ISSN 2472-1972

Proteomics of Children Born After Intracytoplasmic Sperm Injection Reveal Indices of an Adverse Cardiometabolic Profile

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Context: Assisted reproduction technologies (ART), classic *in vitro* fertilization (IVF), and intracytoplasmic sperm injection (ICSI) are increasingly used. Several studies have demonstrated an unfavorable cardiometabolic profile of the ART offspring. Proteomics is a state-of-the-art technology used for the identification of early biomarkers of disease.

Objectives: To investigate the proteomic profile of children born after ICSI compared with naturally conceived (NC) controls in search of cardiometabolic risk markers.

Design: Cross-sectional case-control study: qualitative, comparative proteomic plasma analysis.

Setting: Pediatric Endocrinology and IVF Outpatient Clinics, University of Athens and the Biomedical Research Foundation of the Academy of Athens.

Participants: Forty-two sex- and age-matched couples of ICSI and NC children were assessed. Ten pairs additionally matched for birth weight and twin/single pregnancies were submitted to proteomic analysis.

Intervention: Medical history, clinical examination, and blood biochemical, hormonal, and proteomic analyses.

Main Outcome Measures: (1) Differences in auxological and laboratory data between groups. (2) Differences in plasma proteomic profile in 10 individual pairs and pooled samples.

Results: The ICSI group had shorter gestation, more cesarean sections, smaller birth weight/length, and advanced maternal age. No major differences were observed regarding biochemical markers. Proteomic analysis revealed 19 over- and three underexpressed proteins in ICSI. Most overexpressed proteins are implicated in acute-phase reaction, blood coagulation, complement pathway activation, and iron and lipid metabolism, suggesting a subclinical unfavorable cardiometabolic profile.

Conclusions: This study applies proteomics in ICSI-conceived children, providing evidence for an early adverse cardiometabolic profile and supporting the necessity of their long-term monitoring.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; ART, assisted reproduction technologies; hsCRP, high sensitivity C-reactive protein; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; NC, naturally conceived; RBP, retinol-binding protein; SDS, standard deviation score; SGA, small for gestational age.

Received 15 November 2016
Accepted 22 February 2017
First Published Online 27 February 2017

Freeform/Key Words: proteomics, ICSI, cardiometabolic, metabolism, IVF

Assisted reproductive technologies (ART), comprising classic *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), have significantly expanded over the years, aiming to overcome the growing problem of subfertility. ICSI, a technique first introduced in 1992, was primarily indicated for male subfertility, as it comprises the introduction of a single preselected sperm into the oocyte; however, it has progressively gained significant ground, constituting nowadays ~70% of all ART procedures performed [1, 2].

ART is usually associated with increased parental stress. Although it is still debated whether stress influences ART outcome, it is well established that glucocorticoids, by stimulating hepatic gluconeogenesis, inhibiting insulin actions on skeletal muscle, and potentiating its actions on adipose tissue, are the main mediators of the poor long-term outcome of an adverse intrauterine environment, ultimately promoting sarcopenia and visceral adiposity of the offspring and the occurrence of the metabolic syndrome in later life [3].

Infants born after ART are at higher risk of preterm delivery and intrauterine growth restriction, born small for gestational age (SGA) [4]. SGA, in turn, predisposes to adult disease and is associated with an unfavorable cardiometabolic profile in later life [5]. This alone could raise concern about the long-term health issues of children born after ART and may explain the observed poor cardiometabolic outcome of IVF children [6]. However, the latter might also be the result of the *in vitro* manipulations of the blastocyst and/or an adverse intratubal and/or intrauterine environment. This is in agreement with Barker's fetal origin of adult disease hypothesis, which postulates development of epigenetic alterations occurring in prenatally distressed individuals [7].

Finally, there is increasing evidence for an association between ART and imprinting disorders, such as the Beckwith–Wiedemann or Angelmann syndromes, mostly in children born after ICSI [8] and less frequently after classic IVF [9], raising concern for genomic imprinting disorders and other harmful epigenetic changes in ART [10]. More specifically, reservations have been raised about the overall health of children born after ICSI, as this method has a greater risk for the introduction of a genetic error by bypassing all intrinsic barriers for the fertilization of abnormal gametes, thus eliminating sperm natural selection. Furthermore, embryo culture media and hormonal ovarian hyperstimulation have been shown to influence the imprinting status of some imprinted genes [11]. Indeed, epigenetic changes during the preimplantation period could be a potential mechanism for alterations in growth, development, and metabolism of ART-conceived children.

Proteomics is a technology-driven science, which, in a high-throughput fashion, studies biologic fluid or tissue proteins, their posttranslational modifications, their interactions, changes in their expression levels, and their modifications in response to environmental factors, treatments, or disease [12]. To date, proteomic analysis has provided the opportunity to elucidate complex biological procedures and conditions, including fertility, fertilization, embryo implantation, and pregnancy. The advances in the use of mass spectral profiling in various biological fluids have enabled the determination of markers that may optimize ART outcome, beginning from the evaluation of the male and female gamete to the assessment of embryo quality and endometrial receptivity and, finally, to the prevention of pregnancy complications and disorders in the offspring, frequently, but not exclusively, associated with ART [13]. Therefore, comparative, untargeted proteomic profiling can be expected to reveal potential differences in children conceived by ART compared with naturally conceived (NC) ones.

The objective of this cross-sectional study was to investigate the potential proteomic variation in prepubertal children born after ICSI compared with age- and demographically-matched NC

controls, using medical history and biochemical data, along with untargeted plasma proteomic analysis, in search of clues of early derangement long before biochemical abnormalities become evident. Currently, no other published study exists using proteomics in the exploration of the physiology and pathology of children born after ART.

1. Materials and Methods

A. Study Population

The ICSI group consisted of 42 ICSI-conceived [19 boys (45.23%) and 23 girls (54.76%)] healthy, prepubertal, Caucasian children with a mean age of 6.8 years recruited from the IVF Section of the First Department of Obstetrics–Gynecology of the National and Kapodistrian University of Athens (Table 1). Forty-two age ($P = 0.93$)- and gender ($P = 0.99$)-matched, NC healthy prepubertal, Caucasian children served as controls (NC group) (Table 1). From the initial cohort of the 84 children (42 matched pairs), a subgroup of 20 children (five pairs of girls and five pairs of boys) also matched for birth weight for gestational age, and of them, being twins or singletons, were studied using comparative plasma proteomic analysis.

B. Ethical Issues

This study was approved by the Ethics Committee of the Aghia Sophia Children's Hospital, and children were included in the study only after informed written consent had been obtained from their parents or guardians.

Table 1. Demographic and Auxological Data of ICSI and Normally Conceived (Control) Children

Parameters		N (%)		*P* Value (X^2 Pearson Test)
		ICSI	Control	
Sex	Male (n = 38)	19	19	0.999
	Female (n = 46)	23	23	
No. of fetuses	Single (n = 49)	13	36	<0.0001
	Twin (n = 35)	29	6	
Uneventful pregnancy	No (n = 31)	20	11	0.09
	Yes (n = 49)	22	27	
Breastfeeding	No (n = 23)	11	12	0.86
	Yes (n = 60)	30	30	
Type of delivery	Natural (n = 22)	5	17	0.003
	Cesarean section (n = 62)	37	25	
Birthweight for gestational age	SGA (n = 31)	18	13	0.33
	AGA (n = 47)	22	25	
Age (y)		6.8 ± 2.05	6.8 ± 2.08	0.93[a]
Mother's age (y)		35.6 ± 5.2	31.7 ± 5.3	0.0013[b]
Father's age (y)		38.3 ± 5.4	35.9 ± 5.9	0.06[a]
Gestational age (wk)		35.6 ± 2.8	37.9 ± 1.8	0.0001[a]
Birthweight SDS		−0.39 ± 0.95	0.42 ± 0.9	0.0001[b]
Birth length SDS		−0.24 ± 1.03	0.24 ± 0.9	0.015[a]
Height SDS		0.17 ± 0.92	−0.11 ± 0.99	0.17[b]
Weight SDS		−0.09 ± 0.8	−0.03 ± 0.96	0.7
BMI *z*-score		−0.17 ± 0.95	−0.02 ± 1.04	0.4
Waist/Height (W/H)		0.48 ± 0.04	0.49 ± 0.05	0.5[a]
Waist/Hip ratio (WHR)		0.96 ± 0.06	0.94 ± 0.06	0.4[b]
SBP SDS		0.006 ± 0.9	0.83 ± 1.02	0.0004[b]
DBP SDS		−0.37 ± 0.6	−0.2 ± 0.65	0.2

Abbreviations: AGA, appropriate for gestational age; DBP, diastolic blood pressure; SBP, systolic blood pressure.
[a]Mann–Whitney test.
[b]Normal distribution; two-sample *t* test.

C. Medical History and Clinical Examination

A thorough medical history was obtained from all subjects and their parents. Cause of subfertility, maternal and paternal age at the time of conception, years and number of trials of IVF-ICSI until they achieved a pregnancy, duration and course of gestation, perinatal problems, type of delivery (vaginal delivery or cesarean section), gender, gestational age, twins or singleton pregnancy, birth weight, birth length, head circumference at birth, duration of breastfeeding, as well as the child's personal and family medical history were recorded. Children were classified as SGA or appropriate for gestational age, when birth weight was <10th or between the 10th and 90th centile, respectively, according to the individual centile calculator at www.gestation.net, which takes into account birth weight, gestational age, parity, ethnic group, the child's gender, and the mother's height and weight [14].

Physical examination included height, weight, head, waist and hip circumference, systolic blood pressure and diastolic blood pressure, and pubertal status. Girls with Breast Tanner stage I and boys with testicular size <4 mL were considered prepubertal [15, 16].

D. Blood Sampling

Blood was collected at 8:00 AM from all subjects after an overnight fast; both plasma and serum samples were collected following centrifugation and stored at −80°C until analysis.

Standard blood biochemical, hematological, and hormonal assessments were conducted in the Aghia Sophia Children's Hospital by standard methodology using standard equipment. Specifically, blood chemistry, including serum glucose, total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and iron, was determined using the Siemens Advia 1800 Clinical Chemistry System (Siemens Healthcare Diagnostics, Tarrytown, NY). The lipoprotein Apo A-I, Apo-B, and Lp(a) levels were quantified by means of latex particle-enhanced immunonephelometric assays on the BN ProSpec nephelometer (Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany). Serum insulin, thyrotropin, T3, T4, cortisol, and insulin-like growth factor 1 levels were measured using the automated chemiluminescence Siemens ACS180 System Analyzer (Siemens Healthcare Diagnostics). The intra-assay and interassay coefficient of variation for all variables was <5%, except for the insulin assay, in which the coefficient of variation was <10%. Complete blood counts were performed using the Siemens-ADVIA 120 whole-blood auto-analyzer (Siemens Healthcare Diagnostics). Ferritin levels were measured with the electrochemiluminescence immunoassay using the Roche Elescys 2010 immunoassay analyzer (Roche Diagnostics, Mannheim, Germany). Homeostasis model assessment, as marker of insulin resistance, was calculated according to the original formula [17].

E. Proteomic Serum Profiling

Proteomic analysis was conducted in the Proteomics Research Unit, Biomedical Research Foundation of the Academy of Athens. The collected plasma samples were submitted to proteomic analysis, and the proteomic profiles were compared in pairs. To increase the dynamic range of the plasma proteome and, most importantly, to achieve high-sensitivity detection of low-abundance proteins, two plasma pools from the ICSI and the NC groups (1 μL from each subject) were created and processed following the same methodology.

The plasma protein content was determined using the Bioanalyzer Automated Electrophoresis Station (Agilent Technologies, Waldbornn, Germany) combined with the Protein 200 plus kit (Agilent Technologies), as previously described [18]. The experimental procedure of two-dimensional gel electrophoresis (2-DE) was performed, as previously described [19]. Protein spots were visualized by application of Coomassie Blue G-250 staining solution (Novex, San Diego, CA) on 2-DE gels for 12 h. Gel images were scanned in a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA) and stored on a PC for further analysis.

E-1. Image analysis

Protein spots from all gels analyzed were detected, aligned, matched, and quantified using the PD-Quest v8.0 image-processing software (Bio-Rad), according to the manufacturer's instructions. Manual inspection of the spots was used to verify the accuracy of matching. Spot volume was used as the analysis parameter to quantify protein expression. Selection of protein spots or entire gel regions for mass spectrometry analysis was based upon optical density alteration between the two groups analyzed. A minimum of 1.5-fold change in the expression level was used as a selection criterion, at the $P < 0.05$ level.

E-2. Protein identification by matrix-assisted laser desorption/ionization tandem time of flight mass spectrometer analysis

Protein spots of interest were manually annotated using the Melanie 4.02 software and excised from 2-DE gels using a Proteiner SPII instrument (Bruker Daltonics, Bremen, Germany). Peptide-matching and protein searches were automatically performed with the use of MASCOT Server 2 (Matrix Science). Peptide masses were compared with the theoretical peptide masses of all available proteins from *Homo sapiens* in the SWISS-PROT and TREmBL databases. Stringent criteria were used for protein identification with a maximum allowed mass error of 25 ppm and a minimum of four matching peptides. Probability score with $P < 0.05$ was used as the criterion for affirmative protein identification.

F. Statistical Analysis

Differences of demographic and biochemical data between groups were evaluated by the two-sample Student *t* test or the nonparametric Mann–Whitney *U* test according to the normality of the data. The Pearson χ^2 test was applied to examine the relations between categorical variables. A $P < 0.05$ was considered statistically significant. Analysis was conducted using the STATA-9 statistical software.

Regarding proteomic analysis, to ensure confidence in our experimental approach, we used a design that involved duplicate 2-DE gels per sample to determine analytical variation. Mean densitometry values of all spots corresponding to a specific protein from each pair were first checked for normal distribution using the Kolmogorov–Smirnov/Lillie for test (Stat-Plus 2007 software; AnalystSoft, Vancouver, Canada). Data with normally distributed densitometric values were exported to Microsoft Excel 2007 software and compared with the two-pair *t* test assuming unequal variances. Means of spot intensities for proteins with not normally-distributed values were compared for statistical significance with the Mann–Whitney nonparametric test (GraphPad Instat 3 software; GraphPad Software, La Jolla, CA). Statistical significance (a-level) was defined as <0.05. To control the false discovery rate, individual a-levels for each spot were adjusted following the false discovery rate correction procedure [20].

G. Network Analysis and Functional Evaluation

All identified proteins were used for pathway analysis. Functional relations of the identified proteins were determined by submitting protein entry names to the STRING database. The simplified version of the produced network, which involved both up- and downregulated proteins, was adopted.

2. Results

A. Anthropometric and Biochemical Data

Comparing the ICSI group vs NC controls, mothers' age (35.665.2 vs 31.765.3, $P = 0.0013$), twin pregnancies (29 vs 6, $P = 0.0001$), and cesarean sections (37 vs 25, $P = 0.003$) were significantly higher. Moreover, gestational age (median: 36 vs 38 weeks, $P = 0.0001$), birth

weight standard deviation score (SDS) (0.39 vs 0.42, $P = 0.0001$), and birth length SDS (−0.24 vs 0.24, $P = 0.015$) were significantly lower in ICSI children than controls. No differences were identified regarding gender, pre- or perinatal complications, breastfeeding, or fathers' age (Table 1). Moreover, systolic blood pressure-SDS was significantly lower in ICSI than controls (0.006 vs 0.83, $P = 0.0004$). Among biochemical and hormonal markers, only triiodothyronine (T3) was significantly higher in the ICSI group. In the subgroup of children analyzed using proteomics, T3 remained significantly higher in the ICSI than the control group (Table 2).

B. Proteomic Analysis

Of the 545 spots that were found to be differentially expressed in one-to-one comparisons, 98 were identified with a MASCOT score >50, a score that expresses extended homology or identification at the $P < 0.05$ level. These 98 spots corresponded to 24 different proteins (Table 3). Serum albumin and Ig κ chain C region were not included in subsequent analysis due to their abundance in all samples. Fifteen of these proteins (a1 acid glycoprotein, a2 HS glycoprotein, apolipoprotein A-IV, apolipoprotein E, complement C1, complement factor B, DAN domain family, fibrinogen α chain, fibrinogen γ chain, gelsolin, keratin type I cytoskeleton 10, keratin type I cytoskeleton 9, keratin type II, plasminogen, vitamin D–binding protein) were exclusively overexpressed in the ICSI group, whereas, for the remaining seven, quantification and comparison of optical densities were necessary to reveal the group in which they were overexpressed. To validate and further strengthen our results, we proceeded to the comparison of the proteomic profiles of the pooled samples, following the procedure described earlier. Of the 20 selected spots, 15 were identified, corresponding to six proteins (Table 3). By coevaluating the results of the comparative analysis of both individual and pooled samples, we have compiled a list of 22 differentially expressed proteins, of which 19 were over- and three were underexpressed in the ICSI group (Table 4).

Proteins were classified according to their biological characteristics, using their gene symbol [Knowledgedbase database (http://www.uniprot.org/)]. During analysis, when more

Table 2. Biochemical and Hormonal Data in ICSI and Naturally Conceived (Control) Groups

	Parameters	ICSI	Control	*P* Value (Mann–Whitney Test)
Glucose metabolism	Glucose (mg/dL)	81 ± 7.7	83.7 ± 9.3	0.36
	Insulin (μIU/mL)	5.5 ± 2.5	6.9 ± 6.7	0.9
	Glucose/Insulin	19 ± 15.1	18.9 ± 13.8	0.86
	HOMA	1.1 ± 0.5	1.5 ± 1.8	0.9
Lipid profile	Total cholesterol (mg/dL)	167.7 ± 25.3	172.7 ± 24.5	0.35
	Triglycerides (mg/dL)	45.4 ± 16.5	54.2 ± 22.6	0.07
	HDL (mg/dL)	63.9 ± 8.9	60.8 ± 12	0.17
	LDL (mg/dL)	94.6 ± 21.2	100.9 ± 22	0.18
	ApoA1(mg/dL)	153 ± 21.1	156.1 ± 19.8	0.5
	ApoB (mg/dL)	75.7 ± 14.7	74.2 ± 14.9	0.65
	Lp(a) (mg/dL)	11.8 ± 14.8	15.4 ± 20.3	0.16
Iron metabolism	Ht (%)	39.5 ± 2.9	38.4 ± 2.2	0.07[a]
	Hb (g/dL)	13.1 ± 1.1	12.8 ± 0.8	0.22[a]
	Fe (μg/dL)	71.9 ± 21.7	88.8 ± 38.9	0.1[a]
	Ferritin (μg/L)	40.1 ± 24.6	38.4 ± 17.3	0.7
Hormones	Cortisol (μg/dL)	12.5 ± 6.3	13.9 ± 7.4	0.42
	IGF1 (ng/mL)	193.2 ± 115.3	190.5 ± 92.5	0.58
	T3 (ng/dL)	183.37 ± 27.1	158.58 ± 31.1	<0.0001
	T4 (μg/dL)	8.6 ± 1.11	8.97 ± 1.16	0.06[a]
	TSH (μIU/mL)	3.02 ± 1.3	3.24 ± 1.89	0.96

Abbreviations: HDL, high-density lipoprotein; HOMA, homeostasis model assessment; IGF, insulin-like growth factor; LDL, low-density lipoprotein; TSH, thyrotropin.
[a]Normal distribution; two-sample Student *t* test.

Table 3. List of Proteins That Were Identified by Matrix-Assisted Laser Desorption/Ionization Tandem Time of Flight Mass Spectrometer–Mass Spectrometry From the Spots That Were Selected as Differentially Expressed Between the Two Groups (ICSI and Natural Conception) After Two-Dimensional Electrophoresis (A, Individual Samples; B, Pooled Samples)

Title	Accession	Mascot Score	MS Coverage	Protein MW	pI
A. Individual Samples					
α-1-Acid glycoprotein 1, OS = *Homo sapiens*, GN = ORM1, PE = 1, SV = 2	A1AG1_HUMAN	85	44	23,725	4,80
α-1-Antitrypsin, OS = *Homo sapiens*, GN = SERPINA1, PE = 1, SV = 3	A1AT_HUMAN	168	51	46,878	5,30
α-2-HS-glycoprotein, OS = *Homo sapiens*, GN = AHSG, PE = 1, SV = 1	FETUA_HUMAN	72	23	40,098	5,40
α-2-macroglobulin, OS = *Homo sapiens*, GN = A2M, PE = 1, SV = 3	A2MG_HUMAN	283	34	164,613	6,00
Apolipoprotein A-I, OS = *Homo sapiens*, GN = APOA1, PE = 1, SV = 1	APOA1_HUMAN	274	68	30,759	5,50
Apolipoprotein A-IV, OS = *Homo sapiens*, GN = APOA4, PE = 1, SV = 3	APOA4_HUMAN	73	14	45,371	5,20
Apolipoprotein E, OS = *Homo sapiens*, GN = APOE, PE = 1, SV = 1	APOE_HUMAN	172	61	36,246	5,50
Complement C1s subcomponent, OS = *Homo sapiens*, GN = C1S, PE = 1, SV = 1	C1S_HUMAN	80	18	78,174	4,70
Complement C3, OS = *Homo sapiens*, GN = C3, PE = 1, SV = 2	CO3_HUMAN	256	28	188,569	6,00
Complement factor B, OS = *Homo sapiens*, GN = CFB, PE = 1, SV = 2	CFAB_HUMAN	80	18	86,847	6,70
DAN domain family member 5, OS = *Homo sapiens*, GN = DAND5, PE = 2, SV = 2	DAND5_HUMAN	53	53	20,737	12,30
Fibrinogen α chain, OS = *Homo sapiens*, GN = FGA, PE = 1, SV = 2	FIBA_HUMAN	225	35	95,656	5,60
Fibrinogen γ chain, OS = *Homo sapiens*, GN = FGG, PE = 1, SV = 3	FIBG_HUMAN	216	62	52,106	5,30
Gelsolin, OS = *Homo sapiens*, GN = GSN, PE = 1, SV = 1	GELS_HUMAN	50	8	86,043	5,90
Haptoglobin, OS = *Homo sapiens*, GN = HP, PE = 1, SV = 1	HPT_HUMAN	74	16	45,861	6,10
Ig κ chain C region, OS = *Homo sapiens*, GN = IGKC, PE = 1, SV = 1	IGKC_HUMAN	73	75	11,773	5,50
Keratin, type I cytoskeletal 10, OS = *Homo sapiens*, GN = KRT10, PE = 1, SV = 1	K1C10_HUMAN	68	22	59,020	5,00
Keratin, type I cytoskeletal 9, OS = *Homo sapiens*, GN = KRT9, PE = 1, SV = 1	K1C9_HUMAN	147	41	62,255	5,00
Keratin, type II cytoskeletal 1, OS = *Homo sapiens*, GN = KRT1, PE = 1, SV = 1	K2C1_HUMAN	77	24	66,170	8,82
Plasminogen, OS = *Homo sapiens*, GN = PLG, PE = 1, SV = 2	PLMN_HUMAN	321	43	93,247	7,30
Prothrombin, OS = *Homo sapiens*, GN = F2, PE = 1, SV = 2	THRB_HUMAN	118	21	71,475	5,60
Serotransferrin, OS = *Homo sapiens*, GN = TF, PE = 1, SV = 2	TRFE_HUMAN	366	57	79,280	7,00
Serum albumin, OS = *Homo sapiens*, GN = ALB, PE = 1, SV = 2	ALBU_HUMAN	334	59	71,317	5,90
Vitamin D–binding protein, OS = *Homo sapiens*, GN = GC, PE = 1, SV = 1	VTDB_HUMAN	276	56	54,526	5,30

Table 3. Continued

Title	Accession	Mascot Score	MS Coverage	Protein MW	pI
B. Pooled Samples					
α-1-Antitrypsin, OS = *Homo sapiens*, GN = SERPINA1, PE = 1, SV = 3	A1AT_HUMAN	170	52	46,878	5,3
Apolipoprotein A-I, OS = *Homo sapiens*, GN = APOA1, PE = 1, SV = 1	APOA1_HUMAN	75	36	30,759	5,5
Apolipoprotein A-IV, OS = *Homo sapiens*, GN = APOA4, PE = 1, SV = 3	APOA4_HUMAN	101	30	45,371	5,2
Fibrinogen γ chain, OS = *Homo sapiens*, GN = FGG, PE = 1, SV = 3	FIBG_HUMAN	201	58	52,106	5,3
Serum albumin, OS = *Homo sapiens*, GN = ALB, PE = 1, SV = 2	ALBU_HUMAN	184	42	71,317.00	5,9
Transthyretin, OS = *Homo sapiens*, GN = TTR, PE = 1, SV = 1	TTHY_HUMAN	64	55	15,991	5,4

Abbreviations: GN, gene name; MS, mass spectrometry; MW, molecular weight; OS, organism species; PE, protein existence; pI, isoelectric point; SV, sequence version.
Maximum MASCOT score is presented.

than one assignment for processes occurred, every annotation was taken into consideration in the final results (Fig. 1). All protein identifications were used for pathway analysis. Functional relationships of the identified proteins were determined by submitting protein entry names to the STRING database. The simplified version of the produced network, which involved both up- and downregulated proteins, was adopted (Fig. 2). The main interactions that were identified were between Apo A1, A4, and E, between gelsolin and ApoA1, gelsolin

Table 4. List of Proteins That Were Differentially Expressed Between ICSI and Natural Conception Groups

ICSI	
Overexpressed Proteins	**Underexpressed Proteins**
α-1-Acid glycoprotein	DAN domain family member 5
α-1-Antitrypsin	Fibrinogen α chain
α-2-HS-glycoprotein	Plasminogen
α-2-Macroglobulin	
Apolipoprotein A-I	
Apolipoprotein A-IV	
Apolipoprotein E	
Complement C1s subcomponent	
Complement C3	
Complement factor B	
Fibrinogen γ chain	
Gelsolin	
Haptoglobin	
Ig κ chain C region	
Keratin, type I cytoskeletal 10	
Keratin, type I cytoskeletal 9	
Keratin, type II cytoskeletal 1	
Prothrombin	
Serotransferrin	
Serum albumin	
Transthyretin	
Vitamin D–binding protein	

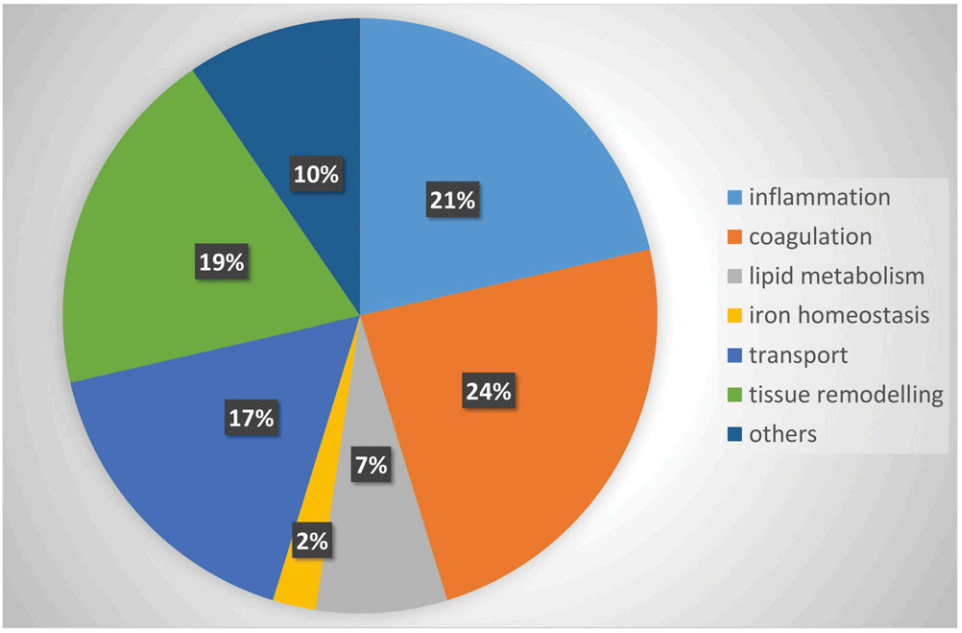

Figure 1. Representation of functional classification of identified, differentially expressed proteins (% total number of identified proteins). Proteins were classified according to their major functions according to the uniprot database.

and transthyretin, A2 macroglobulin and ApoE, A1 antitrypsin and A2 macroglobulin, fibrinogen γ-chain and prothrombin, as well as between transferrin and ApoA1.

3. Discussion

In accordance with most relevant studies, our groups of ICSI-born and NC children did not significantly differ regarding auxological and basic laboratory data [21, 22]. We have confirmed an advanced maternal age, higher incidence of caesarean sections and twin pregnancies, shorter duration of gestation, and lower birth weight for gestational age in the ICSI group, nevertheless, without a concomitant increase in perinatal problems [23, 24]. Notably, higher levels of T3 were observed in our ICSI group. This finding replicated earlier combined analyses of biochemical and metabolomic data in children (10 pairs of ICSI vs NC girls) that were submitted to comparative plasma metabolomic analysis [25]. Higher levels of conversion rates of T4 to T3 in obese patients have been interpreted as a compensatory mechanism to fat accumulation by increasing energy expenditure, a phenomenon possibly mediated by a stimulatory effect of leptin on the activity of 5′ deiodinase [26, 27].

For the proteomic analysis, we carefully selected 10 pairs of children, matched for age and sex, but also for number of embryos (twins-singletons) and weight for gestational age (SGA, appropriate for gestational age), to avoid the confounding effect of these parameters on the metabolic status. Moreover, pubertal children were also excluded from the study, to avoid the impact of this period of natural insulin resistance and best delineate the role of ICSI as a discriminating factor between these two groups as clearly as possible.

Acute-phase proteins (α-1-acid glycoprotein, α-1-antitrypsin, α-2-HS-glycoprotein, complement C1s, complement factor B, haptoglobin, and prothrombin) were significantly overexpressed in the ICSI group. The correlation between the metabolic syndrome and inflammation is well established, as it is believed that the observed increase in proinflammatory cytokines reflects their overproduction from macrophages located in the adipose tissue, directly inducing insulin resistance [28]. Our results are in accordance with the study of Sakka *et al.* [29], who found increased levels of the nontraditional markers of insulin resistance, retinol-binding protein (RBP)4 and neutrophil gelatinase-associated lipocalin, in a group of children born after

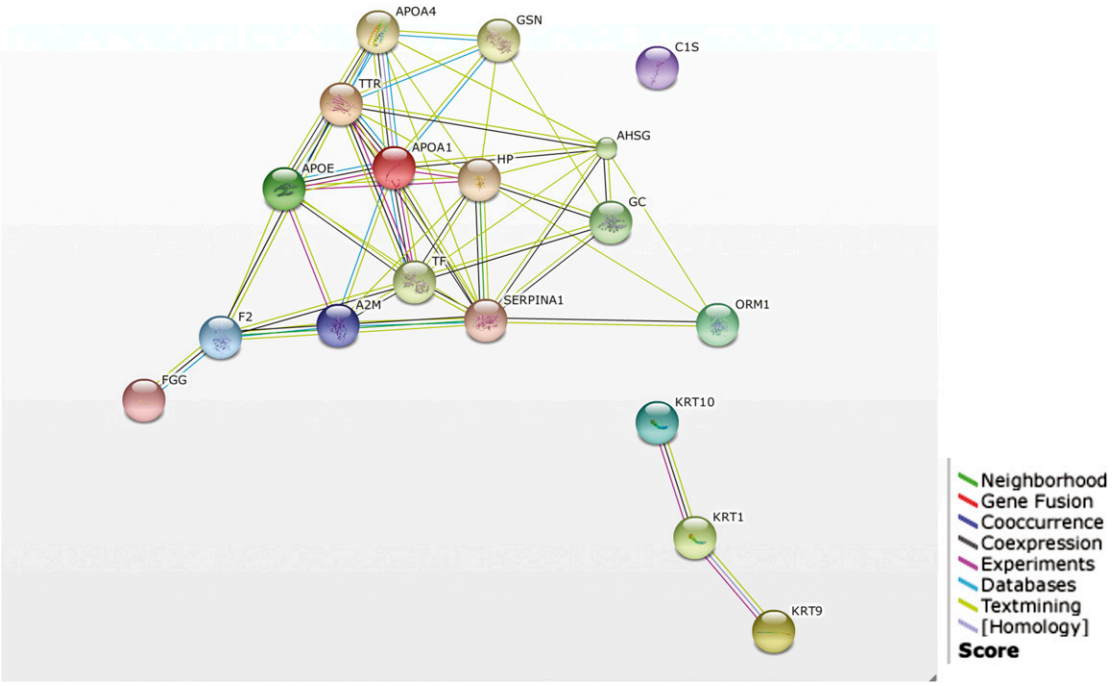

Figure 2. Interaction networks and enriched functional annotations of proteins differentially expressed in examined samples. Thicker network lines demonstrate strong protein relations, as well as neighboring positions. A2M, α2-macroglobulin; AHSG, α2-HS glycoprotein; APOA1, apolipoprotein A-I; APOA4, apolipoprotein A-IV; APOE, apolipoprotein E; C1S, complement component 1; F2, prothrombin; FGG, fibrinogen γ chain; GC, vitamin D–binding protein; GSN, gelsolin; HP, haptoglobin; KRT1, keratin 1 (keratin type II cytoskeletal 1); KRT9, keratin 9; KRT10, keratin 10; ORM1, α1 glycoprotein; SERPINA1, α1-antithrypsin; TF, transferrin; TTR, transthyretin.

IVF. In the same group, high sensitivity C-reactive protein (hsCRP) and interleukin-6 did not significantly differ between the IVF and NC groups, revealing the importance of alternative markers of metabolic disturbance. The results of the metabolomic profiling of ICSI children, already published by our group, were similar [25]. Although the nontraditional inflammatory markers YKL-40 and hsCRP were actually decreased in the initial group of 84 children, the metabolomic analyses of a subset of 10 pairs of girls born after ICSI or normally conceived revealed a panel of 36 metabolites associated with obesity, insulin resistance, and the metabolic syndrome that could characterize and successfully differentiate the female ICSI group, underlining the importance of new technologies in the early detection of metabolic derangements, long before alterations can be observed at the biochemical level [25].

Low-grade inflammation is also closely related to the ART procedures, as consistently increased hsCRP levels have been detected in women undergoing ART, especially in women with the ovarian hyperstimulation syndrome [30, 31]. Some of the proteins that were identified in our study have previously been detected in the proteome of the endometrial fluid, confirming the exposure of the fetus to an adverse, inflammatory environment [32]. Moreover, a2-HS-glycoprotein was increased in intrauterine growth restriction pregnancies, suggesting a possible link between inflammation and impaired intrauterine growth [33].

Proteins of the complement system were also overexpressed in the ICSI group. Complement factors are closely related to lipid metabolism and inflammation and are involved in insulin resistance, dyslipidemia, endothelial dysfunction, and atherosclerosis [34]. Proteomic studies have also revealed the role of the complement system as an important factor of a successful ART outcome [35], further enhancing the close association between ART and inflammation.

Coagulation proteins, another group that was overexpressed in our ICSI children, have been extensively studied in relation to natural human fertilization (ovulation, hatching) and with ART, especially, after ovarian hyperstimulation [36]. Proteomic studies have confirmed the presence of

coagulation proteins in the amniotic fluid, the placenta, and the endometrium and have highlighted their role in premature birth [37]. The underexpression of plasminogen concurs with the enhanced coagulation milieu. The accentuated coagulation profile also suggests an unfavorable metabolic status, as there is a well-established interaction between inflammation and coagulation, leading to an increased cardiometabolic risk, through increased platelet activity, inhibition of fibrinolysis, and promotion of cell–cell interaction [38]. The underexpression of fibrinogen α chain in control samples is a seemingly contradictory finding that requires further validation, especially as it could not be replicated in all pairs studied and was based on the fact that mass spectrometry positively (MASCOT score >50) identified the protein present in a single protein spot (the optical density of this precise spot was superior in the control sample). Moreover, this protein was not identified in the comparison experiments that included the paired pooled samples.

In accordance with these findings, apolipoproteins A1, A4, and E—independent factors of cardiometabolic risk—were overexpressed in the ICSI group [39]. Serotransferrin was also overexpressed. Metabolic syndrome is known to influence iron homeostasis, with one third of the patients with fatty liver exhibiting a dysmetabolic iron overload syndrome, with normal or slightly elevated serotransferrin levels [40].

Pooling of samples and subsequent comparison of the derived pools led to the detection of transthyretin, a low-abundancy protein that was not identified in the paired comparisons. Interestingly, transthyretin, overexpressed in the ICSI group, is also elevated in obese children [41], as well as in preterm babies [42]. The increased transthyretin levels point to a special dysmetabolic profile, granted that transthyretin forms complexes with RBPs, adipokines that promote insulin resistance at the skeletal muscle and liver [43]. In accordance with this, our group previously demonstrated elevated RBP4 concentrations in classic IVF-conceived children [29].

The clinical significance of the overexpression of gelsolin is unclear. Gelsolin is an actin filament severing and capping protein involved in several complex functions, including lipid metabolism. Gelsolin is downregulated by interleukin-4, a cytokine that enhances metabolism and suppresses lipid deposition [44]. Moreover, gelsolin-like capping protein is related to increased carotid intima media thickness, an early index of endothelial dysfunction [45].

The overexpression of vitamin D–binding protein may be coevaluated with the rest of our findings, as polymorphisms of the coding gene correlate with body fat mass, suggesting a possible role in the pathogenesis of obesity [46]. Interestingly, vitamin D and vitamin D–binding protein levels are associated with the expression of amino acid transporters of the placenta, possibly playing a role in intrauterine growth [47]. Finally, the overexpression of keratins should be interpreted with caution, as they often represent contamination of the sample examined. However, type II keratin has been implicated in the lectin pathway of coagulation, and its identification cannot be underappreciated [48].

Very few physiologic data exist regarding the role of DAN domain family 5 protein. It seems to play a role in organogenesis, tissue growth, and differentiation. Interestingly, it also antagonizes the bone morphogenetic protein 4 signaling pathway involved in adipogenesis and cardiovascular development and disease.

All of these differences in the proteomic analysis of the serum of ICSI-born vs NC children at a mean age of ~7 years point to a long-term or even permanent, epigenetically determined modification in protein expression, as a result of the procedures associated with the ICSI method of assisted reproduction.

4. Conclusion: Limitations

This study presents auxological and laboratory data from a well-characterized group of prepubertal ICSI children in comparison with age-matched NC healthy controls. Moreover, this study applies proteomic analysis for the study of children born after ICSI, trying to overcome many confounding factors by carefully selecting case-control pairs.

We have performed a qualitative comparative proteomic analysis; therefore, our results can only approximately delineate the differences between groups. Additionally, our results were characterized by a very low identification rate, probably due to the long time period

between 2-DE and mass spectrometry. However, when analyzing the pooled samples, mass spectrometry immediately followed the 2-DE procedure and led to the identification of almost all selected spots. Finally, one of the major limitations of the technique itself lies in its capacity to detect low-abundant and possibly indicative proteins, even in the presence of significantly higher concentrations of background proteins, including albumin, immunoglobulins, and lipoproteins (selectivity and specificity) from small volumes of clinical specimens [49].

Nevertheless, we have succeeded in identifying a panel of proteins that were consistently either over- or underexpressed in the ICSI group. Most overexpressed proteins are implicated in the acute-phase reaction, blood coagulation, activation of the complement pathway, and iron and lipid metabolism, suggesting an unfavorable cardiometabolic profile of these children, at a subclinical level.

The results of this study underpin the role of proteomics in the early identification of markers of metabolic disturbance in children born after ICSI, long before any derangements become evident at the biochemical level, and highlight the importance of close, long-term monitoring of children born after ICSI, especially regarding cardiometabolic risk.

Acknowledgments

We thank Dr. Aimilia Mantziou for the hormonal assays, Dr. Maria Konsta for the statistical analysis, and Christina Marra for subject recruitment. Finally, we feel grateful toward all the children and their families for collaboration.

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This work was partially supported by the Hellenic Endocrine Society.

Author contributions: G.P.C., C.K.-G., G.T.T., and D.L. conceived and designed the study. I.K., A.G., C.K.-G., and D.L. acquired the data. A.A., A.P., I.K., and G.T.T. performed proteomic analysis. I.P. performed biochemical analysis. I.K., G.T.T., A.A., I.P., C.K.-G., and G.P.C. interpreted the data. I.K., A.G., A.A., and C.K.-G. drafted the manuscript. G.T.T., C.K.-G., and G.P.C. critically revised the manuscript.

Disclosure Summary: The authors have nothing to disclose.

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