subclinical trait present in at risk individuals (Elsabbagh, 2009a).

1. Rational

Yet in the early stages of life (within age of one year) infants are much interested in other people's eyes. Detecting eye gaze is very important from the beginning of development and it has a strong adaptive role. Senju and Johnson (2009b) about the "eye contact effect" phenomenon argue that it *"modulates the concurrent and/or immediately following cognitive processing and/or behavioural response'*. Functional neuroimaging studies have shown that perceiving eye contact increases the activation of the social brain network; especially there are five brain regions involved in response to direct gaze: Fusiform gyrus; Anterior part of the right STS region; Posterior part of right STS region; both Medial prefrontal and orbitofrontal cortex and the Amygdala. Several studies have showed that autism is characterized by an atypical eye contact (Senju & Johnson, 2009a)

As demonstrated in previous studies (Farroni et al. 2002) neonatal sensitivity to mutual gaze is a strong convincing evidence that human beings are able to detect socially relevant information from birth. The symptomatology of autism includes eye contact avoidance and it is well known that mutual gaze is a special stimulus since birth: newborns prefer to look at faces with direct compared to averted gaze and in 4 month-old infants

direct gaze still elicits a larger negativity than averted gaze (Farroni et al., 2002). An atypical response in young children (3-6 years old) diagnosed with ASD has been found using an ERP paradigm (Grice et al., 2005).

A well-known task in visual attention research, has been tested at birth in both groups (HR and LR) using the established paradigm of visual preference: the "eye contact" task with faces with direct and averted gaze (Farroni et al., 2002). Farroni and colleagues (2002) demonstrated sensitivity to direct gaze in typical human newborn infants: they are instantly able to detect that social salient information (i.e. mutual gaze). The behavioral task tests the ability to discriminate between two stimuli; in this case has been used to verify spontaneous preference for mutual over averted gaze as demonstrated in the previous cited study.

The ERP versions of this preference task has already established the presence of group differences between infant sibs and controls around 9 months of age (Elsabbagh et al., 2009): infant sibs show less sensitivity to direct gaze. The behavioral version of this eye gaze task administrated to high risk newborn might provide a measure of the possible abnormal response already at birth. The aim of the first experiment of the current study is to investigate possible differences in the behavioural response pattern (in eye gaze processing)of newborn infants at high risk for ASD, compared to low-risk newborn infants, previously tested by in our neonatal lab.

2. Method

The "eye contact" task tests the ability of newborns (TD and ASD-sibs) to discriminate between "direct gaze" stimulus and "away-oriented gaze" stimulus. The same paradigm and measures from the previous work (Farroni et al., 2002) have been employed for the present experiment.

The employed technique is a classical infant-controlled visual preference composed by two experimental phases. In the first trial the baby is shown a pair of stimuli on the monitor screen (at the same time: one to right and one on the left); in the following trial the same two stimuli are presented again, but exchanged in position. The visual behaviour of participants has been coded and measured.

1. Participants

The responses of the participants of the current experiment, has been compared to the results obtained with the sample of the original study, published on PNAS[30] in 2002 (see Experiment 1, Farroni et al.). For this reason the characteristics of both the experimental groups will be described. The participants of sample utilized as control they all respond to the common criteria of healthy delivery; the newborns form an unselected group in which the genetic risk for ASD has not been evaluated, thus is a set of participants with "mixed or unspecified" degree of risk. For brevity hereafter the sample of the previous work will be defined "at low-risk group" and the group whose data were recently collected will be referred to as "at high-risk"[31].

All the infants submitted to the following experiments (7.1; 7.2; 7.3) and included in the group with undetermined risk for ASD, respond to these selection criteria: an age in days between 1 and 5; a minimum Apgar score of 8 at 5 minutes after birth; a birth-weight ranging 2,600-4,000 grams; to be full term born and free of ocular and neurological, already certified, defects. These criteria, but the age, were not thought to be fulfilled for the high-risk group. In light of the constraints implied in the recruitment procedure the including standards should not be too much strictly, but in our case also the whole sample at genetic risk met the above-mentioned criteria.

For both groups, informed consent from the parents and ethics approval from the Italian Research Ethics Committee have been obtained .

At low-risk sample (LR): 17 healthy newborn infants (7 males and 10 females) aged from 1 to 5 days old (range: 24-120 hours of life); mean: 72 hours). All of them met the screening criteria for healthy delivery listed above. The whole sample has been tested in the maternity ward of the hospital where the neonatal lab, coordinated by Dr. Farroni, is located. The recruitment of newborn infants was made directly in the maternity ward, after birth.

At high-risk sample(HR): 8 human newborn infants (among whose a couple of twins), all within the first 5 days of life (between 55 and 118 hours postnatal age, mean 68 hours). One of them was excluded from the final sample due to a technical error. All of the

[30] *Proceedings of the National Academy of Sciences.*

[31] In the rest of the dissertation will be used the expression "low-risk group" in referring to the control groups of the experiment addressed to the newborns infant siblings (at high-risk), that are at mixed/unspecified genetic risk for autism.

newborns (6 males and 1 female) were later-born siblings of a child who already received a diagnosis of autism.

Infants participants were later-born biological siblings of a child with autism (ASD-Sibs). All of them belongs to simplex families (SIAF: single incidence autism families) in which there is an older sibling yet diagnosed. The proband must to meet criteria for autistic disorder based on DSM-IV and results over the cut off of at least one of the most common standardized diagnostic tool (and validate for Italian population) for assessment of ASD (e.g. the Autism Diagnostic Observation Schedule [ADOS]) and parent diagnostic interviews (e.g. the Autism Diagnostic Interview-Revised [ADI-R] or the Social Communication Questionnaire [SCQ]). At the first visit to the family the clinical diagnosis of the proband was verified examining the certifications produced by clinical services which have in charge the affected child. Given the variegated geographical origin of the participants (different regions of Italy), the local services that parents referred to for the diagnosis were disparate. For this reason, not always there was consistency in diagnostic batteries used by clinicians in the diagnosis of the affected brother. Nonetheless, steps were taken to invite the families, whose children did not undergo at the assessment with the ADOS protocol, to contact an expert in order to execute this more complete and reliable evaluation. Many of them accepted, allow us, in the near future, to evaluate the severity of the symptoms in the proband of the families involved, using the same scale. A standardized score (compared to a cut-off) for older siblings with ASD may be linked to the performances of the later born siblings in our tasks.The recruitment procedure for high risk newborns has been done through the clinical services afferent to the established ad hoc network, the contact with families having a child with ASD and expecting a new born, was taken during the last months of pregnancy. The newborn infants were tested directly in the hospital where the mother has given birth to, with the prior consent of head physician of the maternity ward, or alternatively at the private home of the family, by means of a portable lab equipment.

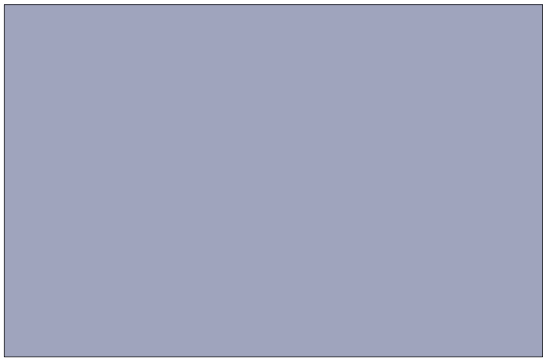


Figure 1 ***Stimuli of Experiment 1 (4th study).***
The pair of pictures displayed to newborn infants.
Adapted from Farroni et al., 2002

1. Stimuli

The infants were shown two pictures depicting the same face, an Asiatic woman model, one on the right and one on the left of the centre of the screen. The two stimuli are full-coloured photographs of the woman (on a grey background), displaying a neutral facial expression, and open eyes looking the centre toward the observator (eye contact, also named "mutual" or "direct gaze") and at sideways ("averted" or "away-oriented" gaze (*see* Errore. L'origine riferimento non è stata trovata.). The oriented gaze stimulus is randomly averted: for half of both samples, shows actress' eyes oriented to the left side, for the rest of the participants she gazes the opposite direction (righ hand side). The measures of the stimuli are summarized below (see illustrated in detail in **Errore. L'origine riferimento non è stata trovata.**):

8.5 cm between center of the screen and inner edges of the images;

distance between face and face is 17 cm

visual angle of face subtended to 27.2° x 41.3°

eye's visual angle is 5.1° x 3.0°

Figure 23 ***Stimuli size and visual angle*** *(adapted from Farroni et al., 2002)**:***

(a) Stimuli in both experiments were color photographic images of female faces directing their gaze straight-on to the viewers (Direct Gaze) or averted to one side (Averted Gaze).

(b) Low-pass filtered versions of the stimuli illustrate the estimated resolution of the images in the visual system according to newborns' average visual acuity. Measures in the figure indicate viewing angles of faces and eyes when fixated or when in the periphery (in brackets).

(c) These pictures illustrate the estimated resolution of the images according to 4-month-old infants' average visual acuity.

1. Setting and Apparatus

The infants of the original study (LR group)were tested in the S. Polo Hospital (Monfalcone, Go, Italy); the high risk sample either in the hospital in which have been given birth or in the private family's house. Independently of where the baby has been tested the features of our apparatus were the same and all the possible expedients were taken in order to minimize any differences in the setting. For this purpose our portable lab replicated exactly all the devices used in the hospital neonatal lab, allowing the experimenters to collect data in the same way. Testing was carried out in a darkened quite room.

During the experimental sessions the infants sat on the experimenter's lap at 30 cm distance from a monitor screen of 60 cm x 34 cm (in 4:3 presentation each peripheral stimulus could subtend about 21° -horizontally -and 35° -vertically- of visual angle). The newborns' eye level was aligned to the centre of the screen at the same height as the actress' eyes. A video camera, placed above the monitor was focused on the infants' face in order to shoot and record baby's eye movement. Filming the participant's face has a twofold goal: 1) to allow the experimenter to monitor newborn's movements, position and possible change of state, by way of a second screen, out of the baby's sight; 2) to record visual fixations of the participants, following to code. During the experimental session caregivers stayed in the room, but were told to not interact with the infant, in order to avoid any kind of interference on the testing procedure.

1. Procedure

Once the newborn is quite, awake and facing the monitor screen, an attention getter appears: a video of a flickering red led. When the baby is attracted to the center and starts to fixate the led, the experimenter presses the key to stop the central attention grabber display and start the presentation of the first trial where the two faces appear side by side. The experiment consists in two presentation of the pictures pair, in which the position of the two stimuli is switched across the sequence. In each phase the stimuli remain on the screen as long as the infant fixate one of them. When the newborn makes a disengage longer than 10 seconds (shift of attention out of the monitor, or closes his/her eyes), the

first trial is interrupted and the second one is launched by the experimenter, preceded by the central attention getter, as long as the baby re-orient the attention toward the screen.

2. Variables/Coding

Videotapes of the baby's eye movements throughout the trial were analyzed by two "blind-to the hypotheses" coders. The dependent variables we used were the total fixation time and the number of orienting responses. The presence of position's biases has been verified, if infants look at one location (i.e. left, right) along the whole session (in which stimuli site are reversed between trails) for more than 85 % of the total fixation time, must be excluded from the final sample for a possible postural bias.

The inter-agreement rate between the coders has been calculated on the 20 % of the whole sample using a two type of coding. The first 60 seconds of the sessions were re-coded in intervals of 3 seconds each. The Pearson correlation was performed on the raw data (r= 88%). Moreover the rate of agreement was verified with the Cohen's k test, using an error's threshold of 200 ms, the results is a good agreement among judges equals to 0,64 and a percentage of agreement[32] equal to 91 %.

1. Analysis and Results

The analyses on the Low-Risk dataset confirmed the absence of stimulus order and gaze direction (in the oriented stimulus) effects, not even interactions between them. Results showed that the fixation times were significantly longer for the face with straight-on gaze than for away-oriented gaze (160.8s versus 63.7s; *see* **Figure 24**). Parametric t-test on log-transformed data shown highly reliable preference for the mutual gaze stimulus (t-test: $t_{16} = 3.211$, $p < 0.01$; Wilcoxon test: $z = 2.580$, $p < 0.01$). Further, the number of orientations was higher towards the direct gaze (mean 17.8) than towards the averted gaze (mean 12.7), supporting the preference already found in fixation times (t-test: $t_{16} = 5.290$, $p < 0.0001$; Wilcoxon test: $z = 3.334$, $p < 0.001$). Preference scores for direct gaze (d) over averted gaze (a) were calculated as (d-a)/(d +a) separately for the looking time and orientation measure. Preference scores significantly differed from zero for both measures ($t_{16} = 3.326$, $p < 0.005$ and $t_{16} = 5.303$, $p < 0.0001$, respectively).

[32] Calculated via the following formula: (N. of true positives + N of true negatives)*100/N. all cases

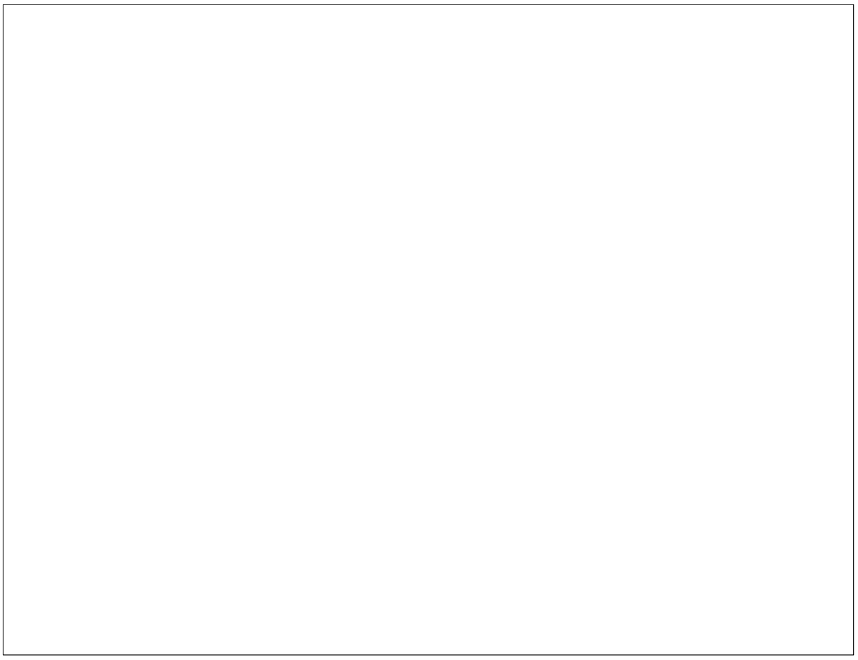
Figure 24 ***Results of the preferential looking study with low-risk newborns.*** *(a) Mean looking times (and SE) spent at the two stimulus types. Newborns spent significantly more time looking at the face with mutual gaze than looking at the face with averted gaze. (b) Mean number of orientations toward each type of stimulus. Adapted from Farroni et al., 2002.*

The raw data were very informative as well: the mutual gaze picture has been looked more times in the whole LR group (17 out of 17) and looked longer by the 88% of the group (only two newborns made more orientations toward the averted gaze picture). The statistical analysis of data coming from the siblings cannot be as much complete as the one made for the LR sample, mainly due to the sample size, thus the description of the preliminary results will be more qualitative than quantitative, the collection of additive data will make possible to operate a more adequate inferential analysis. First of all the High-Risk group (HR) shown the same trend as the LR group that is a preference for direct gaze stimulus, but the behavioral pattern of responses is slightly different in term of engagement. Effectively the fixation time in control newborns (LR group) than in the siblings group (HR sample) is 2.15 times longer for the *averted gaze,* and 2.76 times longer towards *direct gaze*.

Nevertheless the HR subgroup seems to look longer at mutual gaze (mean: 38.7 s, st. dev.= 17.8 s) than averted gaze (mean: 29.6 s, st. dev.= 13.8 s) as the controls, whereas they look a similar number of times the two stimuli: straight-on (mean: 12.0 , st. dev.= 4.9) and averted (mean: 12.6 , st. dev.= 4.8) gaze (see **Figure 25**, **Table 14**, **Figure 26**).

Figure 25 ***Results of the preferential looking study with high-risk newborns.*** *On the right side: mean looking times spent at the two stimulus types. Newborns spent more time looking at the face with mutual gaze than looking at the face with averted gaze. On the left: mean number of orientations toward each type of stimulus.*

Table 14 *Descriptive statistical measures in the two experimental samples.*



The difference between the two stimuli is not significant, the sample is too small to find enough variability to ascertain the effect with parametric and non-parametric measurements (summarized in **Table** 15**2**), but results suggest a trend in the dataset similar to LR sample (with p values near the significance's threshold). Preference scores for two stimuli were calculated for the looking time (t_6= 2.019, p < 0.05) and orientation responses (t_6 = 0.313, p> 0.05): only the fixation time in milliseconds significantly differed from zero in the HR sample. On a more descriptive level, data can be used to generate qualitative inferences. For example 4 out of 6 newborn sibs have looked more at the direct gaze, one infant did not show any preference (rate of fixation = 50%), and only one looked longer the averted gaze stimulus. In terms of attention switching, all the participants, but one, did more saccades toward the photograph showing the eye contact.

Parametric tests on raw data
T-test ➢ on fixation time in milliseconds [ms]: t_6= 1,857, p =.113 ; ➢ t-test on orientation responses: t_6= 0.338 p =0.747 ; ➢ t-test on rate of fixation on total fixation time [%]: t_6= 2.088 , p =.082
Parametric test on log-transformed data
T-test ➢ on rate of fixation on exposition time: t_6 = 2.983, p = .041; ➢ t-test on rate of fixation on total fixation time: t_6 = 2.093, p = .081; ➢ t-test on fixation time [ms]: t_6 = 2.024, p = .089)
Non parametric tests on log-transformed data:
Wilcoxon test ➢ on rate of fixation on exposition time: z =1.826, p = .068 ➢ on rate of fixation on total fixation time: z =1.693, p = .090 ➢ on fixation time [ms]: z= 1.690, p= .091 Sign test ➢ on rate of fixation on exposition time: p = .125 ➢ on rate of fixation on total fixation time: p = .453 ➢ on fixation time [ms]: p= .453

Table 15 Preliminary statistical analyses on high-risk sample's data.

A further analysis that could be execute on the behavioral data collected in the high-risk sample, is the comparison of few measures of center tendency between the two groups (HR vs LR). The difference between any fixation time of the participants of the HR group toward the two stimuli, divided for the standard deviation of the fixation time for each

stimulus in the LR sample[33], produce the rate of variance of the HR subjects than the distribution of responses in infants without history of autism (LR). From this computation is possible to see the distribution of fixation times and number of orientation towards the stimuli in the high risk sample. All the sib infants are situated below the LR average of fixation times both for averted gaze (mean SD: -0.78) as for mutual gaze (mean SD: -1.42), but only in the responses towards the eye contact the gap is major than about 1,20 standard deviations (all participants but one, see **Figure 27**). With regards to the orientation responses: all the participants show a placement under the average of the LR sample's number of saccades for the averted gaze (mean SD: -0.92) and most of them also for the direct gaze (mean SD: -0.12); but in this case the greater variability, compared to the original sample's responses, is in the less number of fixations for the away-oriented gaze (see **Figure 28**).

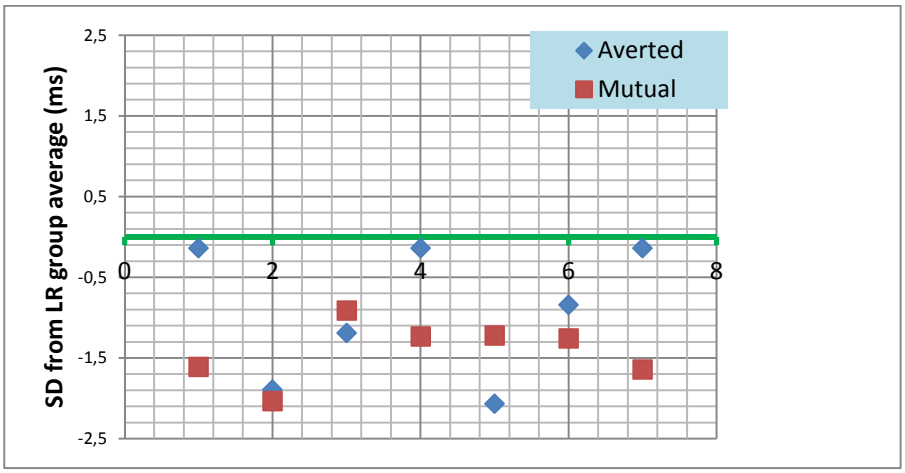


Figure 27 ***Distribution of fixation time responses in the HR group in respect to the average of LR sample (scale in standard deviations).***

[33] $(ms_{\text{HR-participant}} - mean_{\text{LR sample}})/(st.dev._{\text{LR sample}})$

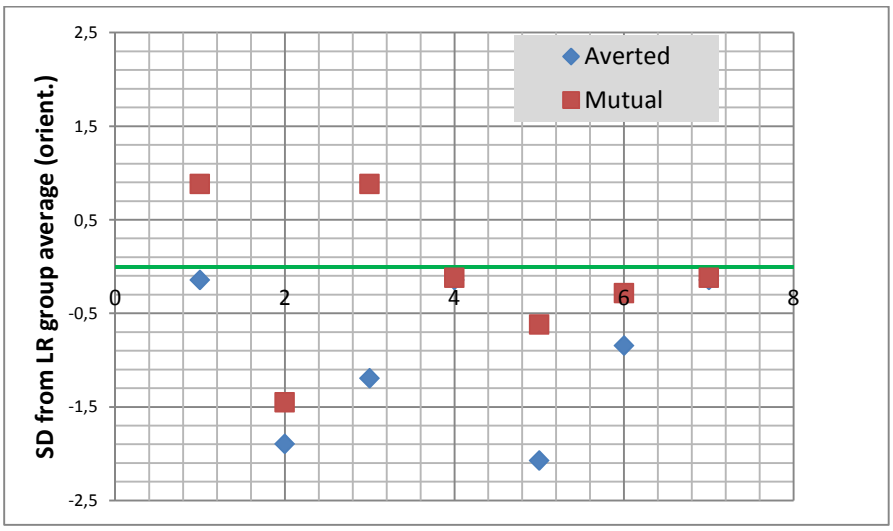


Figure 28 ***Distribution of number of orientations in the HR group in respect to the average of LR sample (scale in standard deviations).***

2. Conclusions (first experiment)

All together these results demonstrate a preferential orienting to direct gaze from birth in both experimental groups, even if for the high-risk group, given, the limited number of data, is better to speak of a tendency (still to confirm on a larger sample with parametric analysis). Thus the preference previously founded in typical newborns does not seem to be altered in the infant siblings (HR), supporting the idea that infants genetically vulnerable to ASD are able to discriminate between gaze averted and mutual gaze, as their low-risk peer; moreover, in the same way of LR newborns, the look longer the more communicative stimulus, the eye contact, might be likewise socially relevant for them. Nevertheless, in spite of this similar trend between the two groups for one of the two measured dependent variable (HR group: longer fixation time for mutual gaze), the number of orientation is not consistent with this tendency and there is a general diminished time of visual exploration during the whole experimental session. This lowered rate of visual attention could be explained by virtue of the social and communicative nature of the stimuli, but also assuming the presence of deficits in the attentional mechanisms (our data suggest in engagement), unconnected from the type of input. Finally the interaction of these factors must be taken in account in the explanation of behavioural differences, emerged in the task, revealed by newborns at high-risk for autism.

4.2 Gap effect in newborn infants with genetic risk of ASD (Experiment 4.2)

From clinical observation of individual with autism, appear clear their difficulty in disengaging the gaze from an object or an activity (3rd criteria for autism diagnosis reported in DSM IV-TR: stereotyped, repetitive behaviors and narrowed interests). In literature there are several evidences about the presence of deficit or atypical skills attention-based in people with autism. Attention is a network of systems which work a different levels and seem located in particular brain areas (Posner, 1988; Posner & Dehaene, 1994). The classification of these systems proposed by Posner and colleagues divides the attention in three principal functions and relative cortical substrate:

1. sustained attention and alertness: sub-cortical area (vigilance system)
2. shift of attention (orienting) and spatial selection of sensory events: posterior area (basic attentional system)
3. voluntary control and detecting process: anterior area (executive system)

The orienting function, driven by the posterior system, is responsible for the visual spatial attention which allow operations such as engagement, disengagement and shift. These processes might be automatic or not and correlated with the nature of the input (i.e. mechanism stimuli-driven). Recently this domain has begun to being investigated also in neuroatypical populations like in Autism Spectrum Disorders. These posterior processes are in most of the cases studied using variants of Posner's (1988) visual cueing task or other paradigms measuring the interference of few variables on global and local attentional processing (i.e. Navon task).

In Plaisted and colleagues (2003a) children with a diagnosis of autism and typically developing children were administrated two version of the Navon task (Navon, 1977): the divided attention task and selective attention task . In the former the participant has to respond when he/she see the target even if no clues about the appearance of the target was given (i.e. local or global). In selective attention task, children were instructed to attend to either the local or the global level. In the first task typically developing children show better performances when the target appeared at the global level, whereas children with ASD made less mistakes when the target appeared at the local level. In the second task

both groups responded faster to the global than to the local target. This study suggest the presence of a heterogeneous profile of attentional abilities in ASD: normal global processing it seemed preserved in one task, but not in the other; possible impairments in mechanisms that inhibit local information in favour of holistic processing are proposed in the paper. The same author has conducted another study on visual attention in children with ASD (Plaisted et al, 2003b) showing a kind of superiority in their performance than typical peers. Using a conjunctive visual search task, in which the target shares one dimension with one set and another dimension with the other set of distracters (e.g. searching for a red X among red T and green X distracters). Plaisted and colleagues found in the ASD group a lack of the typical reduce speed in executing this task, in other words the controls were slower in spot the target, suggesting the idea that the high degree of similarity between flankers (distracters) and target might have a smaller impact on processing in individuals with autism. Also neurophysiological studies have provided data about anatomical abnormalities in autism which could affect functioning resulting in several patterns of visual attention (i.e. attentional phenotypes). In a magnetic resonance imaging (MRI) study, Townsend, Courchesne, and Egaas (1996) found analogies in attentional shifting in individuals with cerebellar abnormalities. Either individuals with autism and those with acquired damage exhibited deficits in reallocating and disengaging attention during a cueing task (slower RTs with valid cues and longer latencies with invalid cues, respectively). The most used paradigm for study spatial attention is the Posner's cueing paradigm, in order to investigate visual orienting in typical and atypical development, a simplified version of the task has been conceived: the *gap-overlap task* (Hood & Atkinson, 1993; Johnson, Posner, & Rothbart, 1991, 1994). the paradigm's adaptation makes possible the spontaneous engage of attention (automatic/involuntary orienting) in infants. The original gap-overlap paradigm, introduced by Johnson, Posner and Rothbart in 1991, measures the difference in saccadic reaction time between two conditions: gap and overlap (to evaluate facilitation and disengage mechanisms, respectively).

The general paradigm consists in the presentation of central and peripheral target/stimuli; the aim is to evaluate the latency reaction times of gaze behaviour. In other words: how much time the individual spend to make a saccade from a central fixation point towards a peripheral stimulus (eye movement to reach the target); the RTs are intended from the onset of the stimulus itself. In the gap condition there is an interval between the offset of the central stimulus and the appearance of the target (variable in literature according to

authors' questions and to the participants age or state); in the overlap condition the central stimulus does not disappear, thus, the peripheral target and the fixation point are visible -simultaneously- all the way through the trial.

This phenomenon, called "gap effect", has been largely demonstrated in literature in adults and in primate at the basis of the saccade generating process there is a neural network, which allow the engage and disengage of attention (Fischer & Weber, 1993). According to literature the gap effect could be explained by virtue of two hypotheses: one based on oculomotor reflexes and the second one on attentional system's mechanisms. The first interpretation is based on the role of a brain structure in the visual attention: the Superior Colliculus (SC). Lesional studies shown the responsibility of SC in the generation of saccades (Schiller, True & Conway, 1980, Schiller, Sandell & Maunsell, 1987 and Schiller & Lee, 1994). The SC is responsible for the integration of signals used for the information processing; SC receive afferents related to eye movements from cognitive e visual centres (cortical and subcortical). The visuo-motor hypothesis highlights how the SC is involved in the control of saccades: when the visual field is free (gap condition) it is able to trigger an orienting behaviour towards a target just appeared; but as long the circuit that allow the generation of saccades is disinhibited by the presence of a previous stimulus, to which the system is already engaged with, the re-allocation of the gaze is prevented (Sparks et al., 2002).

The second theory is based on Posner's findings (disengage-move-engage theory of attention), the RTs are slower (saccades' inhibition) when the covert attention (i.e. without moving eyes) is still involved in the processing (stimulus fixation: engagement), on the contrary occurs a sort of facilitation when the focus of primary attention ceases to exist (stimulus offset: disengagement) consenting the orientation towards a novel target (APT: Attentional Predisengagement Theory, (Kingstone & Klein, 1993) .

Several authors partially ruled out the role of covert visual attention (APT theory) on this attentional pattern; much evidence suggest the presence of two aspect in the gap effect (Forbes & Klein, 1996; Pratt, Bekkering, & Leung, 2000): a motor component and the effect of the fixation's beginning. When there is a gap between offset's fixation and the onset of the following stimulus, the system use this "pause" as an indicator to prepare the eyes' motor response (Ross & Ross, 1980, 1981)[34]. The second component , the fixation

[34] As cited in (Kingstone & Klein, 1993) .

offset effect (FOE), is inherent to the oculomotor system and consists in the competition among two operations, which both activate the Superior Colliculus (SC) producing the inhibition of the first and the disinhibition of the second: orienting and fixating (for the role of SC in reflexive saccades see Munoz & Wurtz, 1992; Reuter-Lorenz et al., 1991)[Errore. Il segnalibro non è definito.].

Yet in the first year of life, in particular between 1 and 3-4 months of age, authors reported difficulties in disengaging attention from one target in order to orient the gaze toward another peripheral target (e.g., Aslin & Salapatek, 1975; Braddick, Atkinson, & Hood, 1996; Johnson, 1990, 1995). This developmental phenomenon has been named "sticky fixation" or obligatory fixation, the interpretations are related to the maturation of brain structures and to the handover of functions between the long-standing subcortical routes to the new-developed cortical areas (for a review see (Goldberg, Maurer, & Lewis, 1997) . The modification of disengage ability might be explained by the maturation cortical networks which affect the role of SC in regulating eye saccades. Hood and Atkinson (1990) found particular patterns of behavioural responses to the gap-overlap experimental conditions, already at 3 and 7 months of life.

Thus given the informative role of the gap-effect in clarifying attentional mechanisms (as orienting and disengaging) and the functioning of neural substrate, and the possibility to investigate over attention (stimulus driven or exogenous) very early in life, the gap-overlap paradigm is a good candidate for the study of these aspects in atypical populations and, most important, in infancy .

1. Rational

Ad demonstrated in the past the gap-overlap paradigm is effective from infancy: gap condition produced shorter latencies, and in overlap condition, instead, the RTs are longer (Hood & Atkinson, 1990). These have been explained in terms of brain development, and also for this reason the gap-overlap task is considered to be sensible to few brain damage during infancy (Hood & Atkinson, 1990) and there are recent publications sustaining the idea that a measure derived from this task may predict later autistic symptoms (e.g. Zwaigenbaum et al. 2005). Thus, there is evidence that the gap task may reveal different performance associated with either the broader autism phenotype (subclinical traits) and/or

that it may provide early markers for the diagnosis of autism (clinical indicators). Kawakubo (2007) hypothesized that the dysfunction of attentional disengagement may contribute to indifference to action observation of others and preoccupation to non-social stimuli in individuals with autism. In this work he provides electrophysiological evidence for deficits in attentional disengagement in adults with autism using the gap overlap task. Yet in childhood these anomalies have been documented, for example Van der Geest and collaborators have demonstrated that children with autism have difficulties in attentional engagement and disengagement mechanisms (Van der Geest et al., 2001). The authors employed the gap overlap paradigm to compare performances of children with ASD and typical children (matched for IQ and age): did not arise any reliable variances among the groups in both condition (gap and overlap), but a reduced gap effect was found in individuals with autism than in the controls. Landry and Bryson (2004) examined young children with autism in regard to their visual attentional mechanisms : shifting and disengage. Their performance in visual orienting tasks were compared to the performances of children with matched Down syndrome and Typically developing children. To measure the ability to disengage and shift attention they proposed respectively trials in which eye movement latency was monitored from the onset of a peripheral stimulus, which either overlapped with, or did not overlap, a pre-existing central (fixation) stimulus. Results have shown that 3–7-year-olds children with autism had marked difficulty disengaging from one of two competing stimuli, as doing as typical 2-month-olds do reaches the peak between 1 and 2 months of life, than it gradually decrease until disappearing: by 4 months of age, infants typically disengage easily (REF?). Descending again with the age of the participants, there is recent evidence on disengaging deficit in very young siblings (ASD-Sibs). Elsabaggh and colleagues (2009a; 2009b) through the use of the same paradigm have demonstrated that 9-month-old infant siblings of children with autism showed longer disengagement latencies relative to the control group (group differences between high-risk and low risk infants).

The proposal for this experiment was to apply the same paradigm in the observation of newborn siblings, because of a previous work (Farroni et al., 1999) which has already established the existence from birth of the “gap effect” (a reliable difference in the mean reaction time between gap and overlap conditions). The ‘gap task’ has been used to assess infants’ visual orienting by several developmental cognitive laboratories (e.g. Johnson et al. 1991, Farroni et al., 1999). The speculative question is: What happens in neonates at high-risk for emergence of a disorder, assuming that this condition imply

disengaging impairments? With the intent to answer to this question the following study has been conducted. The above mentioned attentional task (gap-overlap) has been tested at birth in both groups (HR and LR) using the established paradigm with social (face-like shape) and nosocial (inverted face-like shape) stimuli (Farroni et al., 1999). The task measures the "cost" of disengaging from a central stimulus in order to fixate a peripheral target. This task measures the latency of orienting towards peripheral cues depending on the temporal gap between a central stimulus and the peripheral target and depending, in the case of newborns, on the kind of peripheral target (upright vs inverted face).

4.2.2 Method

As previously described the employed task measure the efficiency of orienting towards a peripheral target by disengage foveal attention and look away from salient or captivating stimuli (Johnson, Posner, & Rothbart, 1991). In particular, in this task the purpose is to explore whether newborn's latency to make a saccade toward a peripheral stimulus, when a central fixation stimulus (a flashing light) is present (overlap condition), is longer than when the central stimulus disappears before the peripheral stimulus appears (gap condition). In the original study, published in 1999 by Farroni, Simion, Umiltà and Dalla Barba, the the "gap-overlap" task was administered to newborn infants using schematic social and non social stimuli (Experiment 1 and Experiment 4, respectively) in order to measure the attentional disengagement mechanisms and how they were affected by the nature of the input (facelike Vs non facelike).

1. Participants

For this experiment the dataset of the low risk sample has been retrieved from the study published on Developmental Science (1999). In the same way of the experiment presented above (7.1), these previous data have been compared with new collected data on a pilot " high-risk" sample.

The sample for this experiment is the same presented in the previous study and that is going to be presented in next study. All the infant siblings who have undergone the experiments 7.1 , 7.2 and 7.3. The experimental protocol included all the three tasks, but not all the participants successfully completed the three sessions. The selection criteria of participants of the low-risk sample were the same presented before (healthy delivery parameters); respective way of recruitment, place of testing and apparatus for participants

of both samples are the same as described in the previous experiment (7.1). Moreover in both groups, informed consent from the parents and ethics approval have been obtained . The only difference between the two groups is that in the original study social and non social stimuli were tested in two separate groups of participants (between groups factor), whereas in the high-risk sample the two categories of stimuli were presented in the same session (within group factor).

At low-risk sample (LR): in the experiment on social stimuli the final sample was composed by 18 healthy newborn infants and in the experiment with non social stimuli was composed by 16 newborns; all the participants were aged from 1 to 5 days old.

At high-risk sample(HR): 8 human newborn infants (among whose a couple of twins), all within the first 5 days of life (between 55 and 118 hours postnatal age, mean 68 hours). The later-born siblings were 6 males and 2 females.

1. Stimuli

The stimuli employed in the experiment are the original ones from Farroni et al., 1999, taken from previous studies (Johnson & Morton 1991; Morton & Johnson, 1991; Valenza et al., 1996; Simion et al., 1998). The peripheral target are two head-shaped, two dimensional white forms, containing three blobs (black squares). The features of both stimuli create a schematic human face upright or inverted face (only the configuration of the inner elements is up-side-down). The fixation stimulus at the centre of the screen is a red flickering circle. The central fixation led is on during the overlap condition and off during the gap condition, the central stimulus remains on or not (see **Figure 29**); this provides independent measures of disengaging (central stimulus 'on') and shifting attention (central stimulus 'off').

Figure 29 ***Stimuli used in Experiment 7.2.*** **(a) "Gap" condition** *sequential presentation: when the central led stops flickering and disappears (fixation's offset), the face-like appears on the left or on the right side (target's onset).* **(b) "Overlap" condition** *parallel presentation of both*

peripheral target and fixation flickering led.

The pictures shown to infants (distance from the screen: 30 cm) were subtending a visual angle of 21° x 35° (horizontally and vertically, respectively); the led was 3° of visual angle and the distance between its external edge and the inner border of the peripheral stimuli was 10°, each blob (black squares) was subtending a visual angle of 1.5°.

There are 8 types of stimuli (4 for each study in the LR sample) obtained through the combination of condition (2: gap and overlap), target's appearance side (2: left or right) and category of stimulus (2: social and non social).

1. Setting and Apparatus

As described in the relative section of the previous experiment (see paragraph 3.2 Method) newborns during the testing were held by an experimenter facing the monitor, when they were quiet and in an alert state the experiment begun; during the presentation of the stimuli their eye movement were recorded by a camera placed above the screen.

1. Procedure

The newborn will be shown several trials of the "gap effect" task with upright and inverted schematic face stimuli. Every trials begun after a fixation time towards the central led of at least 2 seconds, when this criterion was reached the experimenter start the presentation of the target either to the left or right of the midline stimulus; otherwise the presentation was skipped to the next trial. The eight possible stimuli were presented in a pseudo-randomized order: the side of targets' presentation is equiprobable, while conditions and categories of stimuli are presented in sequential blocks in which the identical combination stimulus-condition-side is never presented for more than one time in row (each block contains 16 trial). The order of the blocks is random as well, with 4 cycle of presentation (64 trials); the sequence of blocks is presented in loop until the occurrence of a change of state in the baby and never more than 10 minutes.

1. Variables/Coding

The time spent by every newborn to make a saccade toward the target on periphery were calculated from the recordings of the infants' eye movements. The video were played frame by frame, with 50 frames for every second of real time. The time in frames was multiplied by 20 to get estimation of the latencies in milliseconds. The computation is done by a subtraction: a difference between time in which the eye movement toward the target begins and the onset of the target. For a trial to be coded as valid the newborn must have:

2. fixated the central led for at least 2 seconds;

3. been engaged in central fixation during the appearance of the peripheral stimulus;

4. oriented to the target within 5 seconds from its onset;

5. made the saccade in the same direction of the target after at least 99 milliseconds from its onset (pro-saccade).

Moreover trials were considered invalid if:

1. the infant blinked, look away from the screen or in the opposite direction of the target (anti-saccades) between the fixation's beginning and target's onset.

2. The infant makes a pro-saccade after 5 seconds from the target's onset (failure to disengage).

infants should have been included in the sample only if there were at least two valid latencies for each type of trial (criterion taken from the original study), in other words at least 8 valid trial, two for every condition-stimulus pair. Despite this, given the restricted size of the sample and the inability to replicate the data collection whether not enough informative (e.g. for tiredness of the newborn), it was decided to neglect this criterion in order to be more conservative with regard to the data collected. In that way was possible to analyse the response trend of all the participants, although on a limited number of measurements.

Recordings of the babies' eye movements throughout the trials have been analysed by two coders who were blind the stimuli presented. After the coding was used by experimenter to record the latencies for each stimulus and condition, in order to obtain the mean latency for every participant divided by category of stimulus, by conditions and by the combination of both (condition x stimulus).

1. Analysis and Results

The experiment is a 2 (condition) * 2 (side) *2 (stimulus) within-subjects factorial design. The side of the target emerged to be indifferent in the statistical analyses. In the low risk group (Farroni et al., 1999) the results of the two experiments (facelike stimulus and non-facelike stimulus) confirm the presence of a condition effect when the target is the social stimulus (for mean latencies see: Table 16longer latencies obtained in the overlap context than in the gap one (t-test: $t_{(16)}= 3.95$, $p < 0.001$). On the contrary this effect it is not found in the experiment which used the inverted faces as peripheral target (t-test: $p = 0.30$).

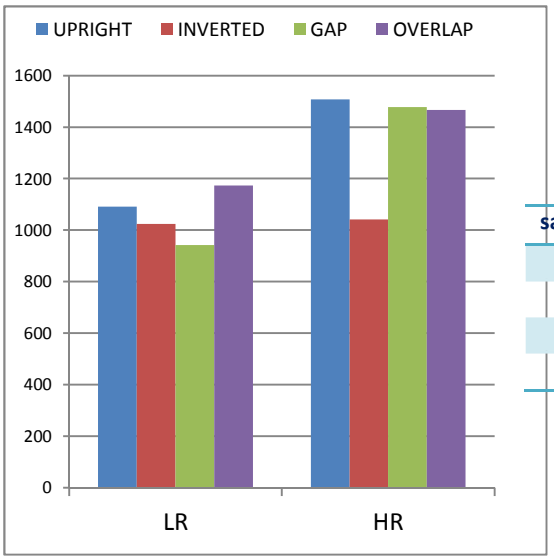
category	SOCIAL TARGET		NON SOCIAL TARGET	
stimuli	UPRIGHT GAP	UPRIGHT OVERLAP	INVERTED GAP	INVERTED OVERLAP
mean latency	874	1308	1011	1038
st. dev.	259	443	388	284
Gap advantage	434		27	

Table 16 ***Latency data in the two experiment on the low-risk sample.***

Given the great difference in results between the two experiment (1st and 4th in Farroni et al., 1999), authors decide to conduct an ANOVA with experiment as between factor and condition as within factor. Results show a condition effect ($F_{(1,31)}= 10.11$, $p < 0.001$) and an interaction experiment*condition ($F_{(1,31)}= 7.82$, $p < 0.01$).

Given the size of the high-risk sample a proper statistical analysis is not possible between groups, thus here will be presented only descriptive and qualitative data. The infant newborn siblings show slower reaction times in initiate saccades towards both the type of stimulus and both the conditions (*see*

Figure 17).



sample	UPRIGHT	INVERTED	GAP	OVERLAP
LR	1091	1024	942	1173
HR	1507	1042	1478	1467

Figure 17 ***Average latency across the two stimuli used and across the two experimental conditions (up) and mean latencies in the two group (right).***

In general, considering the combination stimulus-condition, the newborn siblings performed faster saccades towards the non social stimulus (in both the conditions) and greater latencies when the target was the facelike stimulus. Moreover it does not seem to exist any advantage (in RTs) in the gap context than in the overlap (*see* **Figure 30**).

Figure 30 ***Distribution of mean latencies in high-risk group between stimuli (on the left) and among conditions (on the right).***

A power test within conditions -assuming a power of 0.85 (usually employed in studies with infants)- shows an effect size of 0.73 (large, t= 2.09) and a required sample (to reach the significance level) of 20 participants for the Overlap condition and an effect size of 0.56 (medium, t= 2.04) and a required sample of 30 participants. The same analysis run within stimuli (social Vs. non social) suggest a medium effect size for inverted face (t-test

between gap and overlap conditions) and a very small effect size for the upright stimulus. These non parametric computation support the idea that the trend of responses recorded in the ASD-Sibs is atypical and further data might statistically confirm these tendencies. An qualitative examination of the variance (standard deviations from the low risk performances) between the latencies in the two sample have shown discrepancies as well. As it has been done in the previous experiment for the computation of the variance the following method it has been used: $(ms_{\text{HR-participant}} - \text{mean}_{\text{LR sample}})/(\text{st.dev.}_{\text{LR sample}})$.

As it is possible see from

Figure 31, most of the participants are located very far from the average of the low-risk group. The strongest data are the ones referred to the Inverted-Overlap and to the Upright-Gap trials. In inverted-Overlap the 50% of the HR newborns differ negatively from the LR newborns for more than 2.2 standard deviations, which means that the category of trials towards to at risk newborns make the fastest saccades is the one of them in which typical newborns obtained the longer latencies in (RTs of LR group are 1.75 times slower). Also in Upright-Gap trials, 4 out of 8 babies shown great variances (all but one in positive direction), up to almost 9 standard deviations from LR average; this category of trial is the fundamental in showing the gap effect in the typical sample and the role of social stimulus in strengthen it. In the original study the effect was stronger with the schematic face, here is weaker with the facelike and very strong with the non social stimulus. The latency's RT toward the social stimulus combined with the facilitating condition (gap) in low risk newborns is 1.60 times faster than in HR siblings.

In order to compare the responses of the two groups (LR vs HR) a power test based on the average latency of the two sample (between groups) in the two conditions revealed that the effect size is equals to 0.74 (large, t= 2.00) with a required sample of 34 participants (established power= 0.85) for the Upright-Gap stimulus and equals to 0.99 (very large, t= 2.02) with a required sample of 20 participants for the Inverted-Over stimulus. On the contrary Upright-overlap and Inverted-Gap show very small effects size (for a visual graphic representation see

Figure 33).

stimuli	average variance between LR sample's latencies mean and HR sample's latencies	
Upright Overlap		x= 0.14 sd=2.79
Upright Gap		x= 2.02; sd= 3.72
Inverted Overlap		x= -1.57 sd=2.01
Inverted Gap		x= -0.26 sd=1.76

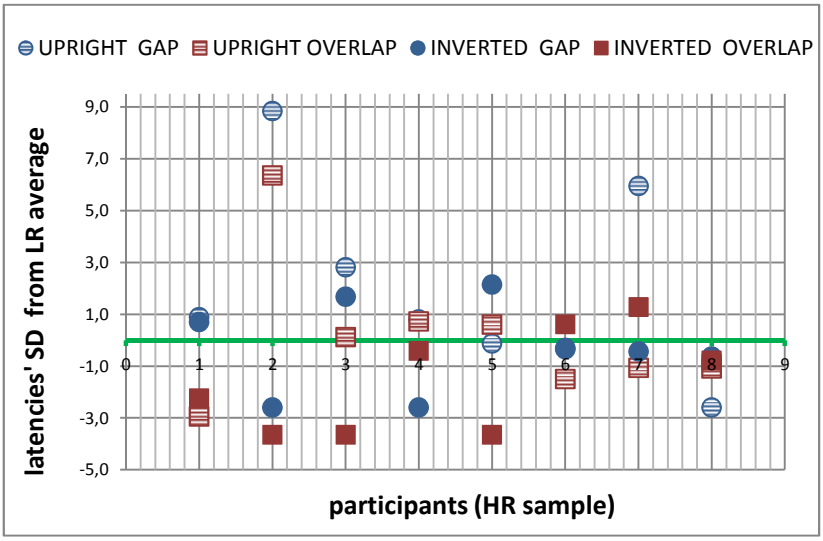


Figure 31 *on the left: average variance (expressed in standard deviation from LR mean) of the latencies in HR group and standard deviation of the distribution of variance scores. On the right: distribution of the mean RT of every participant of the HR sample (in the four stimuli) in regard to the LR averages (horizontal midline).*

These data indirectly suggest the presence of potential discrepancies between typical and at risk newborn infants, especially in their responses to Upright-Gap and Inverted-Overlap trials and the presence of a facilitation effect in the overlap trials than in the gap ones.

Figure 32 ***Average of latencies per each type of stimulus (category X condition) in the High-Risk sample.***

sample	latency	UPRIGHT GAP	UPRIGHT OVERLAP	INVERTED GAP	INVERTED OVERLAP
LR	*mean (ms)*	874	1308	1011	1038
	st.dev	259	443	388	284
HR	*mean (ms)*	1397	1371	909	593
	st.dev	963	1235	681	571

Figure 33 ***Mean RTs and standard deviations in the low risk sample (LR) and in the high-risk sample (HR).***

2. Conclusion (second experiment)

All together these data show that overall RT latency in newborn siblings seems to be greater than RTs in newborns at low-risk. As previously maintained by Blaga & Colombo (2006) the lower speed in disengaging could be affected by the difficulties in processing the midline stimulus, that in this case was quite complex (a flickering red led). According to this hypothesis longer latencies to target in the overlap condition may be attributable to the still ongoing processing of the central fixation stimulus, which requiring more time causes the delay in the reaction times. On a more analytic level the mean latencies in HR group are different: greater in Upright and smaller in Inverted trials. Moreover the gap effect with the social stimulus is absent in ASD-Sibs, on the contrary there is a sort of opposite trend: the conditions seem to affect in a stronger way the eye movements toward the non facelike target. This finding if confirmed by additional data would be coherent with literature arguing the lack of attentional modulation in autism, particularly evident for the social stimuli (see Bird et al., 2006). Another noticeable aspect is that in the LR infants the longest and shortest latencies have been recorded for the upright face, thus within the same stimulus, reinforcing the possibility that the responses are strongly due to the

interaction between the nature of stimulus and attentional mechanisms involved (disengaging vs facilitation). Is not the case in HR newborns who show the more distant RTs in trials belonging to different category of stimulus and different condition. The result of the gap-overlap task suggest presence of an attentional deficit, with failure in disengage, a concept already expressed in literature and supported by several findings on adults and children with ASD and older infant a genetic risk for autism. This deficit, however, seem to be governed by different constraints than in the general population. For example the gap condition is used to measure facilitation mechanisms, but we do not know if the same context elicit facilitation in disengage process as in typical/low risk individuals. The HR sample, here, show a worsen performance when the stimulus was social, confirming the hypothesis of an innate deficit in social orienting (more than in attentional mechanisms) in individual with a family history of ASD. The lack of facilitation in Gap condition might be explained with a poor involvement of the Superior Colliculus in triggering saccades, especially towards communicative target (given the demonstrated salience of faceness from birth). The faster RTs in the overlap context (in particular with non social target) could be explained as an advantage in processing related to the more local-featural looking behavior in ASD. In other words the challenging condition for disengagement (overlap) may be not so demanding for HR infants than LR ones, due to a less interference of the fixation point on the orienting towards the peripheral target. In line to this interpretation, other kind of superiority in perceptual an attentional tasks have been already documented in autism (see Kremner et al 2008). Finally to interpret these data also the atypical visual scanning (i.e. abnormal eye movements, largely investigated) in autism, may contribute to deficits in disengaging and shifting attention, mostly when overt attention, as in this case is required. These results confirm the possibility to investigate early biomarkers of autism through behavioral attentional tasks. Further data and a longitudinal design could make more clear the developmental trajectory of these abilities and the connection with the ASD attentional phenotype, in order to timely categorize possible predictors of the core deficits in autism.

4.3 Preference between biological and non biological agent in newborn infants with genetic risk of ASD (Experiment 4.3)

Passive viewing of biological motion engages extensive regions of the posterior temporal-occipital cortex in humans, particularly within and nearby the superior temporal sulcus

(STS). To identify disruptions in the brain mechanisms for biological motion perception might provide insight into an on-going developmental process whereby early abnormalities in social engagement shape (and are shaped by) the neural processes that support social interactions (Kaiser 2010). Moreover is still unclear how really works the early action processing and which are the inner components of this ability. There are different levels to take in account trying to understand how this process happens.

According to Tomasello, Kruger, and Ratner (1993), three levels of social understanding are recognizable:

1. to perceive the behaviour of animate beings and be able to predict the consequences of the observed behaviours;
2. to understand others' behaviour as goal- directed (conceiving others as intentional agents);
3. to possess Theory Of Mind (TOM): other individuals are conceived as agents whose thoughts and beliefs may differ from those directly inferred from their perceived behaviour.

Thus the purpose of the present experiment was to collect data on visual preference between biological and non biological goal directed actions (See Experiment 3.1, presented in Chapter 3) both on low-risk infants and in siblings in the first days of life. The building of stimuli used and procedure have been described before, thus, here they will be only briefly recalled.

1. Rational

In regard to ASD several studies have suggested the hypothesis of an impaired perception of biological motion (i.e. Moore, 1997, Milne 2002; Kaiser & Shiffrar for a review); since biological movements (i.e. eye gaze) convey social information, it is very important to understand how infant at high- risk of ASD perceive and processes this category of stimuli.

Zwickel (2010) shown that individual with Asperger Syndrome display an impaired process of mentalizing, but they maintain a spontaneous agency perception and spontaneous visual perspective taking. Perceiving others' actions and understand

underlying intentions are precursor on which individuals build the Theory Of Mind (TOM), a cornerstone of social development that seem particularly impaired in individuals with autism

The deficit on Theory Of Mind (TOM) in children with ASD has been ascertained time after time, so it is crucial looking for differences in the behavioural forerunners of TOM, to identify some possible biomarker of the disease. The magnocellular visual system, which contributes to a various aspects of social processing (e.g. subcortical face processing) and is also responsible for the motion processing does not function normally in some children with autism leading to many hypotheses regarding reduced sensitivity of the visual magnocellular system / cortical dorsal stream (Milne et al., 2002; Spencer et al., 2000). Problems of adults with ASD in processing biological motion have been reported by several authors (*see* Dakin & Frith, 2005 *for a review*) and there are very recent data showing visuo-perceptual abnormalities in processing aspects mediated by the magnocellular visual pathway (i.e. luminance contrast) also in young infants at high risk for autism (McCleery, Allman, Carver, & Dobkins, 2007).

In 2009 Klin and colleagues published a work according to toddlers with ASD prefer to attend physical contingencies than to biological motion (a point-lights display study), suggesting that the previous evidence about the more salience of mouth over eyes in individuals with autism might be explained by a modulation of the attention driven by physical than social information. Starting from these data of the literature would be important to figure out if there is an early atypical biological motion processing in ASD. The current study has been conceived with the main idea of comparing visual behavior towards stimuli displaying biological and non-biological features at birth.

2. Method

Infants from both experimental samples (at unspecified risk [LR] and at genetic risk for autism [HR]) were administered a visual preference infant-controlled between two stimuli showing a goal-directed action.

1. Participants

The participants belonging to the low-risk sample have been presented in Chapter 3, in particular the infants have undergone the “coherent” condition of experiment 3.1, were

compared to few infant siblings of the HR sample (a subgroup of newborns enrolled in Experiments 7.1 and 7.2), included in this group because later-born in a family with a child already diagnosed for ASD. Once again selection criteria of participants for the low-risk sample had healthy delivery parameters. Recruitment, setting and apparatus were the same of the previous experiments (7.1 and 7.2); informant consent from parents and ethical approval were obtained.

At low-risk sample (LR): the final sample was composed by 12 healthy newborn infants (age ranging between 1 and 5 days-old), referring to the participants of one of the conditions of experiment 3.1.

At high-risk sample(HR): 6 human newborn infants (among which a couple of twins), all within the first 5 days of life (between 55 and 118 hours postnatal age, mean 68 hours). One of them was excluded from the final sample due to fussiness. The newborn siblings who completed the session were 4 males and 1 female.

1. Stimuli

As stimuli for the experiment have been used the same videoclips described in Chapter 3, employed also in the Nirs studies, consisting in short movies, lasting each 2 minutes, representing and agent came on the stage, approach the target on the center of the surface (a red ball), grasp it and put it down in another location on the stage (peripheral) near the place from where the agent come in on the scene. The whole action (approach-grasping-release) lasts 10 seconds, and it is presented in loop (with an interval of 1.7s from end of one cycle to the beginning of the next one) until the end of the clip. The scene contains two video presented simultaneously, what people can see is a unique scene in which two actions are occurring at the same time. The movie are identical and specular in every aspects but the aspect of the agent and the type of movement that it shows. The appearance of the agent could be human (an arm) or non human (an usually inanimate object); the kind of motion the agents display during the action could be also human-like or non-human like. To obtain this effect it has been used a movie of an arm performing the action in the natural way and a video of a tool moved by a person. Afterwards both the clips have been digitally edited in order to equalize length, luminance and contrast of the pair of clips; moreover from each frame of the tool's video has been erased the presence of the model's

arm and manipulated order and number of frames (via duplication and removal of them)[35]. The two films were presented together according to these criteria:

1. each scene must contain at the beginning only the target of the action;
2. after 1 sec both the agents appeared from the side of the screen (one from left one from right) starting the action.
3. the two clips are specular: the action develops from the center of the screen (target location) towards the side from where agent arrived;
4. the two events are synchronized, each agent spent the same amount of time respectively in reaching, grasping and releasing the object.

The two parallel clips forming the scene were presented to the newborn infants at a distance of 30 cm and subtended a visual angle of 20,4 ° vertically and 23,4 ° horizontally.

Figure 34 ***Stimulus used in Experiment 7.3.*** *On the right side the arm which moves in a natural way (biological kinematics and human aspect); on the left a tool (rod) which is being moved by an unseen person displaying (via digital editing) a mechanical motion (non-human aspect, non biological kinematics). The position of the two agent is switched and mirrored among the first and the second trial, the target of the action is always in the centre of the screen, but the agent are reversed to permit the control of positional biases.*

5. Setting and Apparatus

The setting of the experiment was the neonatal lab of the S. Polo Hospital for the group of low-risk babies, recruited directly in the maternity ward. With regards to sample at genetic risk of autism the place where the observations were conducted was the private house of the family or the hospital in which the younger-siblings of the probands were born; in both cases the team employed the portable equipment which replicates the apparatus of the permanent laboratory. The apparatus was composed by a monitor screen of 27 inch, a

[35] For a more detailed description of the procedure to get the stimuli, see Chapter 3.

camera placed above it, a computer to run the experiment (E-Prime 2.0) and a recorder to register the participant's eye movements.

1. Procedure

Once the newborn was awake and quiet, was placed in front of the presentation monitor, held by the experimenter and the experimental session started. The presentation is composed by two trials: in the first presentation the infants are shown the human agent on one side and the mechanical tool on the other. The presentation of the first phase lasts a maximum of 2 minutes, according to the infant-controlled method the presentation is interrupted as soon as the infant makes a disengage (to look away from the screen or to close his/her eyes) longer than 10 seconds, after a cumulative fixation time of at least 20 seconds (time needed to see the action twice) during which infant must have seen both the agents (left and right), otherwise the presentation continue to permit the newborns to explore both the side of the scene. In absence of a spontaneous disengage of 10 seconds the presentation of the first trial automatically stopped after a cumulative fixation time of 60 seconds, to allow the infants to observe also the second scene before became fuss. The second trial has a maximum length of 2 minutes too, the presentation goes on until the baby makes a 10 seconds disengage , the video stops or he/she get tired.

2. Variables/Coding

The frame by frame videocoding of newborns' eye movements, provided two measures for evaluate the visual preference: the fixation times toward the two agent and the number of orientations in the two trials. The coders also verified the presence of position's biases measuring the total fixation time of each participant towards left and right: only one infant from the LR sample obtained more than 85 % of the total fixation time in one direction, for this reason was excluded from the final sample.

There were two instructed coders, blind to the hypotheses, the inter-agreement rate between them has been calculated on the 20 % of the total experimental group (HR + LR). The data obtained from the recoding shown an high Pearson correlation on the raw data (r= 86%); a K of Cohen equals to 0,61 (using an error's threshold of 500 ms), and a good agreement among judges equal to 86 %.

1. Analysis and Results

The data of the Low risk group have been presented previously (see Chapter 3, coherent condition), therefore they will be just summarize: infants tend to look longer the biological stimulus, but there was a lack of principal effect of the stimulus. Newborns did not prefer hand over tool when the first is moving in a biological way and the second one in a mechanical way (t_{11}= .092, p > .05). Differently than the first two experiments presented in this chapter, for this o ne the collection of data on unselected newborns and high-risk newborns, have been conducted at the same time. For this reason at the time of the testing was still unknown that the study would have been inconclusive on LR newborns. Nevertheless it could be interesting to point out some qualitative inferences emerging from the comparison between the two samples. At the same way of newborn infants with unspecified risk for ASD, the newborns with familial history of autism did not show preference for one of the two agents, but at the first sight it is clear that the exploration's time of the two stimuli is very different: infant siblings spent half of the time than typical newborn in looking the human goal-directed action and 1.5 times less the action performed by the mechanical tool (*see*

Table 2). The discrepancy is stronger in regard to the biological stimulus, resulting in a lack of difference in responses between the two agents, as instead, happens in the LR sample (at least as a trend in fixation times, not yet confirmed by analysis). The Anova and the T-test on the matched pairs executed on the low risk data were not significant, but an high effect size (dz= 0.81 with 0.95 of power, 0.05 of α error probability[36]) suggest the possibility to find a reliable difference in responses towards the two agents, enlarging the sample size to redouble the number of participants. On the contrary the protocol for the power analysis within the high-risk group revealed a very small effect size and percent of change between the two stimuli which would not improve increasing the sample size (Cohen's d= 0.18, 5% of relative change).

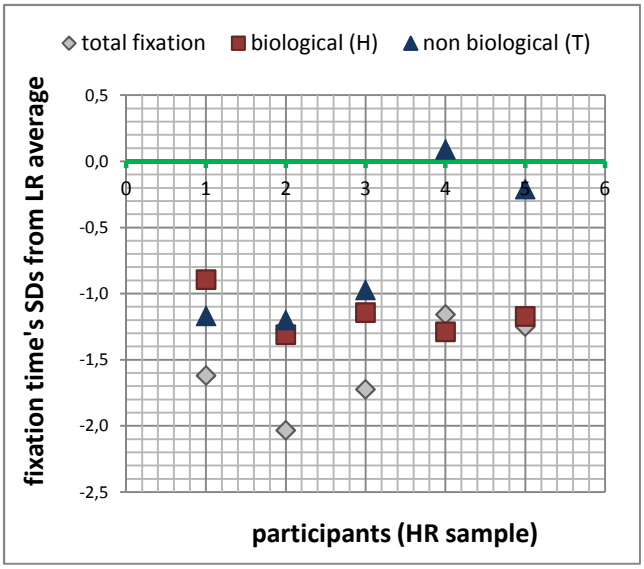
[36] Computed with the Power Protocol Analysis (a priori procedure based on two dependent means) executed by the software G*Power 3.1.2; Franz Faul, Copyrights © 1992-2009 and verified with the spreadsheet to calculate effect size by Thalheimer and Cook (2002).

Low-Risk sample						
	HAND		TOOL		total	
	ms	*orient.*	*ms*	*orient.*	*ms*	*orient.*
mean	58520	21,08	39413	19,17	97933	40,25
st.dev.	26287	5,25	18468	8,32	27838	12,45

High-Risk sample						
	HAND		TOOL		total	
	ms	*orient.*	*ms*	*orient.*	*ms*	*orient.*
mean	27976	6,8	26599	7,40	54575	14,20
st.dev.	4358	2,49	10944	3,65	9969	6,10

Table 18 *Means and Standard Deviations of fixation time and orientations towards the two stimuli and during the whole presentation.*

The examination of the variance computed by the difference between each fixation time of HR participants with the LR average, related to the standard deviation of the low-risk sample has shown that all the siblings looked the biological stimulus less than infants at low risk for ASD, in particular: 4 out of 5 siblings looked the hand at least 1.15 standard deviation less than LR group (average of -1.16 standard deviations) and 4 out of 5 looked the tool less than LR newborns (average: -0.70 standard deviations), thus, in general, infants siblings show a lesser amount of attention towards both the stimuli, but the gap than the LR newborns is more pronounced in regards to the biological stimulus (*see* Figure 35).

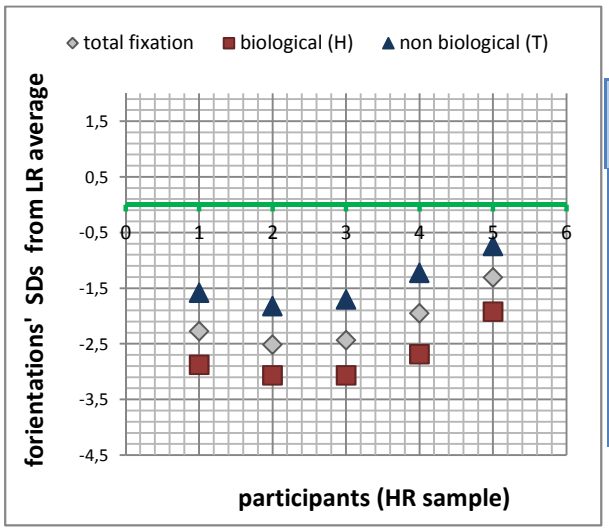


	total fixation	biological (H)	non biological (T)
P1	-1,62	-0,89	-1,17

P2	-2,03	-1,31	-1,20
P3	-1,72	-1,14	-0,97
P4	-1,16	-1,29	0,09
P5	-1,25	-1,17	-0,21
average	**-1,56**	**-1,16**	**-0,69**

Figure 35 *Distribution, in standard deviations, of every participants of the HR sample than the averages (horizontal midline) of total fixation time and relative fixation times (towards the two agents.)*

The distribution of orientation responses in the infants siblings than in the unspecified risk group is also informative: the whole HR sample is located below the low risk average and mostly for the responses related to the biological stimulus, for which the average of standard deviation from LR mean is -2,72 (*see* Figure 36).



	total fixation	biological (H)	non biological (T)
P1	-2,27	-2,87	-1,58
P2	-2,51	-3,06	-1,82
P3	-2,43	-3,06	-1,70
P4	-1,95	-2,68	-1,22
P5	-1,31	-1,92	-0,74
average	**-2,09**	**-2,72**	**-1,41**

Figure 36 *Distribution, in standard deviations, of every participants of the HR sample than the averages (horizontal midline) of total orientation responses and relative orientations (towards the two agents.)*

A power analysis between experimental groups[36], confirmed a very large effect for the stimulus hand (d= 1.44; 109% of change among groups with a very limited need of increase in the samples' dimensions); and a large effect also comparing the fixation time's means towards the mechanical agent (d= 0.81 ; 48% as rate of change between samples). A t-test on independent samples has been conducted to verify the equality of means,

assuming the experimental group (HR or LR) as pooling variable, the results confirmed reliable differences between the samples.

Figure 37 *On the left: total fixation times relative fixation times (toward the stimuli) in milliseconds in the two sample (HR, LR). On the right: Number of orientations towards the two stimuli and all together, divided by experimental group.*

In particular the total time- in milliseconds- spent by newborn siblings in exploring the scenes was minor ($t_{15} = 4.718$, $p = .0001$), especially considering the hand ($t_{15} = 2.537$, $p = .023$), while was not significant the fixation time towards the tool between groups ($t_{15} = 1.433$, $p = .172$). About orientations all the variable were significantly different between high and low risk sample: saccades toward the hand ($t_{15} = 5.741$, $p = .0001$), toward the tool ($t_{15} = 2.999$, $p = .009$), and globally toward both stimuli ($t_{15} = 4.403$, $p = .001$). For a graphical representation see **Errore. L'origine riferimento non è stata trovata.**. These data suggest an effect of the belonging sample (at familial risk or unspecified risk) more on the number of participant's eye movements rather than on the engagement duration (*see*

Figure 38). An general linear model on repeated measures has been run, using sample as between factor and the biological/non biological as within factor (two measures: fixation times and orientations) and total fixation time and total orientations as covariates. The Anova revealed an interaction between the nature of the agent (human or nonhuman) and the total number of orientations ($F_{2, 12} = 6.353$, $p = .013$ and partial Eta square= .541). More than the interaction *bio-non bio X orientations number* another informative date emerge: the interaction *bio-nonbio X sample* is not significant in general ($F_{2, 12} = 2.899$, $p = .095$ and partial Eta square= .325), but the Within-Subjects Contrasts on the separate dependent

variables (fixation time and orientations separately) shown an interaction *bio-nonbio X sample* on the orientation responses ($F_1 = 5.498$, $p = .036$ and partial Eta square= .297).

Figure 38 *Estimated marginal means of orientations and fixation times in the experimental samples.*

4.3.4 Conclusions (third experiment)

To summarize the results of the third experiment it is possible maintain that newborn infant siblings, compared to peer at unspecified familial risk for autism did show few differences and few analogies in their visual behavior with respect to the presentation of two kind of agent: an hand with its natural motion (biological stimulus) and a tool moving in a mechanical manner (non-biological stimulus). Participants belonging to the high-risk sample (HR) did not prefer one agent over the other one, as also the low risk infants did. Despite the lack of a main effect of the stimulus joins the two sample, other characteristics express the degree of variance between newborns with and without genetic risk for ASD. There is a general minor interest in the observation of stimuli demonstrated by total fixation time (HR newborns looked for 50% less time than LR) and by number of orientations (LR turn eyes at the stimuli about 3 times more than HR). All the variables considered (total and relative to the single agents), but the fixation time towards the tool, emerged to be significant different (in negative direction) from the performances effected by low risk newborns; the strongest data are related to the biological stimulus.

4.4 Discussion

From the behavioral patterns in the three experiments emerges a remarkable variability on attentional and visual responses in the high-risk sample compared with the low-risk sample. ASD-sibling newborns have shown atypical patterns in engagement and disengagement behavior all the tasks. In previous a work Batki and collaborators shown that neonates prefer to look at a face with open eyes rather than a face with closed eyes, suggesting the presence of an innate neural mechanism which recognize eye-like stimuli and orient infant's attention to them. At the same way Farroni demonstrated a preference in newborns for mutual gaze over away-oriented gaze (Farroni et al., 2002). Together these data are very powerful in the field of developmental cognitive neuroscience supporting the idea that individuals are socially oriented from birth by virtue of two possible systems: a specific mechanism which mandatory detect eyes (''Eye Direction Detector", [EDD], Baron-Cohen, 1994) from the beginning of life; or a more general appeal for faces which translates in the tendency to orient toward facelike elements, thank to the presence of high-contrast features processed by a subcortical pathway. This very early sensitivity for the gaze, though to be crucial in the affective and social development, may lack in particular conditions characterize by deficits in the social domain, such as the autism spectrum disorder (for a review see Senju and Johnson, 2009). Elettrophysiological data confirm behavioural data about the deficits in face processing in children with ASD: the mean amplitude and latency of face-sensitive ERP components were atypical in people with autism as well in unaffected relatives (Dawson et al., 2005), in particular at risk infants showed a slower P400 response to eye contact than controls (Elsabbagh et al., 2009a). The current study shown a response trend of HR infants very similar to the pattern recorded in newborns at unspecified risk for ASD in terms of direction (the looked more the mutual gaze), but quantitatively they executed a lower number of orientations towards direct gaze and a lower fixation time during the exploration of the stimuli. This minor engagement in the social stimuli presented, is in line with a previous result which indicates that children with ASD process direct gaze as their typically development peers, but they look less the faces in both the conditions (Vivanti et al., 2011); these result has been discussed by authors as an intact capacity of social cues to trigger attention also in individuals with ASD, constrained by the disorganizing power of the mutual gaze stimulus, which affects fixations and orientations. Finally the minor discrepancy between the two stimuli as behavioural measures (number of orientations and fixation times) might be

explained with an inefficient face processing due to defects in the subcortical route in charge for it. Structural abnormality in the amygdala, largely proved in individuals with autism (e.g. Baron-Cohen et al., 2000; Schultz, 2005; Sparks et al., 2002) or in the circuits which send information to it (i.e. magnocellular pathway), also found in young infants at familiar risk for ASD (see McCleery et al., 2007; McCleery et al., 2009) could be the origin of impairments in face processing ability. In the current study this account could clarify the poor differentiation of newborns between mutual and averted gaze: a different sensitivity to contrast and luminance may impair the ability to discriminate between straight on or oriented eyes, or even redirect the attention to others part of the face (i.e. mouth). Beyond brain atypicalities and deficits in visual processing also a lack of motivation to attend social stimuli such as faces may explain the minor time of visual exploration of stimuli in the first experiment (Dawson et al., 2002; 2005).

The second experiment based as well on a previous study (Farroni et al., 1999) run on newborns with the same paradigm (gap-overlap) has been proposed to evaluate in infants at high risk could display differences in the cost of disengaging (RTs) from a central fixation toward a peripheral one. In typical newborns the presence of an adult-like "gap effect" was established from the first study; the aim of the replication addressed to the HR sample, here described, was to evaluate possible dysfunctional mechanisms in visual attention detectable from the first days of life. Moreover manipulating the socialness of the targets (facelike Vs nonfacelike) would have been possible to test the possible charge on performance by the nature of the stimulus. This proposal was in line with previous finding which suggest that early attentional deficit may be precursors to ASD related behaviors (Elsabbagh et al., 2009b). The younger siblings of probands shown a lack of the typical gap effect (absence of facilitation with upright face) and in particular were slower to orient when a face appears in the periphery. In addition the very low rate of valid trials, that represent a strong methodological limit, is also an indirect proof of the abnormalities found in engage and disengage processes (rarely infants made a saccade in the target's direction or reach the stimuli in the valid interval of time). From data emerges a strong effect of the type of stimulus, with longer latencies for face-like and shorter latencies for inverted face and an effect of condition (gap vs overlap) limited to the non social stimulus, in line with the hypothesis of a worsening of performances in social contexts (see Bird et al., 2006; Dawson et al., 1998). These data seem to suggest the presence of domain-specific mechanism, given the disproportion of reaction times between upright and inverted face in the HR sample (stimulus-driven pattern): in infants at risk seem to exists a

disengage problem special for social stimuli. On the other hand the latency's times over the infant siblings were greater in general, coming out in favor of a domain-general attentive impairment in relatives of probands. Moreover the saccade's reaction times in high risk infants could be influenced by a low-level visual orienting impairment, that is a difficulty in doing eye-movements (due to deficits in motor programming). In addition to the HR participants' apparent difficulties in shifting spatial attention (not new in literature: see Courchesne et al., 1994; Townsend et al., 1996), there are few data, in the risk sample, supporting also the superiority of ASD in certain visual tasks (O'Riordan et al , 2002; Dakin & Frith, 2005): in particular than typical peers newborn siblings performed impressively better in the overlap condition than in the gap one (only when the stimulus was non social). The reason of these superior performances might be the less influence of the fixation stimulus as distractor for the orienting process towards the lateral target; in other words the distractor inhibition system works efficiently only in the non social domain. The gap effect is believed to be mediated by Superior Colliculus (SC) activity (e.g., Munoz and Wurtz 1992; Schiller et al. 1987), therefore this absence of the phenomenon in the HR sample suggest a less involvement of SC or the presence of atypical collicular mechanisms. These data are partially in line with previous studies on ASD-Sibs which shown prolonged latencies in disengaging attention and reduced facilitation in gap condition (Elsabbagh et al., 2009).

The last experiment on the perception of human motion in naturalistic scenes is based on studies which demonstrated – using the point-lights-display (PLD) technique- the presence of a predisposition for biological motion detection very early in life (in newborns: Simion, Regolin & Bulf, 2008; at 3 months: Pinto, 2006).Starting from the hypothesis that impairments in select social relevant information might be related to a perceptual deficit in detect biological movement. Several studies already testify the presence of anomalies in processing biological motion in adults with ASD (e.g. Blake et al., 2010), given the yet demonstrated difficulties in visual perception (for a review Dakin & Frith, 2005) and the structural abnormalities, is important investigate this process in newborns at risk. Klin and colleagues in 2003 shown as perception of bio-motion (BM) may be impaired very early in life (in toddlers with ASD) causing cascading effects on social development. Converging lines of evidence support the involvement of specific brain areas in the social perception, especially in processing others' actions and intentions (Allison et al., 2000). The Superior Temporal Sulcus is part of this specialized network and is strongly activated during bio-motion perception; thus, in light of deficits in understanding others' actions and

thoughts, characterizing the ASD, this cortical area could be functionally compromised in autism. Motion perception could be explained in terms of magnocellular/dorsal deficits, but it does not seem to be a sufficient reason for the severity of the impairment: the superior temporal sulcus (STS) is one of the neural basis for motion processing and social perception, thus it has been suggested that abnormalities in that area may contribute to the expression of biological motion-processing deficits. According to several authors there are three main line of research and consequent interpretations, not mutually exclusive, for the action processing in ASD (see also Vanvuchelen et al., 2013): a visual attentiveness hypothesis, a the biological motion preference and the action recognition process. The first idea is based on deficits in visual attention as origin of deficit in perceiving and comprehend actions. The second body of evidence (e.g. Blake et al., 2003; Klin & Jones, 2008; Klin et al., 2003, 2009) has shown that individuals with autism exhibit impairments in perceive biological motion rather other kind of movement, whether displayed with moving point-lights configurations. Finally studies on action imitation in ASD demonstrated that there are not difficulties in the ability to recognize an action (e.g. Charman et al., 1997; Rogers, Bennetto, McEvoy, & Pennington, 1996; Smith & Bryson, 2007; Vanvuchelen et al., 2013).

In the third experiment participants belonging to the high-risk sample (HR) did not prefer one agent over the other one, likewise low risk infants. In particular they show a general reduced interest in the stimuli than their peers at unspecified risk for ASD, mostly towards the biological coherent agent which has been looked significantly less than LR participants. The deficits in ASD in sensory and perceptual information processing could be the cause of less sensitive system to detect biological motion (e.g. Atkinson, 2009 ; Congiu et al., Freitag et al., 2008) and they may originate in delays or imbalance in the development of parvocellular and magnocellular circuits. The behavioural responses pattern found in the infant newborn siblings along the three studies are in line with the categories of perceptual anomalies that have been investigated in ASD up to now: a superiority in local processing (largely demonstrated), impairments in holistic processing (still matter of debate) and compromised motion perception (which interpretation about subserving neural structures is to date unclear).

The data here presented are, however, compromised by several limitations. First of all the sample size, which makes hard the generalization of the results on other infant siblings of an affected child, therefore the current findings require replication with a larger sample. A

second critical issue are the statistical analyses, the size of the sample limits the use of standard methods, so it will be necessary, in future, to create appropriate statistical protocol to verify to apply in prospective studies, making as much reliable as possible the comparison with the low risk participants. In regard to this aspect the above presented studies employed as low risk sample unselected newborns, to convey more significance ti the results would be necessary to recruit newborn infants who have an older sibling (to match also the order of birth) and without first or second-degree relatives with autism. Finally would be crucial go on with the observation in a longitudinal way to map the developmental trajectories of infants at risk in the cognitive domain investigated.

This study conform the possibility to detect few subtle overt atypical behavioural manifestations before the emergence of the full ASD syndrome. The identification of endophenotypes as the attentional one found in these studies could allow experts to execute more precise screening not only based on clinical parameter (but also on behavioural responses to attentive and perceptual tasks) ad to monitor from very early in life infants whit enhanced probability to develop ASD. A failure in social orienting may lead to the disruption of the typical emergence of social brain network (e.g. Johnson et al., 2005; Schultz et al., 2005), supporting the hypothesis that small changes in the typical developmental trajectories during critical period of postnatal life (when the brain is still maturing) could cause the later emergence of neurodevelopmental disorders (Karmiloff-Smith, 1998). Our findings, even if, preliminary and on a limited pilot cohort are a proof that subtle difference between high-risk and low-risk infants are discernible at birth, in opposition to authors who argued that it is not possible identify difference in infants who might be later diagnosed with ASD before 6-12 months of life (Rogers, 2009;Tager-Flusberg, 2010). This study is very innovative in this terms, further investigation on the high risk population in early infancy could help to increase the knowledge about the interaction between development and this variance in behavioural expressions (phenotype), and the nature of the disorder; moreover studying the ontogeny of atypical development will be possible to better understand which are the crucial mechanisms in social development and how the plasticity of the brain could be fundamental in restoring atypical trajectories.

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