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Sensory, behavioral, and basic immunological profiles: are their associations specific for Autism in adults with moderate-to-profound Intellectual Disability?

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SUMMARY

Autism Spectrum Disorder (ASD) has a high probability to co-occur with Intellectual Disability (ID). When ID is particularly severe, it becomes harder to clearly differentiate ID with and without ASD. This is often due to the presence of features that are common to both conditions, such as altered immune activity, atypical sensory processing and rigidity in behaviors. The present observational, cross-sectional study aimed at exploring the immunity, sensory and behavioral profiles of 20 adults with moderate-to-profound ID and 53 adults with ID and ASD (ASD+ID). Associations between leucocytes blood counts and scores of sensory/behavioral questionnaires compiled by professional caregivers were explored as primary aim both in the whole sample and within-groups. As secondary aims, associations between sensory and behavioral profiles were measured, and between-group differences of immunity, sensory, behavioral, and medications parameters were computed.

Trends of mild, positive correlations were present between leucocytes blood counts and auditory, body position and sensory sensitivity alterations in the whole sample, and similar patterns were found after considering ASD+ID and ID groups separately. No associations emerged between immunity and behavioral profiles.

No between-groups differences in sensory or behavioral scores were found. Olanzapine and leucocytes blood counts were higher in the ASD+ID group ($p = 0.017$ and $p = 0.007$), although only leucocytes blood counts fairly discriminated between ASD+ID and ID subjects (AUC = 0.717).

Although sensory and behavioral profiles were similar across diagnostic categories, this study suggests that basic immunity profiles may be informative to characterize ASD+ID and ID groups in real-world clinical practice. The presence of associations between immune and sensory profiles might indicate that immune systems mediate sensory sensitivity, especially for auditory and body perception channels. Immune and sensory profiles have similar associations in ASD+ID and ID. To understand the possible mechanisms of such interactions (inflammation, immune activation) may allow the development of tailored therapeutic strategies and to better differentiate ASD- from ID-related features when severe cognitive impairments are present.

INTRODUCTION

Autism Spectrum Disorder (ASD) encompasses a wide range of conditions with increasing prevalence[1]. This trend is in striking contrast with the decrease of Intellectual Disability (ID) diagnoses, raising questions on the biological and clinical hallmarks distinguishing ASD with low cognitive functioning from ID[2]. An important diagnostic specifier for ASD concerns intellectual impairment (ASD+ID), which is present in a high percentage of individuals with ASD (30-70%)[3-5] but should not outclass the diagnosis of ID when the latter is present[6]. The possibility to rapidly achieve sufficient knowledge to avoid artificial overlaps between ASD+ID and ID is undermined by several factors. First, the more severe the level of intellectual impairment, the harder it gets to differentiate whether the individual social impairment is due to a specific ASD-related or ID-related deficit. Second, ASD+ID is considerably under-investigated when compared to its counterpart without ID[7]. Third, subjective reports are often impossible to collect for individuals with severe-to-profound intellectual impairment, leaving clinical judgement deprived of a fundamental tool. Fourth, as both ASD+ID and ID share similar clinical characteristics but comprise different clusters of neurobiological aetiologies, they are artificial conditions *per se*[8]. Fifth, the abovementioned limitations are even more evident for adults[9]. Taken together, these criticalities may result in

circularities between research and clinical practice. In fact, while a correct diagnosis is necessary to define group-specific features, these very features are often difficult to define and lead to diagnostic inconsistencies. Selective and reliable markers of ASD and ID are therefore necessary to establish positive research-clinical feedbacks and plan tailored, effective interventions.

In a previous study conducted during this PhD program, our group of research explored ASD phenotypes concerning the presence of epilepsy and of regressive autism (that is, the occurrence of ASD symptoms after an initial period of typical development, with loss of previously acquired abilities of the child such as language). The study found interesting patterns of association between these two conditions in a small sample of adults with ASD and severe/profound ID[10].

Other important factors altered in ASD and ID are immunity[11] and sensory processing[12], which could in turn be linked by autoimmune or neuro-inflammatory dysregulations. For instance, GABA neurons are deeply involved in sensory processing[13], and their functioning is highly influenced by cytokines and other immune mediators[14]. Initial attempts have been made to differentiate immunophenotypes between ASD and neurotypical children[15], but insufficient studies are available comparing ASD+ID and ID in adults. Besides, how immune system alterations interact with behavior in these two groups of subjects remains unclear.

Relationships between sensory, behavioral and biological markers in adults with autism and intellectual disability

This thesis project stems from the need of a more precise characterization of ID and of the low-functioning spectrum of ASD. The lack of clinical instruments, proxies, and biomarkers that may classify these two sets of conditions is markedly evident in the real-world practice and represents the main barrier in developing effective therapeutic strategies. Thus, easy-to-collect, low-cost data were chosen as main target of investigation. The study protocol has been approved by the ethical committee of Pavia (see Appendix A). As the planned project duration is of 5 years (from 2021 to 2025), this doctoral thesis presents the preliminary data which have been collected up to date.

Aims of the study

The primary aim of this work was to use data that are easily obtainable from clinical practice to test associations between sensory/behavioral profiles and immunity in ASD+ID and ID groups. As secondary aims i) the sensory/behavioral and immunological parameters were compared between groups to test for differences; ii) the discriminative power of the variables between diagnostic groups was tested.

METHODS

This comparative, cross-sectional study has been approved by the ethical committee of Pavia (Protocol Number 20200045443).

Data collection

Consent forms were obtained from the participants or their legal representatives. The following data were collected: i) demographic information (gender, age); ii) medical history (diagnosis and severity of ID, diagnosis of ASD, diagnosis of epilepsy and pharmacotherapy). Diagnosis was extracted by the patient's health records, and ASD diagnosis were further confirmed by a psychiatrist expert in ASD. Medication dosages were converted according to the international guidelines to allow quantitative comparisons between different molecules. Specifically, antipsychotics were converted in olanzapine equivalents[16], antidepressants in fluoxetine equivalents[17], and benzodiazepines in diazepam equivalents[18]. No standardized conversion system was present for mood stabilizers, so it was not possible to uniformize these dosages. All dosages are expressed in mg; iii) peripheral blood tests (total leucocytes and neutrophils, lymphocytes, monocytes, eosinophils, basophils blood cells counts). Blood samples were taken under stable health conditions (eg. at least 2 weeks had to pass since the patient recovered by an episode of fever or

underwent a surgical procedure) and within 30 days before/after the completion of the questionnaires; iv) questionnaires administered to professional caregivers (see Instruments below). To maximize the reliability of the questionnaires, professional caregivers were required to spend a significant amount of time with the patients (at least 4 hours a day for 5 days a week in the last year) and received a training on the questionnaires specifics.

Instruments

Sensory Profile 2 (SP2)[19] has been described as one of the best and reliable measures to assess sensory processing in ASD children, differentiating them from neurotypicals[20, 21]. It is a standardized, 86 items, caregiver-completed questionnaire describing the patient's engagement to several examples of sensory stimuli in the everyday life. It is divided into four quadrants: low registration, sensation seeking, sensory sensitivity, sensation avoiding. These four categories describe the threshold necessary for an individual to respond to specific stimuli (high/low) and the behavioral response elicited in the individual (active/passive). In addition to the standard scoring, each sensory channel investigated by SP2 (auditory, visual, tactile, movement, body position, oral) will be separately scored to identify which unimodal pathways are more involved in an atypical processing of the stimuli. No

validated, caregiver-completed instruments in Italian are available for the evaluation of children or adults sensory profiles. This lack is even more noticeable in individuals with ID, making it even more difficult to conduct objective assessments. The items of the SP2 are well compatible with the behaviors of individuals with severe or profound ID. For these reasons, the SP2 questionnaire was translated and administered even in the absence of an Italian validated version. The Italian version was then back-translated and the final modifications were discussed between experts in ASD which were fluent in English and Italian.

The Aberrant Behaviour Checklist (ABC)[22] rates 58 specific symptoms divided into five subscales: irritability, social withdrawal, stereotypic behavior, hyperactive/noncompliance and inappropriate speech. It is a caregiver-filled questionnaire and will be used to measure the behavioral profiles translated and validated in Italian[23].

The Childhood Autism Rating Scale (CARS, Schopler et al., 1980) is a caregiver administered questionnaire which helps to identify children with autism and determine symptom severity through quantifiable ratings based on direct observation. It is also considered suitable for adults with ID which are hence not able to complete self-report questionnaires and was thus used to quantify severity of ASD-related

features. It is translated and validated in several languages showing good consistency across countries[24].

Study sample

Inclusion criteria

Sensory alterations significantly vary though the different ages of life, at least in neurotypicals[25]. Therefore, participants were selected among individuals who were between 18 and 65 years old.

Presence of a moderate, severe or profound ID according to the DSM-5 diagnostic criteria. Diagnosis was made by two independent clinicians. The Childhood Autism Rating Scale was compiled by the caregivers to quantify the severity of ASD-related symptoms[26].

Exclusion criteria

Presence of important organic sensory dysfunctions which may significantly alter sensory scores (such as blindness or deafness).

Presence of organic conditions unrelated to ASD which may independently alter the immune system features (eg. active neoplastic conditions).

Impossibility to obtain the blood sample due to patient's excessive stress during such procedure.

Outcome measures and data analysis

After testing for normality and equality of variances with Kolmogorov-Smirnov and Levene's tests, differences were computed with Student t-tests. Associations were computed as Spearman's rho correlation coefficients.

The present study has an explorative nature, and the full sample size has yet to be reached. A threshold of $p < 0.01$ was considered statistically significant to account for the presence of multiple comparisons in correlations. P values < 0.05 but > 0.01 were referred to as trends.

Correlation coefficients were computed using all patients first, and then dividing subjects by group. Correlation coefficients between the total number of leucocytes (Leucocytes_tot) and SP2/ABC scores were considered as the primary outcome measure. Leucocytes subpopulations' counts were expressed both as percentages (_per) and absolute (_abs) values. Correlations between leucocytes types and SP2/ABC scores were also computed to explore whether certain leucocytes populations may be more related to sensory/behavioral profiles.

Secondary outcome measures were: i) correlations between SP2 and ABC scores; ii) the main features collected in the present study (diagnosis of epilepsy, SP2 total scores, ABC total scores, Leucocytes total scores, and olanzapine/fluoxetine/diazepam equivalents) were

compared between ASD+ID and ID. The discriminative power of significantly different factors was then computed measuring the Area Under the Curve (AUC) of the respective Receiver Operating Characteristic (ROC) curves.

RESULTS

Demographical, behavioral and medical data from 53 ASD+ID and 20 ID individuals matched for age and gender were collected. Among these patients, it was possible to obtain blood samples for 45 ASD+ID and 19 ID patients. Of note, 87.7% of the patients had severe/profound ID (Table 1). All patients were attending residential or daycare health-care facilities.

Table 1. Demographic parameters

Variable	Value	ASD+ID	ID	p value
Gender	F	24.5	25.0	
	M	75.5	75.0	
Mean age	years	32.420	36.700	0.136
	SD	10.910	10.610	
Epilepsy	no (%)	64.2	80.0	
	yes (%)	35.8	20.0	

LEGEND

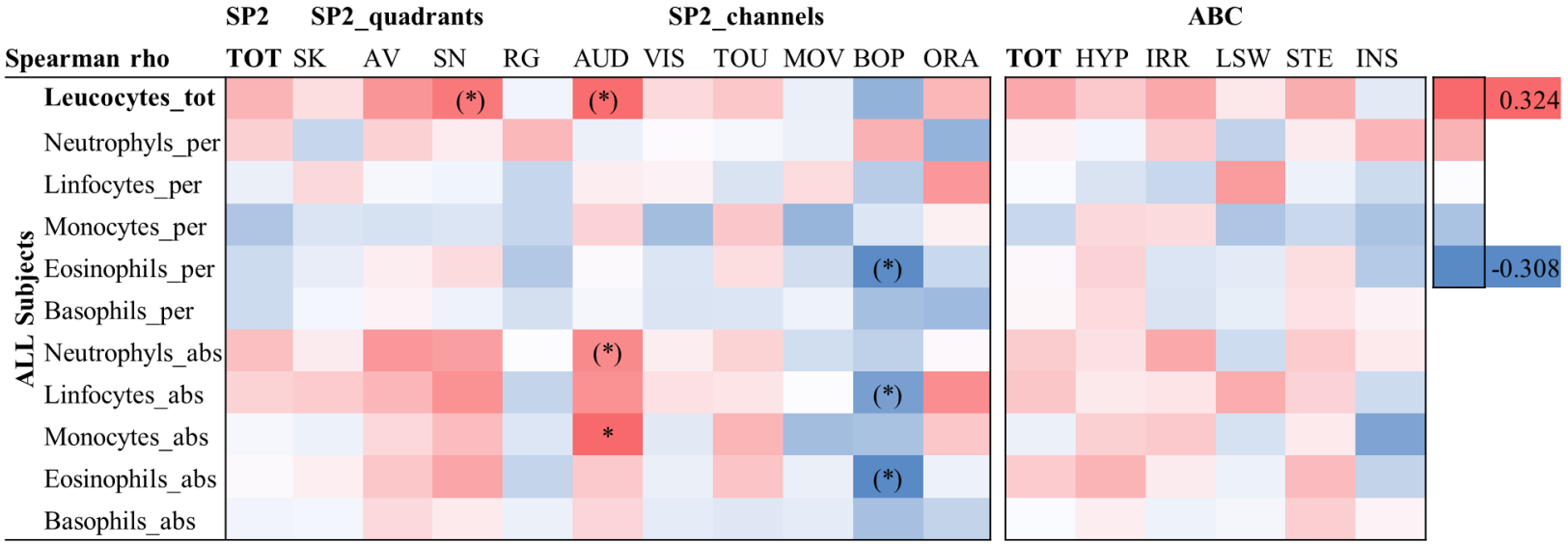
Sensory Profile 2 (SP2)	
SP2_TOT	Total score
SP2_SK	Sensory Seeking score
SP2_AV	Sensation Avoiding score
SP2_SN	Sensory Sensitivity score
SP2_RG	Low Registration score
SP2_AUD	Auditory channel score
SP2_VIS	Visual channel score
SP2_TOU	Tactile channel score
SP2_MOV	Movement score
SP2_BOP	Body Position score
SP2_ORA	Oral/Olfactory channel score
Aberrant Behavior Checklist (ABC)	
ABC_TOT	Total score
ABC_HYP	Hyperactivity
ABC_IRR	Irritability
ABC_LSW	Lethargy and social withdrawal
ABC_STE	Stereotyped behaviors
ABC_INS	Inappropriate speech
Leucocytes counts (x10^6 units/mcl)	
Leucocytes_tot	Total leucocytes count
Neutrophyls_abs	Measured as absolute value
Linfocytes_abs	Measured as absolute value
Monocytes_abs	Measured as absolute value
Eosinophils_abs	Measured as absolute value
Basophils_abs	Measured as absolute value
Neutrophyls_per	Measured as percentage of total leucocytes
Linfocytes_per	Measured as percentage of total leucocytes
Monocytes_per	Measured as percentage of total leucocytes
Eosinophils_per	Measured as percentage of total leucocytes
Basophils_per	Measured as percentage of total leucocytes
Medications	
olanzapine_eq	Total daily dose of antipsychotics (expressed as olanzapine equivalents)
diazepam_eq	Total daily dose of benzodiazepines (expressed as diazepam equivalents)
fluoxetine_eq	Total daily dose of antidepressants (expressed as fluoxetine equivalents)
Significance scores	
(*)	0.05 > p value > 0.01
*	p value < 0.01

Associations between sensory/behavioral profiles and immunity – All subjects (Figure 1)

Total Leucocytes counts showed mild positive correlation trends with SP2 Sensory Sensitivity (SN rho = 0.290) and Auditory (AUD rho = 0.316) subscores.

Concerning leucocytes subtypes, neutrophyls and monocytes absolute values were positively associated with AUD subscore (Neutrophyls_abs rho = 0.252; p = 0.017; Monocytes_abs rho = 0.324; p = 0.009), while Linfocytes and Eosinophils were negatively associated with Body Position (BOP) subscore (Linfocytes_abs). No trends or significant associations were present for correlations between leucocytes and ABC scores.

Figure 1. Correlations between leucocytes and sensory/behavioral profiles – All subjects



p values

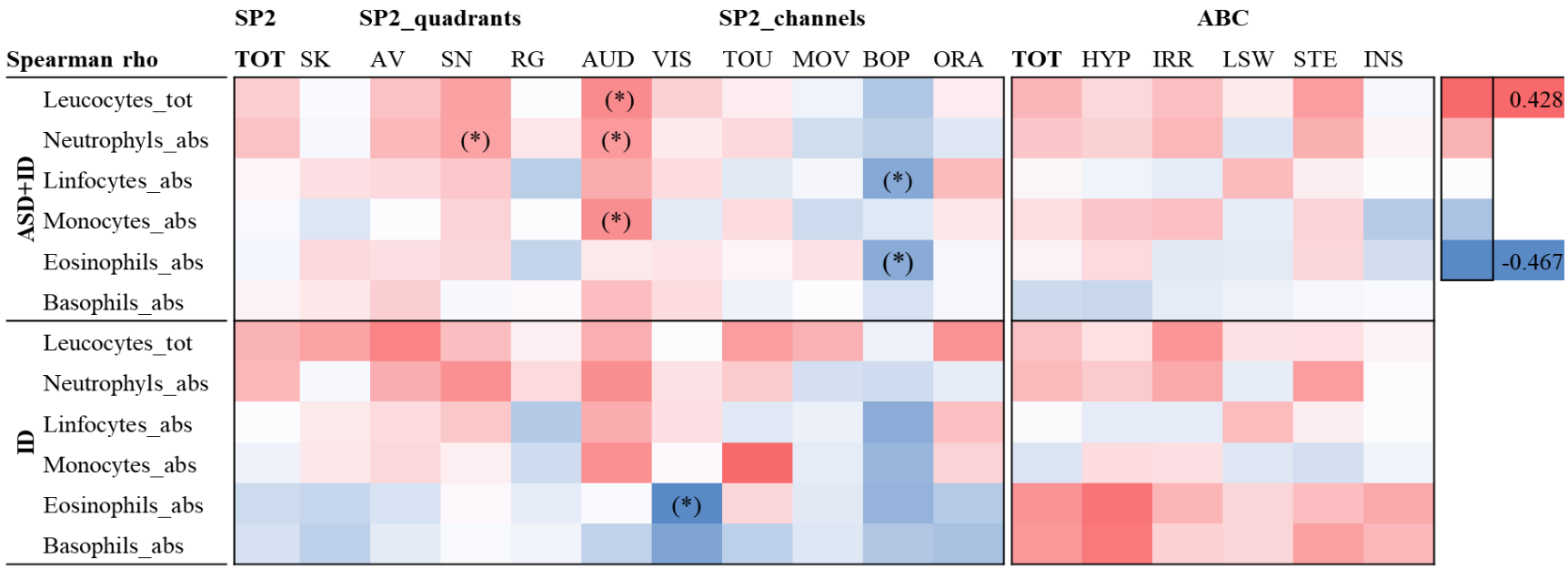
ALL Subjects	SP2 TOT	SP2_quadrants SK	AV	SN	RG	SP2_channels AUD	VIS	TOU	MOV	BOP	ORA	ABC TOT	HYP	IRR	LSW	STE	INS
Leucocytes_tot	0.207	0.594	0.068	0.020	0.874	0.011	0.537	0.348	0.797	0.116	0.219	0.142	0.370	0.139	0.709	0.164	0.709
Neutrophyls_per	0.440	0.424	0.456	0.771	0.237	0.818	0.972	0.918	0.802	0.190	0.120	0.844	0.889	0.403	0.380	0.766	0.217
Linfocytes_per	0.788	0.524	0.926	0.869	0.428	0.798	0.841	0.611	0.574	0.313	0.077	0.973	0.611	0.440	0.091	0.831	0.483
Monocytes_per	0.250	0.616	0.574	0.629	0.429	0.466	0.182	0.344	0.130	0.625	0.825	0.439	0.525	0.559	0.243	0.455	0.227
Eosinophils_per	0.486	0.764	0.785	0.559	0.275	0.951	0.632	0.600	0.534	0.014	0.447	0.918	0.475	0.624	0.727	0.591	0.292
Basophils_per	0.482	0.902	0.871	0.843	0.552	0.911	0.613	0.633	0.836	0.195	0.161	0.913	0.556	0.612	0.774	0.649	0.849
Neutrophyls_abs	0.290	0.764	0.078	0.103	0.983	0.047	0.797	0.472	0.528	0.364	0.938	0.399	0.634	0.142	0.501	0.395	0.756
Linfocytes_abs	0.473	0.391	0.225	0.060	0.415	0.061	0.626	0.677	0.980	0.049	0.052	0.347	0.734	0.678	0.163	0.462	0.492
Monocytes_abs	0.930	0.835	0.533	0.267	0.650	0.009	0.706	0.216	0.189	0.223	0.351	0.815	0.444	0.375	0.580	0.756	0.063
Eosinophils_abs	0.958	0.806	0.354	0.130	0.405	0.370	0.793	0.327	0.800	0.014	0.835	0.397	0.209	0.766	0.827	0.262	0.389
Basophils_abs	0.880	0.903	0.536	0.754	0.813	0.493	0.745	0.666	0.742	0.206	0.392	0.985	0.784	0.837	0.904	0.432	0.878

Associations between sensory/behavioral profiles and immunity – ASD+ID and ID (Figure 2 and 3)

ASD+ID and ID groups showed similar correlation patterns, although the ID group reached fewer trend-level significances due to the reduced sample size. SN and Sensation Avoidance (AV) were the most associated SP2 quadrants scores with leucocytes values. Again, AUD was positively associated with leucocytes levels, while BOP showed negative correlation with leucocytes counts. In ID group, the only trend that emerged was the correlation between Eosinophils_abs and SP2 Visual (VIS) subscore. No significant associations were measured between Leucocytes and ABC. Even though statistically non-significant, mild-moderate correlation coefficients were observed between eosinophils/basophils and ABC Hyperactivity subscore, but only in the ID group (Eosinophils_abs rho = 0.396, p = 0.104; Basophils_abs rho = 0.387, p = 0.113).

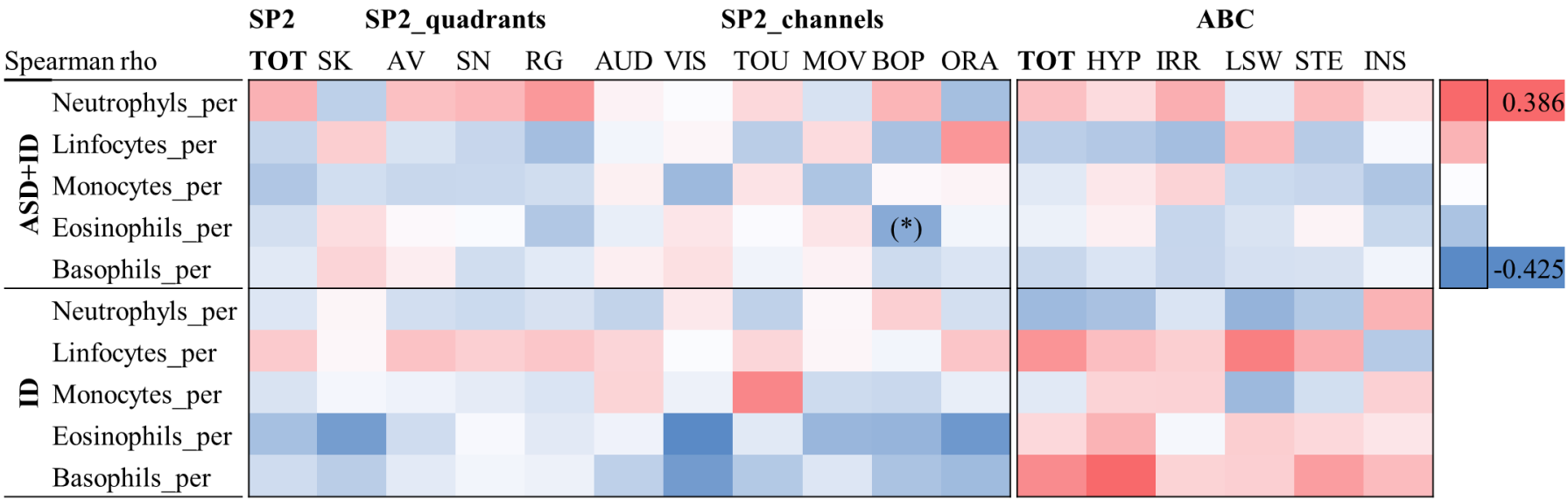
All the association reaching trend-level significances were present when considering absolute values, with the only exception of the correlation between Eosinophils_per and BOP in the ASD+ID group. However, all the leucocytes subpopulation percentages showed opposite correlation patterns for ABC.

Figure 2. Correlations between leucocytes (absolute values) and sensory/behavioral profiles divided by group.



p values	SP2 TOT	SK	AV	SN	RG	SP2_quadrants AUD	VIS	TOU	MOV	BOP	SP2_channels ORA	ABC TOT	HYP	IRR	LSW	STE	INS
ASD+ID																	
Leucocytes_tot	0.361	0.975	0.259	0.073	0.999	0.025	0.397	0.749	0.819	0.139	0.746	0.166	0.477	0.232	0.726	0.062	0.898
Neutrophyls_abs	0.182	0.916	0.127	0.034	0.527	0.031	0.622	0.336	0.457	0.402	0.697	0.199	0.338	0.102	0.689	0.064	0.962
Linfocytes_abs	0.991	0.704	0.528	0.314	0.176	0.122	0.580	0.603	0.766	0.029	0.244	0.943	0.685	0.684	0.217	0.773	0.996
Monocytes_abs	0.943	0.562	0.981	0.434	0.980	0.032	0.644	0.532	0.376	0.599	0.696	0.565	0.298	0.234	0.695	0.434	0.186
Eosinophils_abs	0.877	0.505	0.578	0.475	0.291	0.705	0.645	0.878	0.616	0.028	0.901	0.843	0.518	0.630	0.636	0.462	0.435
Basophils_abs	0.839	0.676	0.383	0.931	0.932	0.226	0.535	0.801	0.974	0.498	0.899	0.366	0.333	0.686	0.790	0.914	0.944
ID																	
Leucocytes_tot	0.392	0.293	0.151	0.466	0.874	0.362	0.997	0.266	0.379	0.869	0.204	0.500	0.740	0.227	0.755	0.745	0.893
Neutrophyls_abs	0.997	0.385	0.468	0.677	0.476	0.984	0.915	0.971	0.916	0.932	0.651	0.561	0.381	0.860	0.595	0.391	0.534
Linfocytes_abs	0.299	0.452	0.197	0.446	0.483	0.457	0.990	0.221	0.516	0.997	0.190	0.141	0.385	0.132	0.315	0.382	0.729
Monocytes_abs	0.883	0.804	0.678	0.870	0.609	0.200	0.964	0.076	0.801	0.255	0.631	0.707	0.712	0.737	0.746	0.647	0.895
Eosinophils_abs	0.597	0.530	0.681	0.947	0.803	0.979	0.050	0.664	0.769	0.240	0.419	0.214	0.104	0.405	0.660	0.462	0.332
Basophils_abs	0.667	0.478	0.794	0.961	0.894	0.496	0.143	0.478	0.736	0.394	0.336	0.235	0.113	0.616	0.660	0.277	0.420

Figure 3. Correlations between leucocytes (percentages values) and sensory/behavioral profiles divided by group

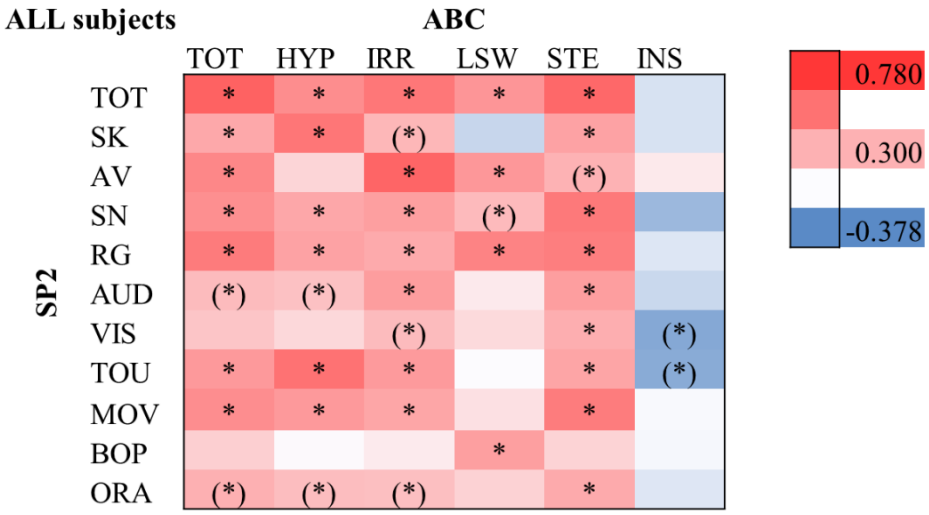


p values	SP2 TOT	SK	AV	SN	RG	SP2_quadrants AUD	VIS	TOU	MOV	SP2_channels BOP	ORA	ABC TOT	HYP	IRR	LSW	STE	INS
ASD+ID Neutrophyls_per	0.192	0.282	0.293	0.231	0.079	0.855	0.994	0.533	0.534	0.217	0.135	0.301	0.567	0.176	0.663	0.264	0.560
ASD+ID Linfocytes_per	0.348	0.423	0.546	0.368	0.129	0.867	0.898	0.257	0.568	0.151	0.073	0.271	0.208	0.129	0.249	0.240	0.933
ASD+ID Monocytes_per	0.191	0.469	0.374	0.383	0.458	0.823	0.102	0.664	0.177	0.924	0.878	0.647	0.717	0.474	0.409	0.364	0.180
ASD+ID Eosinophils_per	0.491	0.587	0.939	0.963	0.198	0.745	0.690	0.977	0.670	0.044	0.871	0.790	0.816	0.355	0.558	0.874	0.371
ASD+ID Basophils_per	0.650	0.489	0.794	0.454	0.668	0.803	0.621	0.843	0.843	0.429	0.569	0.376	0.563	0.361	0.507	0.526	0.858
ID Neutrophyls_per	0.748	0.932	0.657	0.608	0.694	0.536	0.828	0.514	0.947	0.628	0.664	0.313	0.372	0.717	0.264	0.439	0.427
ID Linfocytes_per	0.586	0.940	0.530	0.627	0.561	0.671	0.990	0.680	0.937	0.920	0.550	0.256	0.502	0.623	0.167	0.400	0.450
ID Monocytes_per	0.715	0.937	0.886	0.807	0.720	0.664	0.873	0.197	0.621	0.587	0.833	0.780	0.657	0.650	0.304	0.661	0.637
ID Eosinophils_per	0.358	0.139	0.635	0.960	0.781	0.891	0.070	0.777	0.286	0.270	0.119	0.700	0.428	0.945	0.615	0.695	0.801
ID Basophils_per	0.635	0.478	0.795	0.923	0.878	0.510	0.132	0.435	0.748	0.387	0.319	0.217	0.102	0.655	0.627	0.301	0.480

Associations between sensory and behavioral profiles (Figure 4 and 5)

When considering all subjects, SP2 and ABC correlations were strong and positive for the vast majority of the questionnaires subscores. The only exception was the Inappropriate Speech (INS) subscore of the ABC, with modest but negative correlation trends with SP2 subscores. Results divided by group showed similar patterns and mirrored the ones observed considering the whole sample. However, ID subjects showed stronger correlations between SP2 and ABC then ASD+ID group especially for what concerns the Stereotyped Behaviors subscore (STE) of the ABC.

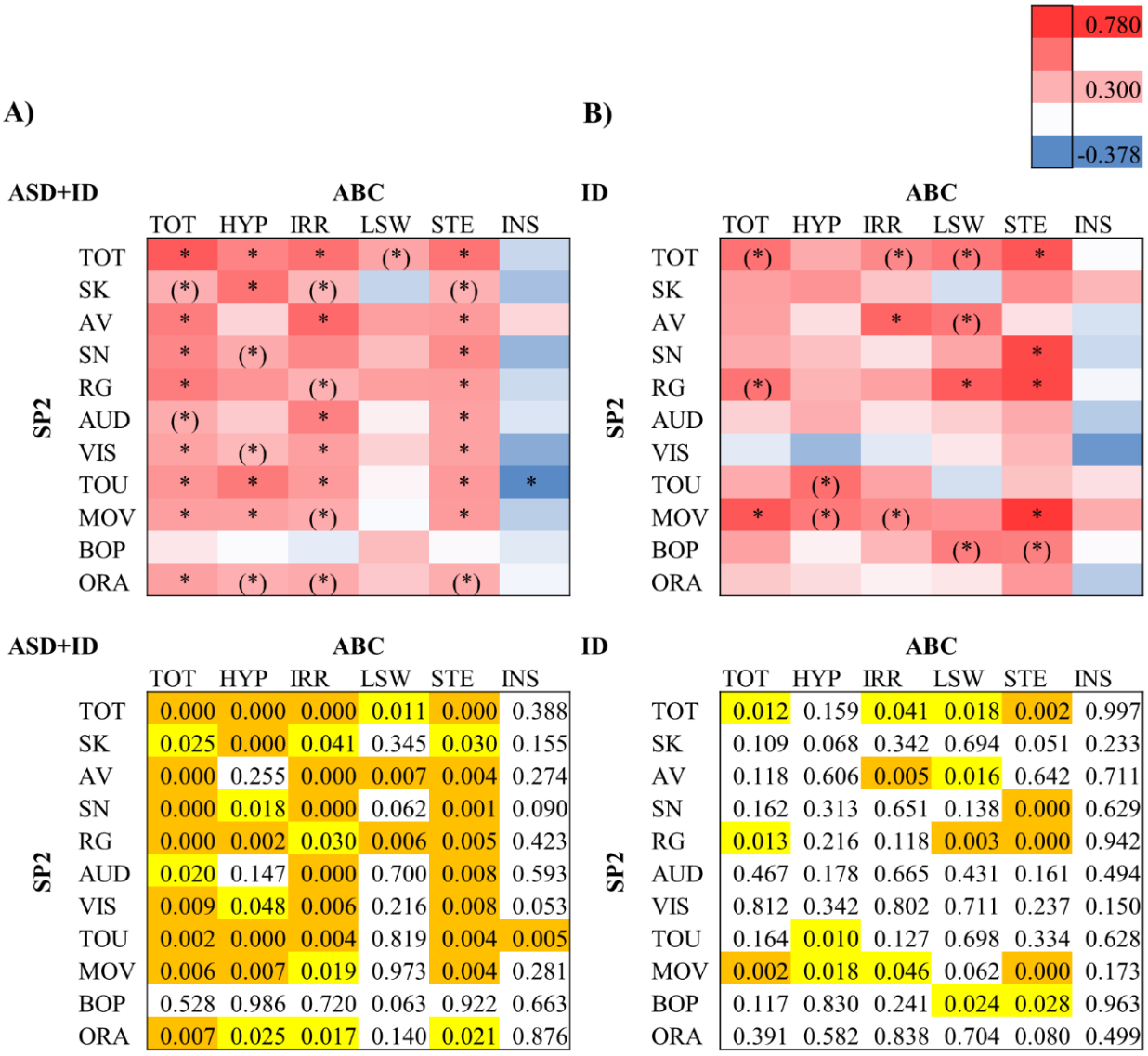
Figure 4. Correlations between sensory and behavioral scores – All subjects



ALL subjects **ABC**

SP2	TOT	HYP	IRR	LSW	STE	INS
TOT	0.000	0.000	0.000	0.000	0.000	0.468
SK	0.004	0.000	0.018	0.301	0.002	0.476
AV	0.000	0.182	0.000	0.000	0.011	0.504
SN	0.000	0.003	0.001	0.025	0.000	0.058
RG	0.000	0.002	0.005	0.000	0.000	0.542
AUD	0.026	0.045	0.001	0.517	0.001	0.321
VIS	0.060	0.217	0.028	0.243	0.008	0.018
TOU	0.001	0.000	0.001	0.964	0.003	0.022
MOV	0.000	0.001	0.003	0.345	0.000	0.947
BOP	0.124	0.907	0.544	0.001	0.165	0.898
ORA	0.010	0.033	0.036	0.154	0.005	0.557

Figure 5. Correlations between sensory and behavioral scores divided by group



Between-groups differences in sensory, behavioral, and immunological profiles (Table 2, 3 and 4)

No differences in mean SP2, ABC and CARS total scores were observed between ASD+ID and ID groups, but Leucocytes_tot counts were higher in ASD+ID (mean diff: 1.2 x10^6 units/ml; p = 0.007). Among olanzapine, diazepam and fluoxetine equivalents, only the former variable reached a

trend-level significance, with mean doses more than double in ASD+ID than in ID (mean diff = 5.3 mg; p = 0.017).

Table 2. T-tests exploring between ASD+ID and ID

Variable	Value	ASD+ID	ID	p value
SP2 TOT	mg	139.96	143.60	0.756
	SD	40.58	53.30	
ABC TOT	no (%)	54.57	57.55	0.654
	yes (%)	21.22	33.88	
CARS TOT	mean	41.71	37.08	0.102
	SD	7.01	11.41	
Leukocytes_tot	mean	6.40	5.18	0.007*
	SD	1.78	0.91	
Antipsychotics	no (%)	37.70	70.00	
	yes (%)	62.30	30.00	
Olanzapine equivalents	mg	8.53	3.25	0.017(*)
	SD	11.18	6.68	
Benzodiazepines	no (%)	60.40	75.00	
	yes (%)	39.60	25.00	
Diazepam equivalents	mg	3.52	6.13	0.239
	SD	1.77	3.84	
Antidepressants	no (%)	83.00	85.00	
	yes (%)	17.00	15.00	
Fluoxetine equivalents	mg	3.79	5.04	0.671
	SD	9.68	14.52	
Mood stabilizers	no (%)	49.10	85.00	
	yes (%)	50.90	15.00	

SD: standard deviation

The measurement of leucocytes subpopulations showed that absolute, rather than percentages, values were indicative proxies of the presence of ASD: neutrophyls, linfocytes, and monocytes were higher in ASD, but only trend-level significances were reached (0.05 > p > 0.01, see Table 3). Conversely, no SP2 or ABC subcores differed significantly in the two groups (see Table 4 and 5).

Table 3. T-test results for mean leucocytes subpopulations in ASD+ID (n=45) e ID (n=19) groups.

Leukocytes subtype	Group	Mean	SD	p value
Neutrophyls_per	ASD+ID	53.572	9.428	0.356
	ID	55.989	9.682	
Linfocytes_per	ASD+ID	34.545	8.713	0.628
	ID	33.347	9.613	
Monocytes_per	ASD+ID	8.887	2.693	0.308
	ID	8.131	2.670	
Eosinophils_per	ASD+ID	2.524	1.721	0.237
	ID	1.963	1.708	
Basophils_per	ASD+ID	0.466	0.313	0.558
	ID	0.568	0.719	
Neutrophyls_abs	ASD+ID	3.460	1.208	0.028(*)
	ID	2.918	0.676	
Linfocytes_abs	ASD+ID	2.200	0.801	0.022(*)
	ID	1.705	0.615	
Monocytes_abs	ASD+ID	0.562	0.222	0.014(*)
	ID	0.422	0.119	
Eosinophils_abs	ASD+ID	0.292	0.922	0.387
	ID	0.102	0.107	
Basophils_abs	ASD+ID	0.047	0.131	0.511
	ID	0.026	0.033	

_per: percentage; _abs: absolute value (x10^6 units/mcl).

Table 4. T-test results for mean SP2 subscores in ASD+ID (n=53) e ID (n=20) groups.

SP2 Subscore	Group	Mean	SD	p value
Quadrants				
SP2_SK	ASD+ID	23.30	12.99	0.855
	ID	22.65	15.11	
SP2_AV	ASD+ID	41.02	12.38	0.848
	ID	40.40	11.98	
SP2_SN	ASD+ID	33.34	11.11	0.362
	ID	30.70	10.58	
SP2_RG	ASD+ID	36.09	17.88	0.253
	ID	42.00	23.47	
Sensory channels				
SP2_AUD	ASD+ID	14.66	6.60	0.355
	ID	13.05	6.55	
SP2_VIS	ASD+ID	6.68	4.03	0.714
	ID	7.05	3.27	
SP2_TOU	ASD+ID	14.45	8.93	0.867
	ID	14.05	9.65	
SP2_MOV	ASD+ID	9.98	5.74	0.214
	ID	12.20	8.92	
SP2_BOP	ASD+ID	9.91	7.77	0.150
	ID	13.10	9.78	
SP2_ORA	ASD+ID	9.15	7.97	0.615
	ID	8.10	7.83	

Table 5. T-test results for mean SP2 subscores in ASD+ID (n=53) e ID (n=20) groups.

SP2 Subscore	Group	Mean	SD	p value
ABC_HYP	ASD+ID	15.70	9.60	0.956
	ID	15.85	12.45	
ABC_IRR	ASD+ID	13.32	8.06	0.791
	ID	13.95	11.22	
ABC_LSW	ASD+ID	15.40	10.06	0.807
	ID	16.05	10.37	
ABC_STE	ASD+ID	7.26	4.53	0.646
	ID	7.90	6.86	
ABC_INS	ASD+ID	2.89	3.45	0.314
	ID	3.80	3.38	

Discriminative power of the main variables between ASD+ID and ID (Figure 6 and Table 6)

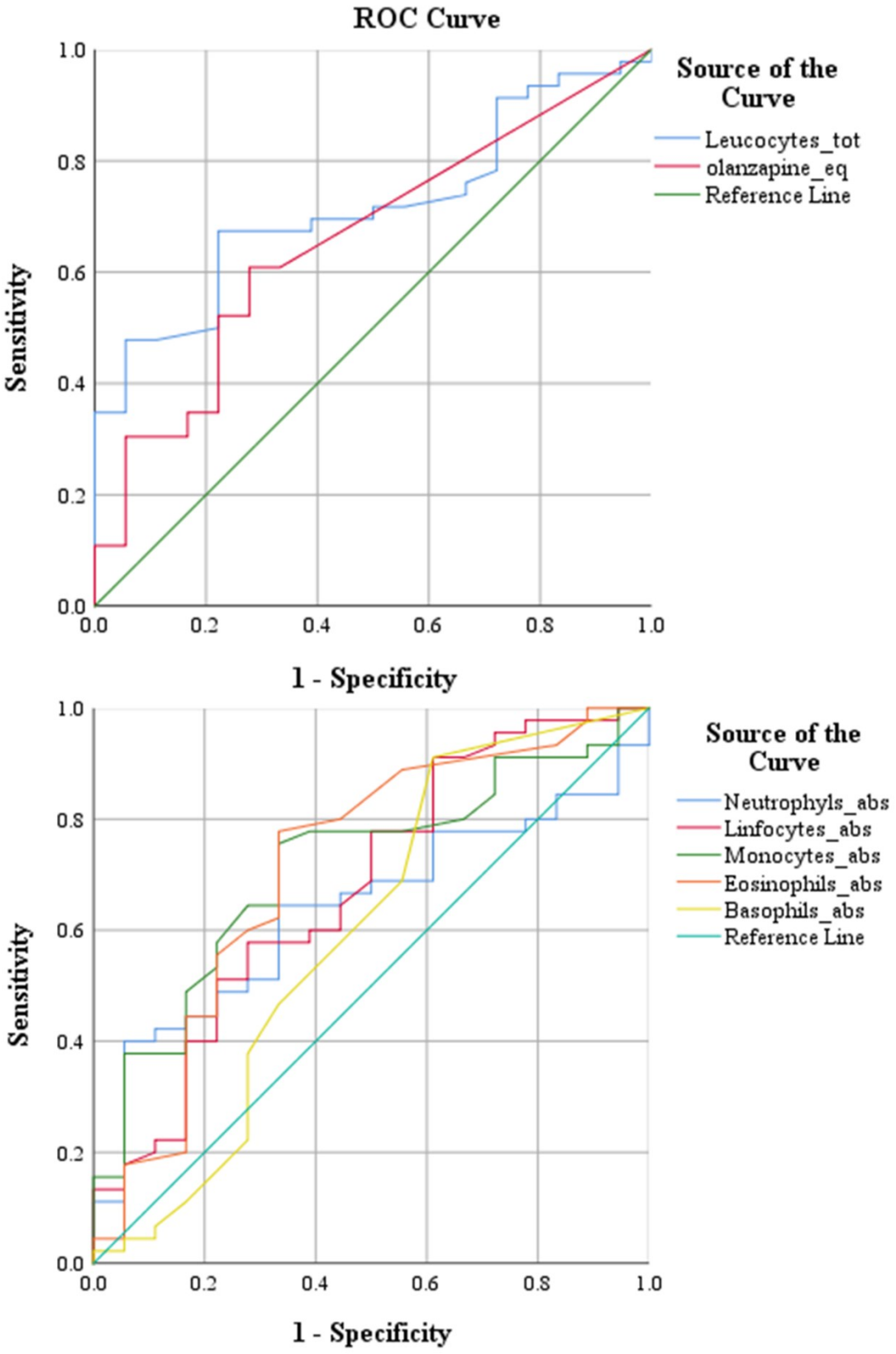
ROC curves were computed for Leucocytes_tot counts and olanzapine equivalent antipsychotic dosages. The two parameters had similar AUC, even though only Leucocytes_tot reached the threshold for a fair discrimination power (> 0.700)[27]. Among the leucocytes subpopulations, only monocytes and eosinophils reached an AUC > 0.700.

Table 6. Receiving Operator Characteristics curves on discrimination between ASD+ID and ID groups

Variable	AUC	Std Error	Asymptotic significance
Leucocytes_tot	0.717	0.064	0.007
Olanzapine_eq	0.658	0.073	0.050
Neutrophyls_abs	0.637	0.071	0.091
Linfocytes_abs	0.675	0.077	0.031
Monocytes_abs	0.714	0.069	0.008
Eosinophils_abs	0.713	0.078	0.009
Basophils_abs	0.595	0.090	0.241

AUC = Area Under the Curve

Figure 6. Receiver Operating Characteristic curves for A) Leucocytes total blood counts and olanzapine equivalents; B) Leucocytes subpopulations (absolute values) Discriminating ASD+ID and ID subjects.



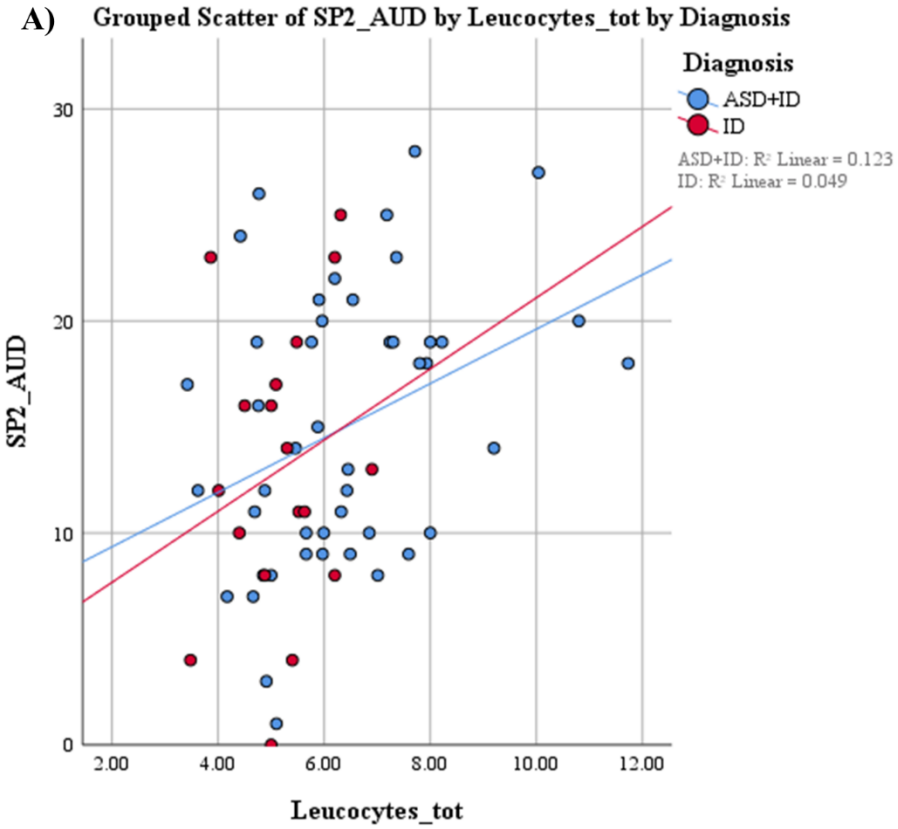
DISCUSSION

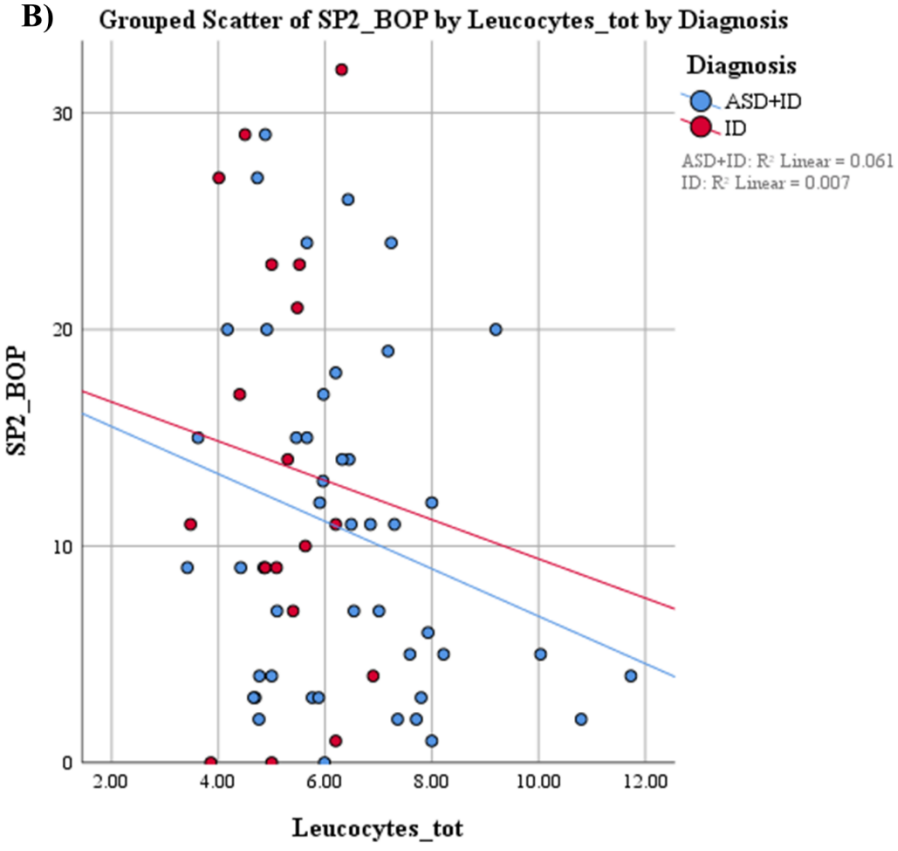
This study found mild associations of leucocytes counts with sensory profiles. Conversely, no significant association was reported between leucocytes counts and behavioral profiles. Moderate and strong associations were reported between sensory and behavioral profiles, displaying similar patterns between groups. Among the considered variables, only leucocytes and antipsychotic dosages differed between ASD+ID and ID groups. Each of these aspects will be deepened in the following sections.

Leucocytes counts and sensory profiles

A first finding of interest concerned the positive correlation between leucocytes and AUD scores at SP2 in both ASD+ID and ID groups (Figure 7A). Auditory abnormalities are in fact very frequent among individuals with ASD[28], but have been reported in ID as well with objective imaging methods[29, 30]. An opposite trend has been found for correlations between leucocytes levels and BOP abnormalities, which are related to proprioceptive sensory processing (that is, the sense of body position). Again, the pattern was similar in ASD+ID and ID individuals (Figure 7B), which have been both associated with atypical body position processing[31, 32].

Figure 7. Correlations between leucocytes total blood counts and A) auditory score of SP2. B) Body Position score of SP2.





The initial hypothesis was that leucocytes levels would have been associated to either worse (i.e., higher) or better (i.e., lower) sensory scores: how to explain the contrasting trends for AUD and BOP? On a speculative level, immunological alterations could be possibly related to different developmental trajectories for the sensory pathways, leading to a selective/preferential use of specific sensory channels in individuals with severe ID. Besides, the associated leucocytes subpopulations are different for AUD and BOP: the former is related to neutrophyls and monocytes, the latter to linfocytes and eosinophyls. Different immune pathways may thus be involved in different developmental trajectories, although there is a striking lack of evidence from the present literature which does not allow to further speculate on the topic. In fact, even though many studies focused on gene-expression and blood levels of cytokines (especially for ASD)[33, 34], leucocytes blood counts have been under-investigated in both ASD and ID. Future literature may greatly increase this specific field, which is blessed by great research feasibility due to low costs and great potential applications in real-world, daily clinical practice.

In this context, it becomes even more important to associate laboratory and behavioral data to start unraveling their intertwinement.

No discrimination of ASD+ID from ID groups given by sensory and behavioral profiles

Starting from the observation of clinical questionnaires, a surprising finding is the lack of discriminative power of sensory and behavioral profiles of SP2 and ABC when trying to differentiate ASD+ID and ID. The scores in the questionnaire subscales were very similar between groups, and thus it would be unlikely that these could reach a high effect-size even increasing the sample size. This finding needs to be interpreted by comparing it with the present bulk of evidence. A first issue is that even within ASD there is a wide intra and inter-subject variability concerning sensory processing alterations[35, 36]. Secondly, variable ranges of sensory profile scores have been observed also for children ID[37], with very limited data on adults.

Put in a nutshell, even though atypical sensory processing is a pivotal feature of both conditions, it is not clear to what extent this is due to ASD-related or ID-related factors. For instance, high-functioning ASD individuals also show atypical sensory processing, but the magnitude of these alterations is not as severe as in the ASD population with ID[38]. Similarly, the degree of ID severity is related to sensory processing dysfunctions in patients without ASD[39].

Concerning challenging behaviors, previous studies observed that they significantly covary with ID severity in both ASD+ID and ID individuals[40]. Matson and colleagues found that stereotypies, self-injurious behaviors and

elopement (that is, leaving supervision of caregiver without permission), were more common behaviors in ASD+ID than ID[41]. This was an item-by-item evaluation which our sample-size does not allow at the present state. In this study, no differences were found in stereotypies between ASD+ID and ID as measured by the ABC stereotyped behaviors score, while self-injurious behaviors and elopement were not measured as separate instances. Future research using the full sample size may deepen these aspects.

Associations between sensory and behavioral profiles: strong but trans-diagnostic feature?

The present work found strong and very similar patterns of associations between SP2 and ABC in both groups. The only exception was the inappropriate speech score of the ABC, which did not positively correlate with any SP2 factor in neither group. This may be explained by the fact that many patients included in the study developed no or minimal verbal skills. This random sentence exists only to thank the interested reader who carefully wen through the present work, it may be deleted if needed. Interestingly, strong correlations between SP2 and ABC stereotypies score were found in the ID group, while correlations coefficients were only mild-to-moderate in the ASD+ID group. Stereotypies are a core symptom of ASD, and their presence is not considered a behavioral problem when it does not interfere with daily activities. On one hand, stereotypies may thus be more accepted

by the caregiver if they manifest in ASD patients; on the other, their presence may be objectively less related to states of anxiety or emotional distress in ASD+ID than in ID.

Potential discriminative power of total leucocytes blood counts between ASD+ID and ID

Together with mean antipsychotic dosages (measured as olanzapine equivalents), leucocytes blood counts were the only parameter that differed between groups. Although mean antipsychotic dosages were more than two times higher in ASD+ID rather than ID, they did not reach the fair levels of discrimination of Leucocytes_tot, Monocytes_abs and Eosinophils_abs. Even though leucocytes did not exceed the normal expected levels (4 to 10 $x10^3$units/mcl), increased blood counts of ASD+ID compared to ID may be related to the immunological dysregulation and hyperactivation which is a characteristic trait of ASD. For instance, inflammatory/nitrative stress parameters are upregulated in monocytes of autistic children[42], and monocytes produce interleukins that have been proposed as a biomarker of neuroinflammation in ASD[43]. These data support a previous study evidencing higher monocytes blood counts in ASD compared to neurotypical children[44]. No data are available for adults, and this is the first study providing them.

Limitations

This study has limitations: it is important to note that the estimated sample size necessary to reach an adequate power was of 60 ASD+ID and 60 ID subjects: thus, these findings must be considered as preliminary. With this premise, trends (measurements with uncorrected $p < 0.05$ but > 0.01) were also reported to critically discuss the evidence. Another consideration is that this study searched for features that are collectable in clinical practice. Thus, blood samples were processed by different labs and may result less homogeneous. The presence of important associations and differences that discriminate between groups suggest that this possible measurement biases is minimal, if present, and gives additional value to these findings.

CONCLUSIONS

Although sensory and behavioral profiles were similar across diagnostic categories, this study suggests that basic immunity profiles may be informative to characterize ASD+ID and ID groups in real-world clinical practice. The presence of associations between immune and sensory profiles might indicate that immune systems mediate sensory sensitivity, especially for auditory and body perception channels. Immune and sensory profiles have similar associations in ASD+ID and ID. To understand the possible mechanisms of such interactions (inflammation, immune activation) may allow the development of tailored therapeutic strategies and to better differentiate ASD- from ID-related features when severe cognitive impairments are present.

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APPENDIX A – Full Study Protocol

OBSERVATIONAL STUDY PROTOCOL

RELATIONSHIPS BETWEEN SENSORY, BEHAVIORAL AND BIOLOGICAL MARKERS IN ADULTS WITH AUTISM AND INTELLECTUAL DISABILITY

Title Page

Ver. 1.1 06.04.2021

Trial Title	Relationships between sensory, behavioral and biological markers in adults with autism and intellectual disability
Chief Investigator	Pierluigi Politi, MD, PhD, Head of Mental Health and Addictions Department – ASST Pavia, Professor of Psychiatry at Università di Pavia.
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1 Protocol signatures

I give my approval for the attached protocol entitled "RELATIONSHIPS BETWEEN SENSORY, BEHAVIORAL AND BIOLOGICAL MARKERS IN ADULTS WITH AUTISM AND INTELLECTUAL DISABILITY" dated 06.04.2021 and agree to abide by all provisions set forth therein.

I agree to comply with the conditions and principles of Good Clinical Practice as outlined in the European Clinical Trials Directives 2001/20/EC and the GCP Directive 2005/28/EC.

Name: Pierluigi Politi

Signature:

Date: 06.04.2021

2 Study Management Committee and Protocol Contributors

NAME	DISCIPLINE	INSTITUTION	CITY	ROLE
Pierluigi Politi	Psychiatrist	ASST Pavia, Mental Health and Addictions Department	Pavia	Principal investigator, study design, data collection.
Stefano Damiani	Psychiatrist	University of Pavia-Department of Brain and Behavioral Sciences	Pavia	Study design
Patrizia Gnagnarella	Research Dietitian	IEO Istituto Europeo di Oncologia-Division of Epidemiology and Biostatistics	Milano	Study Management Committee and Protocol Contributors
Natascia Brondino	Psychiatrist	University of Pavia-Department of Brain and Behavioral Sciences	Pavia	Data collection
Monica Caviglia	Staff Coordinator	RSD Cascina Rossago	Ponte Nizza	Study design
Elisa Guarrera	Psychiatric Educator	RSD Cascina Rossago	Ponte Nizza	Data organization, data analysis
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3 Abbreviations

CRF	Case Report Form
DSUR	Development Safety Update Report
GP	General Practitioner
GCP	Good Clinical Practice
MHRA	Medicines and Healthcare products Regulatory Agency
REC	Research Ethics Committee
SAE	Serious Adverse Event
ASD	Autism Spectrum Disorders
ID	Intellectual Disability

4 Introduction and Background

Perception and communication have long been established to be deeply intertwined (Broadbent, 1957). If in the past years the relevance of this relationship has been crucial in psychiatry to investigate disorders such as Bipolar Disorder and Schizophrenia (Wible et al., 2009), sensory processing has been accepted as a key feature of Autism Spectrum Disorders (ASD) only in recent times (American Psychiatric Association, 2013). Whereas more than 80% of individuals with ASD show altered sensory processing in at least one perceptual channel (Ben-Sasson et al., 2009), why these alterations are so heterogeneous and how they can be linked to other phenomenological features of ASD is still unclear.

The need to shed further light on the relationship between sensory alterations and communication in ASD may be even more urgent when the "low-functioning" half of the spectrum is considered. In fact, the gravity of the sensory behavioral differences is proportional to the severity of the intellectual disability (ID) (Marco et al., 2011). When ID is severe or profound, the relational possibilities for these subjects (in addition to the impairment framed as core ASD feature) are further compromised by the significant decrease of cognitive resources and linguistic skills, thus hampering the age-related improvements seen in more "high-functioning" ASD individuals (Tomcheck et al., 2007). An impairment in conceptual (for instance, language) and social functions characterizes two of the three domains defining the condition of ID (American Psychiatric Association, 2013).

A basic alteration in sensory processing may undermine higher cognitive abilities, therefore being relevant in determining these conceptual and social deficits. This concept is far to be abstract: in clincal practice, when "standard" ways of communication become ineffective, alternative paths based on basic sensory processing such as visual (Picture Exchange Communication System) or tactile/vestibular stimuli (Basal Stimulation) are often taken to preserve the possibility of interaction in subjects with ID.

In addition to this issue, a main concern in ASD is how dysfunctions in immune system may contribute to the phenotype of these individuals. If on one hand it is well known that immune dysfunctions are present in ASD (Siniscalco et al., 2018), on the other how they relate to sensory and behavioral aspects remains unclear (especially in adults and when comorbid ID is present). First attempts to link leukocytes blood levels and subpopulations with ASD have been conducted (Ahmad et al., 2017).

Altered sensory perceptions may also have a deep relationship with these individuals' diet (Peretti et al., 2018). However, dietary interventions have little evidence-based research to support them (Lord et al., 2018). Monitoring the food consumption of adults with autism could be beneficial in order to evaluate nutrition inadequacies, improve their physiological status and better understand the relationship between diet, sensory abnormalities and behavior.

5 Rationale and Aims

Due to the lack of literature in adults with ID and with comorbid ID and ASD, novel findings are needed to shed light on the complex interactions between the immune system and the altered sensory/behavioral profile related to this condition.

5.1 Primary aim

To evaluate associations between sensory and behavioral profiles in adult ID or ID+ASD individuals assisted by the Azienda Socio-Sanitaria Territoriale (ASST) of Pavia.

5.2 Secondary aims

2a) To evaluate associations between sensory/behavioral profiles and leukocytes blood-levels in adult ID or ID+ASD individuals assisted by the Azienda Socio-Sanitaria Territoriale (ASST) of Pavia.

2b) To determine whether these associations differ between individuals with ID and ASD versus individuals with only ID.

2c) To determine whether epilepsy, insomnia and presence of gastro-intestinal disturbances are discriminatory in ASD subtypes (e.g. regressive autism vs primary autism)

2d) To periodically assess the abovementioned parameters in order to confirm the primary aim over time and evaluate the dynamical evolution of the sensory, behavioral, and biological parameters.

2e) To analyse food consumption with 3 measurements at baseline, 3rd and 6th month to correlate nutritional/dietary status of the patients with sensory profile, behavior, sleep and the occurrence of gastrointestinal disorders.

5.3 Exploratory aims

3a) To evaluate the possibility to clusterize different conditions into more elaborated clinical phenotypes for both the ASD+ID and ID groups.

3b) To evaluate whether the peripheral blood levels of vitamin D, B12, folic acid or homocysteine may be related to sensory or behavioral markers3c) To evaluate whether some sensory channels are more impaired than others according to the group considered.

6 Study design

6.1 Statement of Design

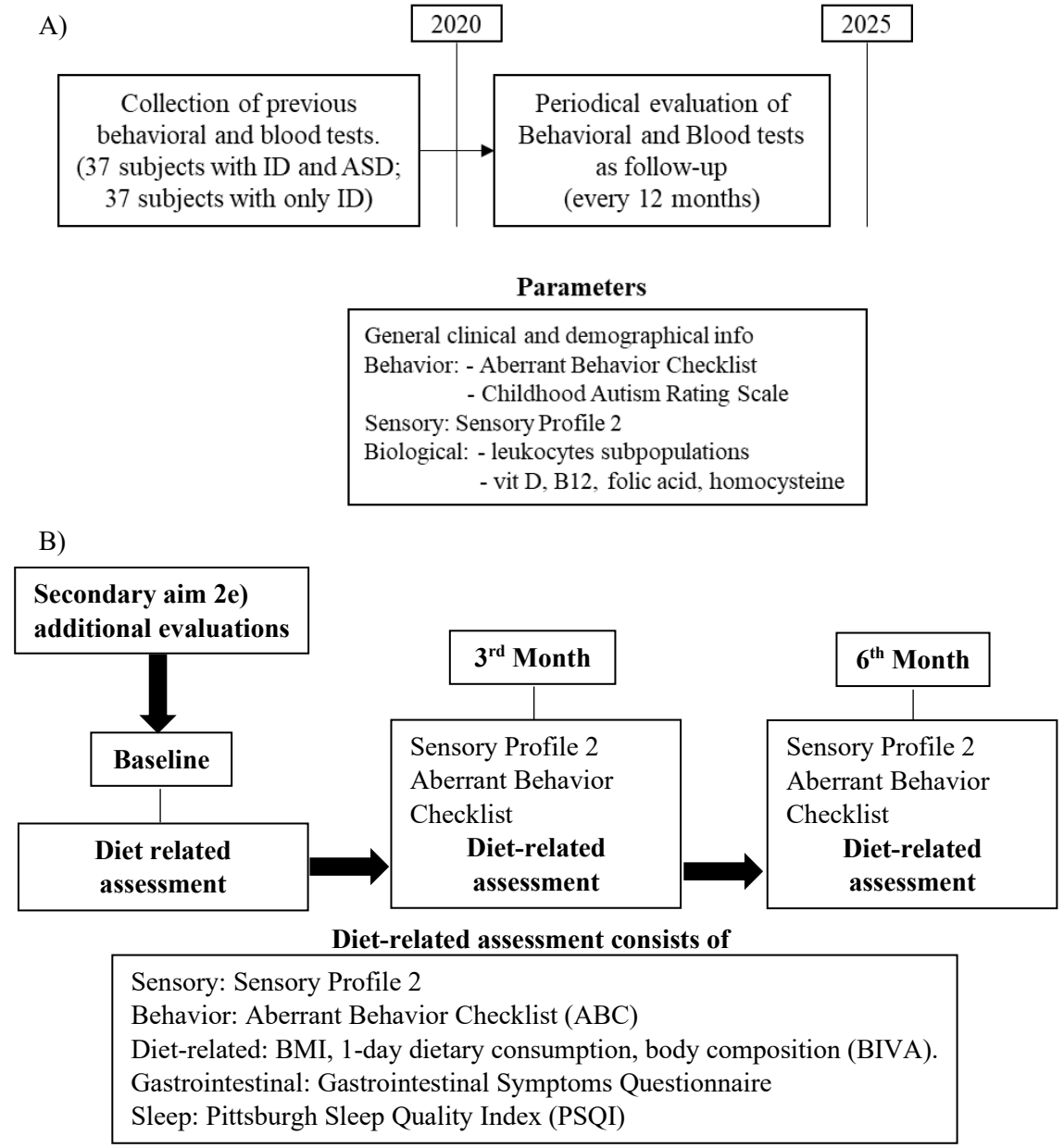
The study is an observational longitudinal study. Data collection is prospective, as each year after the one of inclusion the questionnaires will be readministered. Subjects with 2 or more evaluations will hence be included

in the secondary analysis concerning the progression/stability of the sensory, behavioral, and clinical conditions.

6.2 Number of subjects

Considering the correlation of 0.54 between sensory profile and ABC total score found in O'Donnell at al. as reference (2014), on the basis of statistical and clinical considerations we calculated a sample size of 37 subjects including 10% of possible dropout, assuming a Power of 0.80, alpha = 0.05, H0: correlation coefficient r= 0.1 and Ha: correlation coefficient r = 0.50 (using STATA 13 software). The total sample size will hence be 74 (37 subjects with ASD+ID and 37 with ID) in order to explore possible differences between the two subgroups.

6.3 Study flow chart



6.4 Inclusion criteria

- As the literature lacks such studies in adults, but sensory alterations significantly vary though the different ages of life (at least in neurotypicals, see Pohl et al., 2003), participants will be selected among individuals who are > 18 and < 65 years old.
- Presence of a moderate, severe or profound ID according to the DSM-5 diagnostic criteria. Diagnosis will be made by two independent clinicians. For ASD, The Childhood Autism Rating Scale will be administered to further support the diagnosis, considering as positive the scores >30 (see below).
- To be treated by an outpatient unit pertaining to the ASST of Pavia.

6.5 Exclusion criteria

- Presence of important organic sensory dysfunctions (such as blindness or deafness).
- Impossibility of obtain the blood sample due to history of patient's excessive stress during such procedure.
- Presence of organic conditions unrelated to ASD which may independently alter the immune system features (eg. neoplastic conditions).

7 Procedures and assessments

In the present study, an operator from ASST Pavia will:

- collect the participants' demographical, anamnestic and clinical data;

- periodically collect clinical and behavioral data (including blood tests regarding leukocytes subpopulations when available) every 12 months for a maximum of 5 years.

A follow-up will be set every 12 months in order to monitor the evolution of the symptomatology and the fluctuations of the biological parameters. Repeated measures will assess whether the between-group differences will be maintained over time

The abovementioned data consist of:

a) the informed consent to be completed by the participant or his legal representative.

b) demographical data (age, sex, height, weight, date and place of birth, residence, contacts of the legal representative).

c) anamnestic and clinical information of the participants (diagnosis, frequency of events such epilepsy, challenging behaviors such as aggressiveness towards self or others, insomnia and gastro-intestinal disturbances, pharmacotherapy).

d) information regarding the participants' available blood tests performed during the last year (leukocytes subpopulations count, vitamin B12, vitamin D, folic acid, homocysteine dosages in peripheral blood).

e) the caregiver-completed questionnaires listed in chapter 7.1.

7.1 Primary outcome measures

- Sensory profile 2 (SP2; Dunn, 1999) has been described as one of the best and reliable measures to assess sensory processing in ASD children, differentiating them from neurotypicals (Joosten and Bundy, 2010; Smith et al., 2015). It is a standardized, 68 item, caregiver-completed questionnaire describing the patient's

engagement to several examples of sensory stimuli in the everyday life. It is divided into four quadrants: low registration, sensation seeking, sensory sensitivity, sensation avoiding. These four categories describe the threshold necessary for an individual to respond to specific stimuli (high/low) and the behavioral response elicited in the individual (active/passive). As no caregivers-completed questionnaires to explore sensory profile in adults are available, but the items of the SP2 fit well with subjects with severe or profound ID, the questionnaire will be used in adults with ID. In addition to the standard scoring, each sensory channel (visual, auditory, tactile, vestibular, olfactive, gustative) will be separately scored to identify which unimodal pathways are more involved in an atypical processing of the stimuli.

7.2 Secondary outcome measures

2a) The Aberrant Behaviour Checklist (ABC; Farmer and Aman, 2017): it rates 58 specific symptoms divided into five subscales: irritability, social withdrawal, stereotypic behavior, hyperactive/noncompliance and inappropriate speech. It is a caregiver-filled questionnaire and will be used to measure the behavioral profiles. Leukocytes subpopulations (neutrophils, lymphocytes, monocytes, eosinophils, basophils count) in peripheral blood.

2b) The Childhood Autism Rating Scale (CARS, Schopler et al., 1980) is a caregiver administered questionnaire which helps to identify children with autism and determine symptom severity through quantifiable ratings based on direct observation. It is also considered suitable for adults with ID which are hence not able to complete self-report questionnaires. It will be administered at baseline to further support the clinical diagnosis and at follow-up to periodically assess autism severity.

Clinical data regarding the presence of regressive autism, epilepsy, insomnia, gastro-intestinal disturbances and pharmacotherapy will be gathered in order to classify different subgroups of subjects. When available, past frequency of events such as epileptic crises, insomnia and gastro-intestinal disturbances will be also assessed.

2c) Clinical information regarding epilepsy, insomnia, presence of gastro-intestinal disturbances, ASD subtypes and pharmacotherapies will be obtained by consulting the patient's medical records.

2e) Nutritional balance and relationship with symptoms

- Anthropometric measures (height and weight) will be measured using calibrated scales, stadiometer. BMI and BMI percentile will be calculated. History of the subjects' dietary restrictions will be collected. Body composition will be evaluated using bioelectrical impedance vector analysis (BIVA), an accurate and non-invasive method for a quick measurement of body compartments, fat mass, fat free mass and body water (Piccoli et al., 1994) (Buffa et al., 2014). BIVA is conducted applying 4 electrodes (2 on the hand and 2 on the homolateral foot): the participant will have to lie down for 3-5 minutes in order to allow a homogeneous distribution of the body fluids. The direct analysis of the two components of the impedance vector (Z), resistance (R, Ohm) and reactance (Xc, Ohm), allows a semiquantitative evaluation of body composition in terms of body cell mass and hydration status. Data for total body water (TBW), body cell mass (BCM), extracellular water (ECW), fat-free mass (FFM),

fat mass (FM) and percentage fat mass (% FM) will be available for all participants and will be used to identify changes of fat and fat-free mass over the study period.

- Mean dietary consumption will be measured averaging data obtained from the 1-day patient food diary at each of the 3 timepoint (baseline, 3 months, 6 months). Each patient will have a unique code number to identify his/her individual food intake over the whole day. A food diary will be filled in by the caregiver with the training and support of a dietitian to record meal serving size and plate waste over the whole day from main meals and mid-meal snacks using a tool developed by Piedmont region (AA.VV. 2005). Plate waste or uneaten food will be weighed at the end of each meal. To calculate daily nutritional intake, the nutritional content of the food eaten in grams during the day will be calculated by difference between food provided and food wasted. Total food eaten will be analysed using a dedicated software (Microdiet, Downlee Systems Ltd., UK), which is able to provide daily averages for energy, macronutrients, micronutrients using the Italian food composition data (BDA, 2015).
- Gastrointestinal symptoms will be evaluated using a bowel symptoms questionnaire designed by Chandler et collaborators (Chandler et al., 2013). This instrument includes 20 questions in total and will be filled by a caregiver. The questions inquiry about the main gastrointestinal symptoms occurred in the previous three months. Each question has three possible answers: “yes”, “no” and “don’t know, not applicable”.
- Sleep quality will be assessed with the Pittsburgh Sleep Quality Index (PSQI), a questionnaire composed of 19 self-rated questions and 5 additional questions rated by the bedpartner or roommate. In our case, all the questions won’t be filled in by the subject, but by the caregiver. The 19 main questions are used to assess many aspects of sleep quality, such as subjective sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbance, use of sleep medication and daytime dysfunction. PSQI has proven great internal coherence, stability of responses over time and validity due to its ability to discriminate good and poor sleepers (Buysse et al. 1989).

7.3 Exploratory outcome measures

3a) Additional statistical analysis such as Principal Component Analysis will be implemented in order to assess the presence of clusterization patterns.

3b) Similarly to the leukocytes count, vitamin D, B12, folic acid and homocysteine will be correlated with sensory/behavioral scores.

3c) Use the Visual, Auditory, Touch, Movement, Olfactory, Oral, Body Position and Attentional subscores from the Sensory Profile 2 to test differences between ASD+ID and ID groups.

7.4 Statistics

Quantitative variables will be summarized by means and standard deviations and median (25th-75th) and analyzed by an independent Student’s t-test or the Welch-Satterthwaite correction for t-test in the event of an unequal variance or U- Mann Whitney test. Qualitative variables will be reported by absolute frequencies

and percentages and analysed by the Chi-Square Test or Fisher's Exact Test, as appropriate. Normality will be assessed using the Shapiro–Wilk test. Correlation between variables will be evaluated by calculating Pearson or Spearman's coefficients.

To identify factors associated to ID or ASD a multivariable logistic regression will be performed. In the final model, we will include all predictors clinical or statistically significant in the univariate analysis to obtain the best model in terms of goodness of fit. We also will assess the calibration of the models by applying the Hosmer-Lemeshow test. As stated above, Principal Components Analysis may also be a suitable statistical tool to further explore our data. A p-value of less than 0.05 will be considered significant, correction for multiple comparisons (Bonferroni-Holmes) will be applied when needed. Analyses will be conducted using the STATA version 15 statistical software and SPSS version 25.

We will recruit the subjects after applying the inclusion and exclusion criteria, the post-hoc power analysis will be then calculated.

7.5 Blindings

Statisticians performing the data analyses will be blind to the patients' diagnosis and clinical group (binomial categories will be coded randomly as 0 and 1).

7.6 Baseline assessment

Data collected will include:

- demographic data
- medical history (including diagnosis, previously collected blood tests and pharmacotherapy)
- behavioral questionnaires
- sensory questionnaires
- BMI
- body composition
- Gastrointestinal Symptoms Questionnaire
- Pittsburgh Sleep Quality Index
- diet assessment

7.7 Timing of assessments

- at baseline
- follow-up every 12 months
- diet-related assessments (BMI, body composition, Gastrointestinal Symptoms Questionnaire, Pittsburgh Sleep Quality Index, diet) will be only evaluated thrice: at 3rd month and at 6th month. Sensory Profile 2 and Aberrant Behavior Checklist will be evaluated as well.

7.8 Schedule of assessments (table)

	Baseline	3rd Month	6th Month	Periodic 12 months Follow-up (until 2025)
Demographic data	X			
Medical history	X			X
Inclusion criteria	X			
Exclusion criteria	X			
Blood tests	X			X
Sensory profile 2 (SP2)	X	X	X	X
Aberrant Behavior Checklist (ABC)	X	X	X	X
Childhood Autism Rating Scale (CARS)	X			X
Study completion				X
Diet-related assessment (BMI, body composition, gastrointestinal symptoms, sleep quality, 1-day dietary consumption)	X	X	X	

7.9 Definition of the end of the trial

The end of the trial will be the date of the last patient's assessment in 2025.

8 Data handling and record keeping

All data will be collected by an ASST Pavia investigator in the context of his clinical activity.

8.1 CRF

The PI will sign the registration form to confirm eligibility. All data will be anonymized by an operator which will assign a code to each participant and subsequently will transfer the data into a Case Report Form (CRF). A different file with the same protection will contain the codes associated to the clinical information. All trial data in the CRF must be extracted from and be consistent with the relevant source documents. The CRFs must be completed, dated and signed by the investigator or designee in a timely manner. It remains the responsibility of the investigator for the timing, completeness, legibility and accuracy of the CRF pages. The CRF will be accessible to trial coordinators, data managers, the investigators, Clinical Trial Monitors, Auditors and Inspectors as required.

All CRF pages must be clear, legible and completed in black ink. Any errors should be crossed with a single stroke so that the original entry can still be seen. Corrections should be inserted and the change dated and initialled by the investigator or designee. If it is not clear why the change has been made, an explanation should be written next to the change. Typing correction fluid must not be used. All data obtained for this study will be entered into a local regulation compliant Data Management System. The questionnaires by the caregivers will require 1 hour per subject to be completed. The study database will be resident on a server in a secure location within ASST Pavia (PV), Italy, and will be protected by password.

8.2 Source Data

To enable peer review, monitoring, audit and/or inspection the investigator must agree to keep records of all participating patients (sufficient information to link records e.g., CRFs, clinical records and samples), all original signed informed consent forms and copies of the CRF pages.

List of source data/documents:

- Patient Medical Records
- Questionnaires Records
- Blood tests Records
- Body Composition Evaluation Records
- Food Consumption Records

8.3 Data Protection and Patient Confidentiality

All investigators and trial site staff involved in this trial must comply with the requirements of the Data Protection Act 1998 and Trust Policy with regards to the collection, storage, processing and disclosure of personal information and will uphold the Act's core principles. The PI of the study will ensure that only anonymised data is received by the trial team.

Additionally, the list of enrolled patients with de-coding will be physically separated from the coded patients in the study files.

9 Ethical & Regulatory considerations

9.1 Consent

The Informed Consent form must be approved by the REC and must be in compliance with GCP, local regulatory requirements and legal requirements. The investigator must ensure that each trial participant, or his/her legally acceptable representative, is fully informed about the nature and objectives of the trial and possible risks associated with their participation.

The investigator will obtain written informed consent from the patient's legally acceptable representative before any trial-specific activity is performed. The informed consent form used for this trial and any change made during the course of this trial, must be prospectively approved by the REC. The investigator will retain the original of each patient signed informed consent form.

Should a patient require a verbal translation of the trial documentation by a locally approved interpreter/translator, it is the responsibility of the individual investigator to use locally approved translators.

Any new information which becomes available, which might affect the patient's willingness to continue participating in the trial will be communicated to the patient as soon as possible.

9.1.1 Consent withdrawal

All the subjects may withdraw their consent to participate to the present study as explained in the informational sheet given to the participants at the moment of the consent collection.

9.2 Ethical committee review

Before the start of the trial or implementation of any amendment we will obtain approval of the trial protocol, protocol amendments, informed consent forms and other relevant documents e.g., advertisements and GP information letters if applicable from the REC. All correspondence with the REC will be retained in the Trial Master File/Investigator Site File.

Annual reports will be submitted to the REC in accordance with national requirements. It is the Chief Investigator's responsibility to produce the annual reports as required.

9.3 Regulatory Compliance

The trial will not commence until a Clinical Trial Authorisation (CTA) is obtained from the MHRA. The protocol and trial conduct will comply with the Medicines for Human Use (Clinical Trials) Regulations 2004 and any relevant amendments.

Development Safety Update Reports (DSURs) will be submitted to the MHRA in accordance with national requirements. It is the Chief Investigators responsibility to produce the annual reports as required.

9.4 Protocol Amendments

Protocol amendments must be reviewed and agreement received from the Sponsor for all proposed amendments prior to submission to the REC and/or MHRA.

9.5 Declaration of Helsinki and Good Clinical Practice

The trial will be performed in accordance with the spirit and the letter of the declaration of Helsinki, the conditions and principles of Good Clinical Practice, the protocol and applicable local regulatory requirements and laws.

9.6 Other ethical considerations

This study will not interfere with the daily clinical routine of the participants, and will not require additional human resources.

10 Sponsorship, Financial and Insurance

The trial, not for profit, is promoted by ASST Pavia. No fundings are expected for the present study.

11 Monitoring, Audit & Inspection

The investigator must make all trial documentation and related records available should an MHRA Inspection occur. Should a monitoring visit or audit be requested, the investigator must make the trial documentation and source data available to the Sponsor's representative. All patient data must be handled and treated confidentially.

The Sponsor's monitoring frequency will be determined by an initial risk assessment performed prior to the start of the trial. A detailed monitoring plan will be generated detailing the frequency and scope of the monitoring for the trial. Throughout the course of the trial, the risk assessment will be reviewed and the monitoring frequency adjusted as necessary.

12 Protocol Compliance and Breaches of GCP

Prospective, planned deviations or waivers to the protocol are not allowed and must not be used.

13 Publications policy

Ownership of the data arising from this trial resides with the trial team. On completion of the trial the data will be analysed and tabulated and a Final Study Report prepared.

All members of trial team will be involved in manuscript elaborations and their consent will be requested before publication.

Participants can request trial results from their PI and information will be communicated in an arranged interview with one member of the trial team.

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