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2 **Boolean analysis of the transcriptomic data to identify novel biomarkers of IVIG response**
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Abstract

Intravenous immunoglobulin (IVIG) is used to treat several autoimmune and inflammatory diseases, but some patients are refractory to IVIG and require alternative treatments. Identifying a biomarker that could segregate IVIG responders from non-responders has been a subject of intense research. Unfortunately, previous transcriptomic studies aimed at addressing IVIG resistance have failed to predict a biomarker that could identify IVIG-non-responders. Therefore, we used a novel data mining technique on the publicly available transcriptomic data of Kawasaki disease (KD) patients treated with IVIG to identify potential biomarkers of IVIG response. By studying the boolean patterns hidden in the expression profiles of KD patients undergoing IVIG therapy, we have identified new metabolic pathways implicated in IVIG resistance in KD. These pathways could be used as biomarkers to segregate IVIG non-responders from responders prior to IVIG infusion. Also, boolean analysis of the transcriptomic data could be further extended to identify a universal biomarker that might predict IVIG response in other autoimmune diseases.

Keywords: IVIG, Biomarkers, Autoimmune diseases, Immune metabolism, Therapy, Transcriptome, Boolean patterns

55

56 **Highlights:**

57

- 58 • Not all autoimmune patients respond to intravenous immunoglobulin (IVIG)
59 immunotherapy.
- 60
- 61 • Several studies have attempted to identify biomarkers of IVIG response.
- 62
- 63 • Specificity and/or sensitivity of identified biomarkers of IVIG response are the major
64 issues.
- 65
- 66 • Boolean analysis of the transcriptomic data could identify novel biomarkers of IVIG
67 response.
- 68
- 69 • Boolean approach identified several metabolic and signaling pathways implicated in
70 IVIG resistance.
- 71

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73

74 **1. Introduction**

75 Intravenous Immunoglobulin (IVIG) is a therapeutic normal human Immunoglobulin G
76 (IgG) prepared from the pooled plasma of several thousand healthy donors. Although, initially
77 used in the immunoglobulin (Ig) replacement therapy of primary immunodeficiency (PID)
78 patients, currently high-dose (1-2g/kg) of IVIG is used for the treatment of diverse autoimmune
79 and inflammatory diseases [1]. IVIG is used as a first line therapy in several autoimmune
80 diseases such as Guillain-Barré syndrome (GBS), Chronic inflammatory demyelinating
81 polyneuropathy (CIDP), Idiopathic thrombocytopenia purpura (ITP), Kawasaki disease (KD),
82 Autoimmune blistering disease, Inflammatory myopathies and others [2-4]. The beneficial
83 effects of IVIG are mediated via diverse mechanisms [5, 6].

84

85 Autoimmunity stems from the inability of immune system to differentiate self-antigens
86 from the foreign antigens. Mounting aberrant immune response to self-antigens has been linked
87 to over 80 inflammatory disorders, collectively known as autoimmune diseases [7]. IVIG is used
88 as first line therapy for treating several autoimmune diseases. However, not all autoimmune
89 patients respond to IVIG therapy. Resistance to IVIG therapy has been reported in many
90 autoimmune diseases including KD, ITP, CIDP and GBS [8-12].

91

92 **2. Biomarkers of IVIG response**

93 Several studies have attempted to identify biomarkers of IVIG response in various
94 autoimmune diseases. In accordance with the therapeutic use of IVIG in autoimmune and
95 inflammatory diseases, most of these markers followed the trend of either inflammatory cells like
96 platelet, lymphocyte and neutrophil counts or molecules that are the hall marks of inflammation
97 such as alanine aminotransferase, matrix metalloproteinase-8 C-reactive protein, neutrophil-
98 derived elastase, inflammatory cytokines and chemokines or their receptors (IL-6, IL-1 β , TNF,
99 G-CSF, CCR2), damage-associated molecular patterns like High mobility group box protein 1
100 (HMGB-1), S100 calcium-binding protein A8 (S100A8), S100A9 [13-19]. However, specificity
101 and/or sensitivity of these markers are the major issues. Even genetic studies on polymorphism
102 (like activating and inhibitory Fc γ receptors (Fc γ R), Phospholipase A2 Group VII) have been
103 attempted to predict IVIG response [20-22]. But those findings were not reproducible in all the
104 patient groups of given pathology.

105

106 Majority of the studies on the quest for the biomarkers of IVIG response have been
107 focused on KD patients. KD is an acute systemic vasculitis, which affect children under 5 years

108 old. Around
109 10-20% of KD patients are refractory to IVIG and are at high risk of developing coronary artery
110 aneurysms [10]. Several prediction models such as Kobayashi, Egami, Kawamura, Sano and
111 Formosa have been designed to address IVIG resistance in Japanese KD patients [23-27].
112 However, these models failed to predict IVIG resistance in other ethnicities, and also are not
113 sensitive to predict IVIG resistance in other autoimmune diseases [28].

114

115 **3. DNA Microarrays for the identification molecular biomarkers**

116 One of the classical techniques used to identify molecular biomarker is microarray [29,
117 30], which provides a snapshot of all the biological processes taking place inside a cell. DNA
118 microarray is used to study the transcriptional profiles associated with broad range of diseases to
119 identify disease specific molecular biomarker(s) [31-35]. Unfortunately, the number of
120 microarray datasets available in the public databases to study IVIG response is limited, thereby
121 emphasizing the need to revisit these datasets and seek for hidden patterns using novel data
122 mining techniques.

123

124 Several data analysis techniques are used to understand the biological meaning from the
125 microarray data. Initially, numerical score based gene ranking was used to correlate a gene with
126 a disease, thereby the gene that is highly expressed in a disease is considered to be implicated in
127 then pathogenesis [36, 37]. Later, an enhanced version of such gene selection technique was used
128 in clustering algorithms to identify huge list of genes exhibiting similar expression levels,
129 comparing the expression levels of such genes with healthy subjects helps us to identify the
130 genes associated with the disease [38]. Nowadays, sophisticated algorithms have been developed

131 to study complex transcriptional regulatory mechanisms such as co-expression, gene activation,
132 and inhibition using microarray data [39]. Although, the current data mining techniques provide
133 insights into complex gene regulatory mechanism, their ability in predicting biomarker(s), which
134 could segregate IVIG non-responders from responders remain limited.

135

136 **4. Boolean analysis of the transcriptomic data to identify novel biomarkers of IVIG** 137 **response**

138 In this study, we have used a novel boolean approach to segregate IVIG non-responders
139 from responders, and identified that cellular metabolism in peripheral blood mononuclear cells
140 plays a vital role in IVIG resistance. Using the GEO database, the expression profile of the data
141 was extracted and normalized using the quantile method [40]. Log-transformation of the gene
142 expression data was performed. Statistical analysis of gene expression data was based on the
143 single-factor Analysis of variance (ANOVA). Sorted matrix (as a tab-delimited text file) was
144 used for performing the heat map which was generated by selecting gene filtering parameters
145 (FDR threshold < 0.05 and fold change threshold =1). Clustering was performed on the sorted
146 matrix using TM4- MEV software (MultiExperiment Viewer, Dan- Farber Cancer Institute,
147 Boston, MA) [41]. Data underwent Z- score normalization so that parameters with vastly
148 different ranges could be compared directly. K-Mean clustering by using Pearson correlation as
149 the distance metric was performed within parameters.

150

151 To identify the biomarkers that could distinguish IVIG responders from non-responders
152 we studied the boolean behaviors in nearly 40,000 genes. The expression profile of the KD
153 patients undergoing IVIG therapy was extracted from the GEO database (GSE18606). By

154 converting the microarray readout into 0's and 1's ($>0 = 1$; $<0 = 0$), we were able to filter the
155 genes, which exhibit switch like behavior (on/off) specific for the treatment condition (**Figure**
156 **1**). Later, we purified these genes that exhibit boolean behavior by manually removing the false
157 positives with the aid of BART [42]. Using this approach, we were able to identify several genes
158 exhibiting boolean behavior specific to IVIG responders and non-responders. Later, we
159 performed enrichment analysis on these genes using EnrichR [43] and identified that cellular
160 metabolism is implicated in IVIG resistance (**Table 1**).

161

162 **5. Boolean approach sheds light on metabolic and signaling pathways specific to IVIG** 163 **responders and non-responders**

164 Based on the enrichment analysis, we identified that several metabolic and signaling
165 pathways are implicated in IVIG resistance (**Figure 1**). We focused our analyses on the
166 pathways that are linked to immune response. Also, to ensure accurate annotation, we selected
167 the pathways with P values lesser than 0.009. In IVIG non-responders, interferon (IFN)
168 regulatory factor 3 (IRF3)-mediated activation of type 1 IFN was turned on. Genes that increase
169 myeloid cell number, and that promote pyrimidine metabolism, abnormal plasmacytoid dendritic
170 cell physiology were also turned on (**Table 1**) (**Figure 1**). Of interest, though several genes
171 (fifteen) were turned on in non-responder patients, these genes contribute to limited set of
172 pathways. On the other hand, genes that modulate lymphocyte migration and Th17 response
173 were switched off (**Table 1**). Interestingly, the genes that mediate palmitate biosynthesis were
174 also switched off in IVIG non-responders (**Table 1**). Palmitate is a saturated fatty acid that acts
175 as agonist for Toll-like receptor 4 (TLR4) receptor and promotes inflammation by activating
176 macrophages and innate immune cells [44, 45]. Previous studies have highlighted the importance

177 of inflammation in IVIG resistance [25]. In IVIG responders, the genes that mediate
178 mitochondrial calcium uptake and several aspects of mitochondria mediated adenosine
179 triphosphate (ATP) production were switched off (**Table 1**). Interestingly, though the number
180 genes switched off in responder patients with boolean behavior were small (six), these genes
181 contribute to many pathways, particularly mitochondrial functions. The genes that mediate
182 degranulation of mast cells were also switched off (**Table 1**). This suggest that suppression of
183 mitochondrial function and inflammation plays a vital role in inducing immune homeostasis in
184 IVIG responders.

185

186 **6. Boolean behavior of genes could not be identified using conventional data analysis** 187 **algorithms**

188 There are several tools available to study the differentially expressed genes in a
189 microarray dataset, but they are not developed to specifically study boolean behaviors in a
190 microarray dataset. We performed data analysis on GSE18606 dataset using GEO2R and BART
191 to study whether algorithms developed to study differentially expressed genes in a microarray
192 dataset were able to shed light on boolean behavior, but both GEO2R and BART failed to index
193 the genes exhibiting boolean behavior as differentially expressed. We also used MEV to
194 performed K mean clustering, where we grouped the 40,000 genes into different clusters. We
195 generated 25, 50, 75 and 1000 different clusters to see if clustering algorithm could group the
196 genes that exhibit boolean behavior, but these genes were distributed across different clusters
197 (**Table 2**).

198

199 **7. Conclusion and perspectives**

200 IVIG is used as first line therapy in several autoimmune and inflammatory diseases, but
201 not all patients respond to IVIG treatment. Identification of biomarker that could segregate IVIG
202 responders from non-responders remains a challenge. Patients who exhibit IVIG resistance need
203 to undergo alternative therapy, thus identifying these patients in the early stages of disease will
204 help clinicians to initiate an alternative treatment strategy. Microarray is a sophisticated
205 technique that sheds lights on the complex processes occurring inside a cell and helps in
206 identifying disease specific biomarkers. There are several software's available to understand the
207 biological meaning hidden in the microarray data, each tool provides insights into the distinct
208 biological processes taking place inside the cell. Despite these advancements in microarray
209 technology, identifying a biomarker, which could segregate IVIG responders from non-
210 responders remains a challenge. In this study we have used a novel data analysis technique,
211 where we screened the genes that exhibit boolean or switch like behavior specific to IVIG
212 responders and non-responders. Using this boolean approach we have identified that cellular
213 metabolism plays a vital role in IVIG resistance. The KD patients who responded to IVIG
214 therapy exhibited limited mitochondrial activity and low inflammation. Whereas, KD patients
215 resistant to IVIG therapy had increased expression of genes that promote inflammation,
216 abnormal regulation of Th17 response.

217

218 Taking into consideration the role of cellular metabolism in influencing the success of
219 IVIG therapy, diagnostic assays could be developed to rapidly study these parameters in KD
220 patients. This could help clinicians in identifying the IVIG non-responders at an early stage and
221 to initiate alternative therapies to reduce the morbidity and associated therapeutic costs [46].
222 However, the role of cellular metabolism in the outcome of IVIG therapy in other autoimmune

223 diseases need to be studied to discover a universal biomarker. Currently, dimethyl fumarate, a
224 small molecule targeting glycolytic pathway have been approved to treat multiple sclerosis [47-
225 49]. Since mitochondria mediated ATP production was suppressed in IVIG responders, small
226 molecules capable of selectively inhibiting mitochondrial function in immune cells could be used
227 along with IVIG to treat IVIG non-responders.

228

229

230 **Declaration of Competing Interest :**

231

232 JB and SVK received grants from CSL Behring France for the research work on the mechanisms

233 of action of intravenous immunoglobulin.

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380

381 **Figure Legend**

382

383 **Figure 1. Genes and the major pathways that exhibit switch like behavior (on/off) in**

384 **Kawasaki disease patients specific for the IVIG treatment condition.** Several genes in IVIG

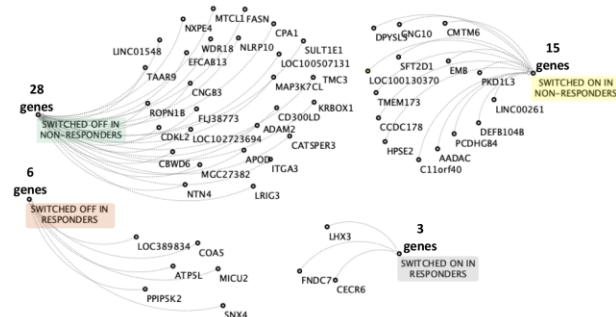
385 resistant and responder Kawasaki disease patients display switch like behavior (on/off). The

386 cluster number of those genes are highlighted on the right side of the figure. The key pathways

387 that are associated with those genes are also listed.

388

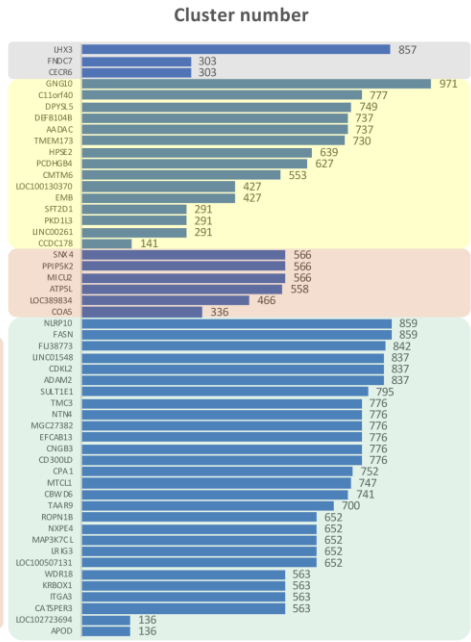
389



PATHWAYS SWITCHED OFF IN NON-RESPONDERS
 Delta Np63 pathway
 negative regulation of inflammatory response (GO:0050728)
 positive regulation of protein localization to membrane (GO:1905477)
 negative regulation of lymphocyte migration (GO:2000402)
 positive regulation of microtubule motor activity (GO:2000576)
 Integrin-mediated cell adhesion
 palmitate biosynthesis Homo sapiens PWY-5994
 regulation of monocyte chemotactic protein-1 production (GO:0071637)
 regulation of T-helper 17 type immune response (GO:2000316)
 intracellular cGMP activated cation channel activity (GO:0005223)
 trace-amine receptor activity (GO:0001594)

PATHWAYS SWITCHED ON IN NON-RESPONDERS
 MP:0008608 increased circulating interleukin-13 level
 Proton-coupled monocarboxylate transport Homo sapiens R-HSA-433692
 IRF3 mediated activation of type 1 IFN Homo sapiens R-HSA-1606341
 positive regulation of triglyceride catabolic process (GO:0010898)
 MP:0013663 increased myeloid cell number
 MP:0008769 abnormal plasmacytoid dendritic cell physiology
 Pyrimidine Metabolism Homo sapiens P02771

PATHWAYS SWITCHED OFF IN RESPONDERS
 Inositol pyrophosphates biosynthesis Homo sapiens PWY-6369
 positive regulation of mast cell degranulation (GO:0043306)
 mitochondrial calcium uptake (GO:0036444)
 proton-transporting ATP synthase activity, rotational mechanism (GO:0046933)
 Chemiosmotic coupling formation of ATP
 energy coupled proton transport, down electrochemical gradient (GO:0015985)
 Formation of ATP by chemiosmotic coupling Homo sapiens R-HSA-163210
 positive regulation of ATP synthesis (GO:0046887)
 mitochondrial respiratory chain complex IV assembly (GO:0033617)
 mitochondrial ATP synthesis coupled proton transport (GO:0042776)
 mitochondrial calcium ion transmembrane transport (GO:0006851)
 mitochondrial respiratory chain complex IV biogenesis (GO:0097034)
 adenosine ribonucleotides de novo biosynthesis Homo sapiens PWY-7219
 ATPase activity, coupled to transmembrane movement of ions, rotational mechanism (GO:0044769)
 endocytic recycling (GO:0032456)
 cation-transporting ATPase activity (GO:0019829)
 Endosomal Recycling



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Table 1: Major pathways specific to IVIG responders and non-responders in Kawasaki disease

Main pathways that are switched off in non-responder patients

Name	P-value	Adjusted p-value	Odds Ratio	Combined score	Source
Delta Np63 pathway	0,001965	1	30,4	189,43	Bioplanet 2019
Negative regulation of inflammatory response (GO:0050728)	0,005315	1	18,32	95,92	GO biological process 2018
Positive regulation of protein localization to membrane (GO:1905477)	0,007787	1	15,04	73,01	GO biological process 2018
Negative regulation of lymphocyte migration (GO:2000402)	0,008372	1	119,05	569,39	GO biological process 2018

Positive regulation of microtubule motor activity (GO:2000576)	0,008372	1	119,05	569,39	GO biological process 2018
Integrin-mediated cell adhesion	0,008596	1	14,29	67,95	Bioplanet 2019
Palmitate biosynthesis Homo sapiens PWY-5994	0,00976	1	102,04	472,39	HumanCyc 2016
Regulation of monocyte chemotactic protein-1 production (GO:0071637)	0,00976	1	102,04	472,39	GO biological process 2018
Regulation of T-helper 17 type immune response (GO:2000316)	0,00976	1	102,04	472,39	GO biological process 2018
Intracellular cGMP activated cation channel activity (GO:0005223)	0,00976	1	102,04	472,39	GO molecular function 2018
Trace-amine receptor activity (GO:0001594)	0,00976	1	102,04	472,39	GO molecular function 2018

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Key pathways that are switched off in responder patients

Name	P-value	Adjusted p-value	Odds Ratio	Combined score	Source
Inositol pyrophosphates biosynthesis Homo sapiens PWY-6369	0,002098	0,319	476,19	2936,47	HumanCyc 2016
Positive regulation of mast cell degranulation (GO:0043306)	0,002098	1	476,19	2936,47	GO biological process 2018
Mitochondrial calcium uptake (GO:0036444)	0,002697	1	370,37	2190,93	GO biological process 2018
Proton-transporting ATP synthase activity, rotational mechanism (GO:0046933)	0,003296	1	303,03	1731,85	GO molecular function 2018

Chemiosmotic coupling formation of ATP	0,003894	1	256,41	1422,64	Bioplanet 2019
Energy coupled proton transport, down electrochemical gradient (GO:0015985)	0,003894	1	256,41	1422,64	GO biological process 2018
Formation of ATP by chemiosmotic coupling Homo sapiens R-HSA-163210	0,004791	1	208,33	1112,71	Reactome 2016
Positive regulation of hormone secretion (GO:0046887)	0,00509	1	196,08	1035,4	GO biological process 2018
Mitochondrial respiratory chain complex IV assembly (GO:0033617)	0,005986	1	166,67	853,06	GO biological process 2018
Mitochondrial ATP synthesis coupled proton transport (GO:0042776)	0,006284	1	158,73	804,72	GO biological process 2018
Mitochondrial calcium ion transmembrane transport (GO:0006851)	0,006284	1	158,73	804,72	GO biological process 2018
Mitochondrial respiratory chain complex IV biogenesis (GO:0097034)	0,006284	1	158,73	804,72	GO biological process 2018
Adenosine ribonucleotides de novo biosynthesis Homo sapiens PWY-7219	0,007477	0,5683	133,33	652,78	HumanCyc 2017
ATPase activity, coupled to transmembrane movement of ions, rotational mechanism (GO:0044769)	0,007477	1	133,33	652,78	GO molecular function 2018
Endocytic recycling (GO:0032456)	0,008074	1	123,46	594,96	GO biological process 2018
Cation-transporting ATPase activity (GO:0019829)	0,00867	1	114,94	545,74	GO molecular function 2018
Endosomal Recycling	0,009563	1	104,17	484,36	Elsevier pathway collection

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Important pathways that are switched on in non-responder patients

Name	P-value	Adjusted p-value	Odds Ratio	Combined score	Source
MP:0008608 increased circulating interleukin-13 level	0,003745	1	266,67	1489,98	MGI mammalian phenotype level 4 2019
Proton-coupled monocarboxylate transport Homo sapiens R-HSA-433692	0,004492	1	222,22	1201,21	Reactome 2016
IRF3 mediated activation of type 1 IFN Homo sapiens R-HSA-1606341	0,004492	1	222,22	1201,21	Reactome 2016
Positive regulation of triglyceride catabolic process (GO:0010898)	0,005239	1	190,48	1000,31	GO biological process 2018
MP:0013663 increased myeloid cell number	0,005985	1	166,67	853,08	MGI mammalian phenotype level 4 2019
MP:0008769 abnormal plasmacytoid dendritic cell physiology	0,006731	1	148,15	740,89	MGI mammalian phenotype level 4 2019
Pyrimidine Metabolism Homo sapiens P02771	0,007476	0,8373	133,33	652,8	Panther 2016

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Table 2: General characteristics and parameters of K-Medians Clustering

Clusters	KMC mode	Iterations	Converged	Pearson Correlation
1000	Calculated Means	24	TRUE	TRUE
25	Calculated Means	100	TRUE	TRUE
50	Calculated Means	69	TRUE	TRUE
75	Calculated Means	66	TRUE	TRUE

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