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Research paper

New insight of human-IgH 3′regulatory regions in immunoglobulins switch

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ABSTRACT

Background: Several studies in animal models have demonstrated the role of the 3′ Regulatory Region (3′RR) in the B cell maturation in mammals. In healthy humans, the concentration of each class of circulating immunoglobulins (Igs) has stable but different levels, due to several control mechanisms that also involve a duplicated version of the 3′RR on the chromosome 14 (chr14). The classes' equilibrium can be altered during infections and in other pathological conditions.
Material and methods: We studied the concentrations of IgA, IgM, IgG classes and IgG subclasses in a cohort of 1235 people having immunoglobulin concentrations within normal range to determine the presence of any correlation between the Igs serum concentrations, age and ratio among Ig classes and IgG subclasses in healthy humans. Furthermore, we assessed the concentrations of IgE and the allelic frequency of 3′RR1 hs1.2 enhancer in a group of 115 subjects with high levels of circulating IgE due to acute exacerbation of allergic asthma and in a control group of 118 healthy subjects.
Results: In both children and adult subjects, the concentrations of the four IgG subclasses decreased from IgG1 to IgG4. Furthermore, the 3′RR1 enhancer hs1.2 alleles contribute to the control of the IgG subclasses levels, but it does not affect the IgE levels.
Conclusion: The 3′RR1 controls IgG and IgE through different mechanisms, only in the IgG case involving the hs1.2 alleles. Thus, considering the IgH constant genes loci on the chromosome 14 and the multiple steps of switch that rearrange the whole region, we found that in humans the classes of Igs are modulated by mechanisms involving a complex interaction and transition between 3′RR1 and 3′RR2, also in physiological conditions.

1. Introduction

In mammals, immunoglobulin (Ig) heavy chain (IgH) production is under the control of a large network of cell-interactions and is modulated by specific cytokines. The B-cell regulatory system is involved in all immune reactions, from infections to inflammation and autoimmunity (Wang et al., 2020). The Ig heavy chain cluster has a 3′Regulatory Region (3′RR) that plays a pivotal role in Ig class switch,

Abbreviations: 3′RR, 3′ Regulatory Region; chr, chromosome; Ig, Immunoglobulin; IgH, Immunoglobulin heavy chain; PBMC, Peripheral Blood Mononuclear Cells; PCR, Polymerase Chain Reaction.

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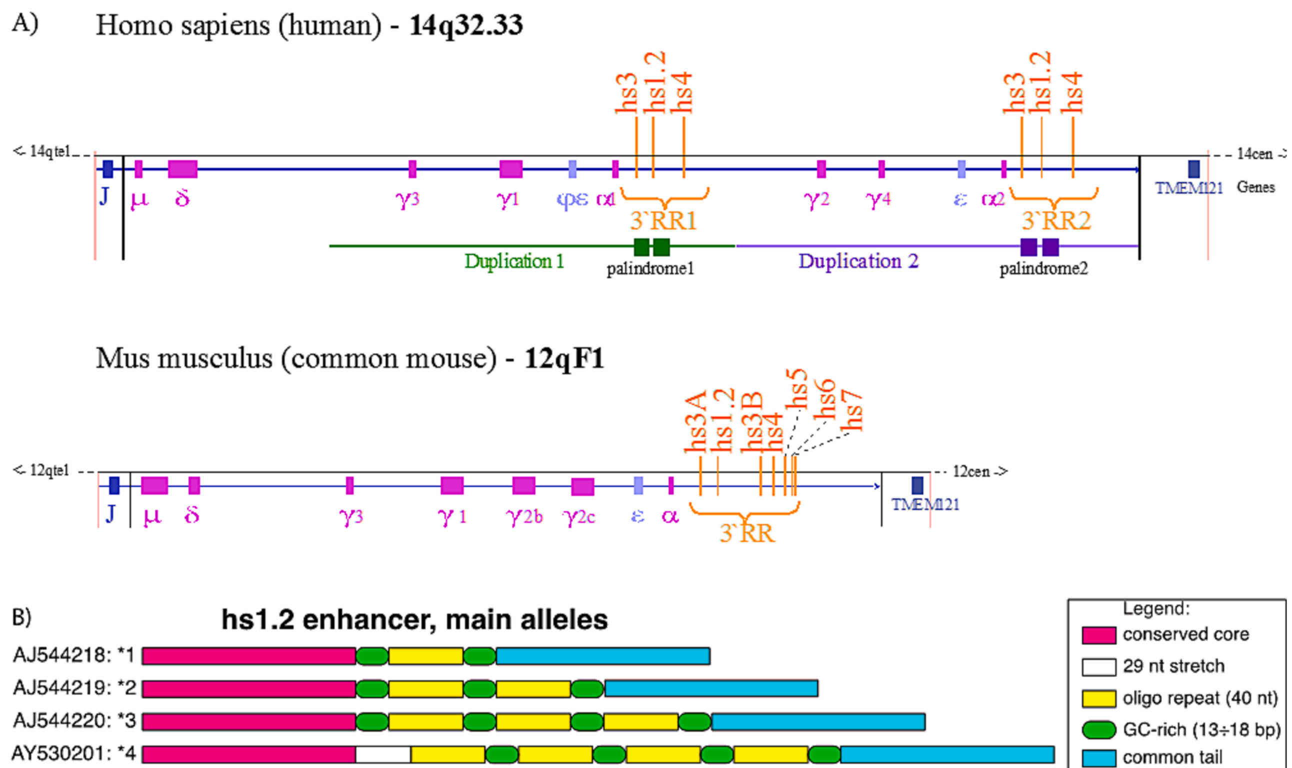


Fig. 1. Map of the Constant heavy region in human and mouse (A) and scheme of the four main alleles of the human hs1.2 enhancer (B). A) The different genetic structures in human and mouse are shown. An internal duplication occurred in human, possibly inducing different constrains of regulation for isotype switch and plasma cell production. B) The human hs1.2 enhancer consists of an evolutionary conserved core sequence (purple box), followed by a variable number of tandem repeated elements, i.e. a sequence harboring transcription binding sites (yellow boxes) and a shorter CG-rich region (green boxes). Moreover, a 29 bp sequence (white box) has been identified that may or not be present between the core and the other elements, rising the number of the allelic polymorphisms of this enhancer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

maturation and expression (Vincent-Fabert et al., 2010; Kim et al., 2016).

B-cell isotype switch modulates the adaptive immune response (Stavnezer and Schrader, 2014). This process involves a series of events: initially, the activation that induces deaminase and, finally, the joining of the variable-chain region with one of the downstream *IgH* constant-chain genes on the same chromosome (14q32 in human, scheme of the cluster region in Fig. 1A). These phenomena lead B cells to become mature antibody-secreting plasma cells. The mechanisms leading to these rearrangements are determined by chromatin conformational changes as methylation, demethylation, binding to nuclear factors, remodelling events of the activated chromatin region (Santos et al., 2019; Garot et al., 2016; Thomas-Claudepierre et al., 2016; Spiegel et al., 2021).

The Ig heavy chain cluster with the internal 3′RR can lead to several 3D structures shown to be crucial in the regulation of mouse and human immune responses (Birshtein et al., 1997; Cogné, 2013). The different genetic structures in humans and mouse depend on an internal duplication possibly inducing different constrains of regulation for isotype switch and plasma cell production (Fig. 1A).

The chromatin 3D change of dislocation is necessary for B cell maturation and control (Birshtein et al., 1997). The highly repetitive donor and acceptor switch (S) regions come into contact inducing activation of intronic (I) promoters, that are located upstream of each *IgH* constant region gene (Thomas-Claudepierre et al., 2016; Delgado-Benito et al., 2018). The spatial conformation of chromatin is finally determined by the interaction with specific DNA binding proteins that induce a proper position in the nuclear environment (Delgado-Benito et al., 2018; Michaelson et al., 1996). Considering the different chromosomal location of the heavy chain constant genes and different steps of switch, we hypothesized that 3′RR1 control of IgG and IgE could happen through different mechanisms. In fact, Igγ and Igε genes are located very far from each other respect to 3′RR1 (Fig. 1A). This distance implies three-dimensional organization that changes during activation. The four γ genes are located on both sides of 3′RR1 and the ε gene lies in the 3′ terminal part of the Ig heavy chain cluster far from 3′RR1 and close to 3′RR2 (Fig. 1A). The 3′RRs harbour three enhancers: DNase-hypersensitive region 3 (hs3), hs1.2, and hs4. The central 3′RR1 hs1.2 enhancer consists of a specific polymorphic structure presenting one to four copies of a 40 bp satellite, separated by GC rich spacers of different number of nucleotides (Fig. 1B). The 40 bp element is capable of carrying the consensus for several transcription factors, such as Sp1 and NFκB (Cianci et al., 2018).

Studies in past years have documented that many IgG-mediated diseases are differently related to the four main alleles of the 3′RR1 hs1.2 enhancer, located downstream of Alpha-1 gene (Fig. 1B) [e.g. (Cianci et al., 2018; Aupetit et al., 2000; Mills et al., 1997), suggesting a critical role of this enhancer in Igs expression, perhaps through interactions with transcription factors, hormones and xenobiotics (Jones et al., 2016; Salisbury and Sulentic, 2015).

In the human *IgH* cluster region, a segmental duplication harbours 3 constant region genes and a pseudo-epsilon gene between the mu enhancer (Emu) and the 3′RR-1, and 4 constant region genes, so including a still functional epsilon gene, between the two 3′RR copies (Fig. 1A).

This map prompted us to hypothesize that the position of the *IgH* genes may exert a possible influence on the expression of 4 IgG subclasses and that would differ for IgE. To test the hypothesis that the classes' expression is differently regulated, we analysed the concentration of four IgG subclasses and of the IgE class in two cohorts of subjects, i.e. individuals with immunoglobulin concentrations within normal range and asthmatic patients. In the latter group of patients, we have

Table 1
Study population, first cohort.

	Total	0 ≥ 4 years old	5 ≥ 6 years old	7 ≥ 16 years old	≥17 years old
Males	701	210	135	309	47
Females	534	148	98	212	76
Total	1235	358	233	521	123

also considered the possible influence of the 3′RR1 hs1.2 alleles on IgE production.

2. Materials and methods

2.1. Study populations

The first cohort comprised a total of 1235 individuals with immunoglobulin concentrations within normal range (701 males and 534 females). This group included: 1112 children from Bambino Gesù Children's Hospital (Table 1), in Rome (654 males and 458 females, mean age 7.11 ± 4.22 years old; 358 ≥ 4 years, 233 from 5 ≥ 6 years, 521 from 7 ≥ 16 years) and 123 adults (47 males and 76 females, ≥17 years, mean age 40.92 ± 20.71 years). These individuals were either volunteer healthy students or employees of the Medical School of the University Hospital of Tor Vergata in Rome.

The second cohort was made of 115 children (70 males and 45 females, mean age 7.9 ± 3.2 years) who presented with high levels of circulating IgE due to acute exacerbation of allergic asthma from the San Pietro Hospital Fatebenefratelli of Rome. As control group for these allergic patients, we enrolled 118 volunteer healthy adult subjects (73 males and 45 females, mean age 26.2 ± 5.1 years) among medical students at the University of Roma Tor Vergata. All subjects were from the same geographical area (Rome, Italy).

Data from titration of Igs classes and IgG subclasses of 1112 children have been provided by the Clinical Laboratory at the Bambino Gesù Children's Hospital, limited to children with immunoglobulin concentrations within the normal reference range. Data from titration of Igs classes of 115 allergic asthma children from the San Pietro Hospital Fatebenefratelli of Rome have been provided by the Hospital Clinical Laboratory.

Peripheral blood samples were collected from each participant, both adult volunteers and allergic asthma children. Mononuclear cells (PBMC) were separated according to standard procedures (Cianci et al., 2012). B and T cell subsets were evaluated by cytofluorimetric count, performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Surface molecules were detected by using monoclonal anti-CD3, CD4, CD8, CD19, or CD16/56 antibodies, in different combinations (Cianci et al., 2012).

Concentrations (mg/dl) of IgG and the subclasses IgG1, IgG2, IgG3 and IgG4 have been determined by a nephelometric assay (BN ProSpec, Siemens Healthineers Diagnostics, German). An immunoturbidimetric method was used for IgA, IgG e IgM (Architect c16000, Abbott Laboratories, 1921 Hurd Drive Irving, TX 75038-4313); chemiluminescence was performed for IgE using Immulite 2000, Siemens Healthcare Diagnostics, Germany.

2.2. PCR assay

Aliquotes of blood samples from 118 adult volunteers and 115 asthmatic patients were used to perform a selective PCR to amplify the hs1.2-A region as described previously (D'Addabbo et al., 2018) (Gene Bank AJ544218, AJ544219, AJ544220, AJ544221). Genomic DNA was extracted by standard methods (D'Addabbo et al., 2018). A second nested PCR was performed to amplify the polymorphic core of the enhancer hs1.2. To avoid carryover of genomic DNA from the first reaction, 1/50 of the volume of the first PCR was used for the nested PCR. The primers for the PCR have been reported in Giambra et al. (Giambra et al., 2006). PCR products were analysed on a 3.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA). Control reactions corresponding to diluted genomic DNA to show absence of amplification of carry-over were performed with 1 and 5 ng of total genomic DNA and resulted in no visible amplification in those conditions on gel agarose electrophoresis (Giambra et al., 2006).

2.3. Ethics

Informed consent was obtained from all subjects involved in the study and from parents of minors enrolled. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Tor Vergata University Hospital (ID number 41.17) of Rome.

2.4. Statistical analysis

Descriptive analyses are presented as mean (standard deviation), median (interquartile range [IQR]) or absolute and relative frequency, as appropriate. Data were analysed for normality of distribution using the Shapiro-Wilk W test and nonparametric tests have been chosen for the comparisons between and among groups. Mann-Whitney *U* test was used for the comparison between two groups, while the Kruskal-Wallis for the comparison of several groups. $\chi 2$ test was used for categorical data. A p value < 0.05 was considered statistically significant. Statistical analysis was performed using the Statistical Package IC STATA, release 15.1.

3. Results

3.1. Igs concentrations

The concentration (mg/dl) of IgM, IgA, IgG and IgG subclasses was assessed for all 1235 subjects.

Since there is a change in antibody levels and expression of Igs isotypes in children younger than 5 years compared to children older than 5 years through adults (D'Addabbo et al., 2018), subjects were divided

Table 2
Serum concentrations of all Igs classes and subclasses.

Igs subclasses	All subjects (n = 1235)	0 ≥ 4 years old (n = 358)	7 ≥ 16 years old (n = 521)	≥17 years old (n = 123)	p-value*
IgG	959.0 (754.0,1174.0)	746.0 (582.0,918.5)	1074.0 (874.0,1318.0)	1102.5 (977.5,1260.0)	<0.001
IgG1	680.0 (549.0,843.0)	577.0 (455.0,725.0)	745.0 (608.0,935.0)	672.0 (609.0,818.0)	<0.001
IgG2	165.0 (111.0,234.0)	108.5 (86.0,140.0)	200.0 (152.0,258.0)	297.0 (222.0,381.0)	<0.001
IgG3	35.0 (25.0,50.0)	28.0 (20.0,39.0)	42.0 (29.0,59.0)	38.0 (31.0,53.0)	<0.001
IgG4	26.0 (9.0,69.0)	10.0 (3.0,27.0)	40.0 (14.0,96.0)	46.0 (27.0,86.0)	<0.001
IgA	95.0 (52.0,152.0)	49.0 (26.0,79.0)	120.5 (78.0,171.5)	198.0 (143.0,265.0)	<0.001
IgM	97.0 (72.0,130.0)	86.0 (65.0,111.0)	99.0 (75.0,140.0)	115.0 (70.5,157.5)	<0.001

Median values (and interquartile range) of total IgG, IgG subclasses and of other classes of Igs in children ≤ 4 years, 7 ≥ 16 years of age and adults ≥ 17 years. The values were expressed in mg/dl.

* Kruskal-Wallis test.

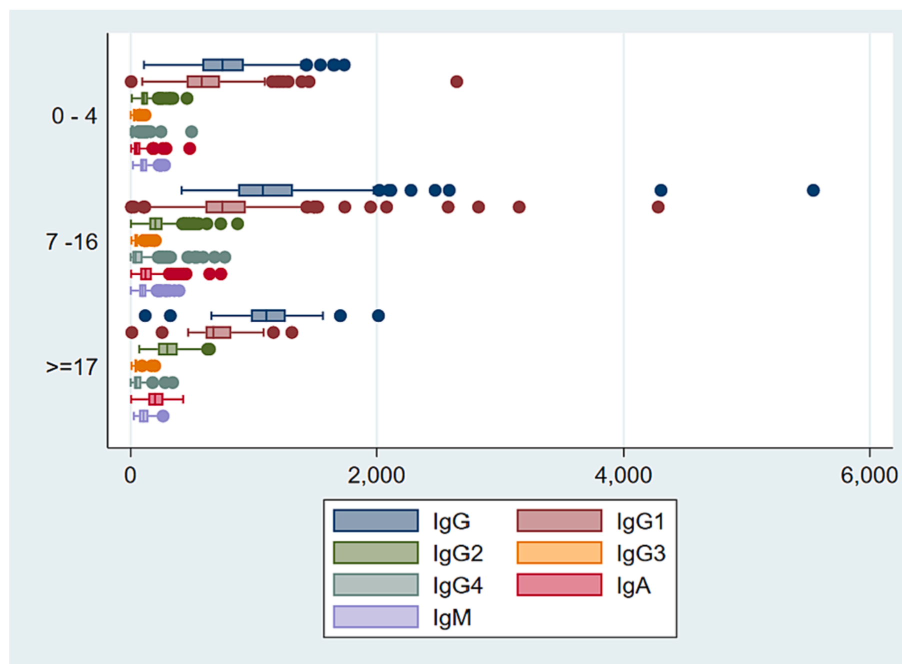
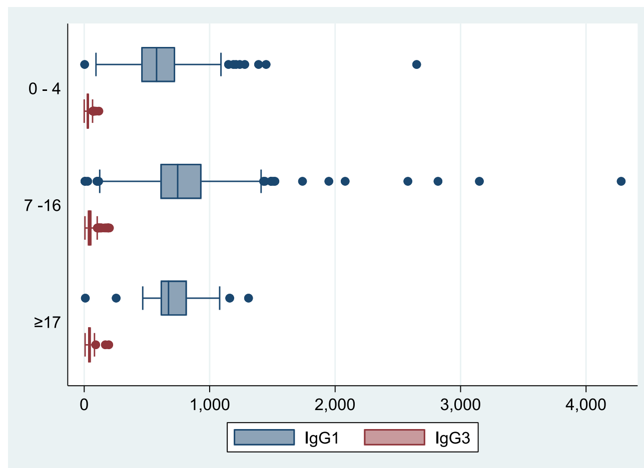


Fig. 2. Median values (and interquartile range) of total IgG, IgG subclasses, IgA and IgM in children < 4 years, 7 > 16 years of age and adults > 17 years.



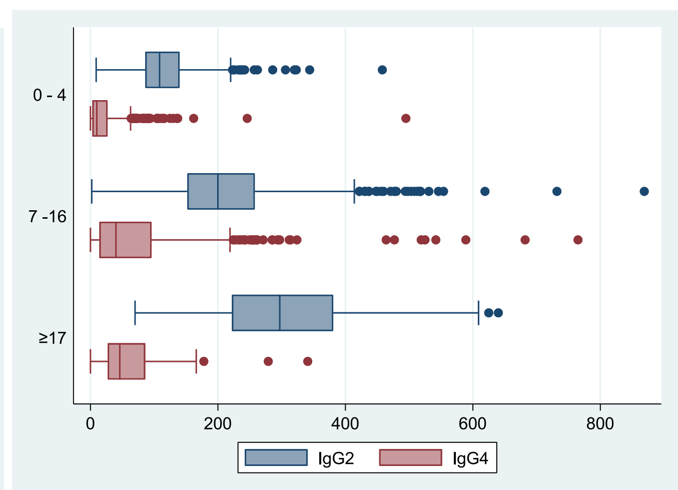


Fig. 3. Differences between serum IgG1 and IgG3 and IgG2 and IgG4. Igγ1 and Igγ3 genes subclasses in the IgH map are on the same side respect to 3′RR1. IgG3 concentrations are lower as compared to IgG1 for each age class (*$p < 0.001$). Igγ2 and Igγ4 genes subclasses are on the other side in the IgH map of 3′RR1. IgG2 concentrations are higher than IgG4 for each age class (*$p < 0.001$) *(Mann-Whitney.test).

into three age groups: 1) ≤ 4 years, 2) 7 ≥ 16 years and 3) older than 17 years. 233 subjects aged 5 ≤ 6 years were not considered for this analysis.

As shown in Table 2, the serum Ig concentrations of all classes and subclasses are significantly lower in patients aged ≤ 4 years compared to those ≥ 7 years or to subjects ≥ 17 years. There is a physiological increase of the Ig concentrations with age and these differences were statistically significant among all age groups ($p < 0.001$) (Fig. 2), with a not significant exception observed for IgG1 and IgG3 in 7 ≥ 16 and ≥ 17 age groups (higher in subjects aged 7 ≥ 16 years than in those ≥ 17 years, but in any case lower in subjects aged 0 ≥ 4 years). No differences were found between males and females in any group (data not shown).

Comparing the IgG subclasses, serum concentration decreased from IgG1 to IgG4 (i.e. IgG1 > IgG2 > IgG3 > IgG4), a trend that is maintained in all age groups, with the only exception of IgG4 in subjects ≥ 17 years old, whose median value is higher than IgG3 median one (Table 2). Comparison of IgG1 and IgG3 subclasses levels, whose genes in the IgH genomic map are on the same side respect to 3′RR1, shows a strong consistent difference in all groups: IgG3 concentrations are lower compared to IgG1 in each age group ($p < 0.001$). This is true also for IgG2 and IgG4, whose genes are on the other side of 3′RR1 in the IgH map, with IgG2 values higher than IgG4 in each age group ($p < 0.001$) (Fig. 3).

3.2. *IgE concentrations in asthma patients and analysis of hs1.2 allelic frequencies*

We measured IgE concentrations (mg/dl) and frequencies of the four hs1.2 enhancer alleles within the 3′RR1 in 115 subjects with acute exacerbation of allergic asthma and 118 healthy controls. Neither the patients with asthma nor the healthy controls show noticeable defects in the B- and T-cell compartments, as evaluated by cytofluorimetric counts

Table 3
Number of observed hs1.2 alleles in asthma subjects and healthy controls.

	Healthy controls		Asthma subjects	
	Observed	%	Observed	%
Allele 1	68	28.82	79	34.35
Allele 2	140	59.32	127	55.22
Allele 3	1	0.42	3	1.3
Allele 4	27	11.44	21	9.13
Total genotypes	236	100	230	100

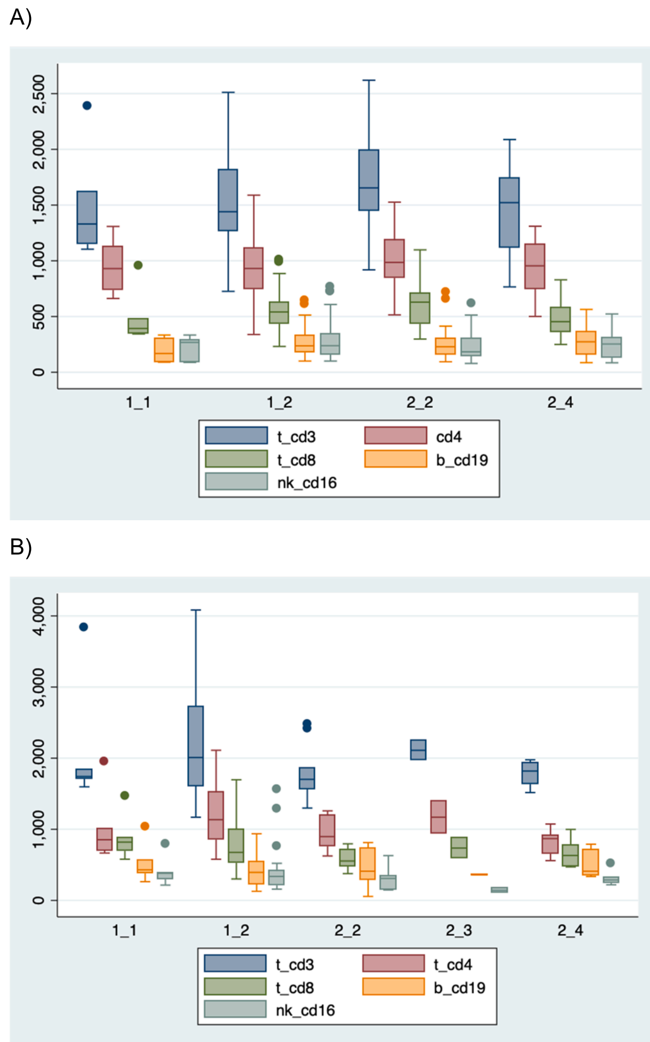


Fig. 4. Median values (and interquartile range) of CD3, CD4, CD8, CD19 or CD16/56 cells for all genotypes in control individuals (A) and in asthmatic patients (B).

of B- and T-cell subsets.

The circulating IgE concentrations were in the normal range for healthy subjects, while they were significantly higher in subjects with acute asthma (Median values [IQR]: 50.5 [119] vs 239 [519]; $p < 0.001$) (Figure S1). No significant differences were found between asthmatic patients and controls in allele frequencies of the 3′RR1 hs1.2, as shown in Table 3.

The two groups displayed similar genotype frequency, either in homozygosis or in heterozygosis. None of the genotypes were associated with a statistically significant level of IgG, IgM, and IgA both in healthy controls ($p = 0.56$, 0.42 and 0.16, respectively) and in patients with asthma ($p = 0.36$, 0.32 and 0.06, respectively).

IgG and IgM concentrations in subjects homozygous for alleles 1 or 2 showed no statistically significant differences in patients with asthma ($p = 0.73$ and $p = 0.66$, respectively) as compared to controls ($p = 0.16$ and $p = 0.69$, respectively). However, there is a significant difference in IgA levels in subjects homozygous for allele 1 (117 mg/dl.) vs allele 2 (184 mg/dl) in controls ($p = 0.01$). This difference is not present in patients with asthma ($p = 0.599$). In asthmatic patients and in control individuals, there was no significant difference in the number of CD3, CD4, CD19 or CD16/56 cells for all genotypes (Fig. 4). A significant difference was shown only in CD8 cells of asthmatic patients homozygous for allele 1 (820 cell/μl) vs allele 2 (554 cell/μl) ($p = 0.02$).

4. Discussion

In humans, the genomic organization of the Ig constant region heavy-chain cluster (IgH) is structurally different from rodents. In fact, a segmental duplication, spanning from the γ gene to the α gene and including the entire 3′RR, occurred during evolution and was found in primates. The two copies of the 3′RR (Fig. 1A) in human ancestor could have acquired different functions. We hypothesize that this complex genetic organization contributes to the regulation of the human Ig heavy chain genes through mechanisms involving alternatively the two 3′RRs. The first mechanism is related to the 3′RR1 hs1.2 alleles (Fig. 1B) that have been associated to several autoimmune diseases and to IgM production (Cianci et al., 2018; Aupetit et al., 2000). The second mechanism could be related to the different regulation induced by 3′RR1 and 3′RR2 because of their difference in chromosomal location. The 3′RR1 is downstream to γ3, γ1 and α1 genes and a pseudogene version of the ε class, while 3′RR2 is near the 3′ terminal part of the cluster, downstream to γ2, γ4, a functional ε and α2 genes (Fig. 1A). Indeed, the class switch versus γ2/γ4 is obtained through the deletion of 3′RR1 along with Emu and the genomic part in between, when the variable regions join to the constant one, so ablating the segmental duplication. Consequently, IgH expression in plasma cells after the isotype switch of γ2, γ4, ε and α2 cannot be regulated anymore by 3′RR1. Thus, the 3′RR2 is the sole regulatory region that can participate in the control of expression of these 4 genes at the 3′ of the cluster and the influence of 3′RR1 hs1.2 alleles on the expression of these four genes at this stage can be excluded. It is worth noting that 3′RR2 has not the same variability in its hs1.2 enhancer as the 3′RR1 copy of the enhancer, like is seen in populations from Africa and Europe (Serone et al., 2014; Giambra et al., 2005).

It was found that the higher serum concentration in IgG1 and IgG2 is the result of a greater number of specific plasma cells switched rather than a greater secretion rate of immunoglobulins among the committed plasma cells (Blanco et al., 2018). The commitment of plasma cells towards IgG3 or IgG4 can have same functioning regulatory regions as for IgG1 and IgG2.

Differently from the findings in several diseases with autoimmune components [e.g., (Cianci et al., 2018; Aupetit et al., 2000), we found no association between the 3′RR1 hs1.2 polymorphic alleles and asthma, an allergic immune disease related to IgE dysregulation. In humans, there is only one active ε constant region gene, despite the internal duplication on the IgH region, because of a gene loss through pseudogenization in the second ε gene locus. Thus, it results that there are no alternative switches to produce IgE for comparison. The IgE production by mature B lymphocytes follows the entire genomic deletion of the heavy chain introns, including the 3′RR1, up to the ε constant gene region, and the remaining region can be transcriptionally modulated only by the 3′RR2 (Fig. 1) (Giambra et al., 2005).

Consistently with the difference between allergic reactions and immune reactions in several immune-mediated diseases, the IgE production seems to follow a different pathway compared to other isotypes (Looney et al., 2016). Genomic comparison among multiple mammalian species support the suggestion that maintaining the IgE expression under a threshold level could be a crucial step for fitness. Indeed, to our

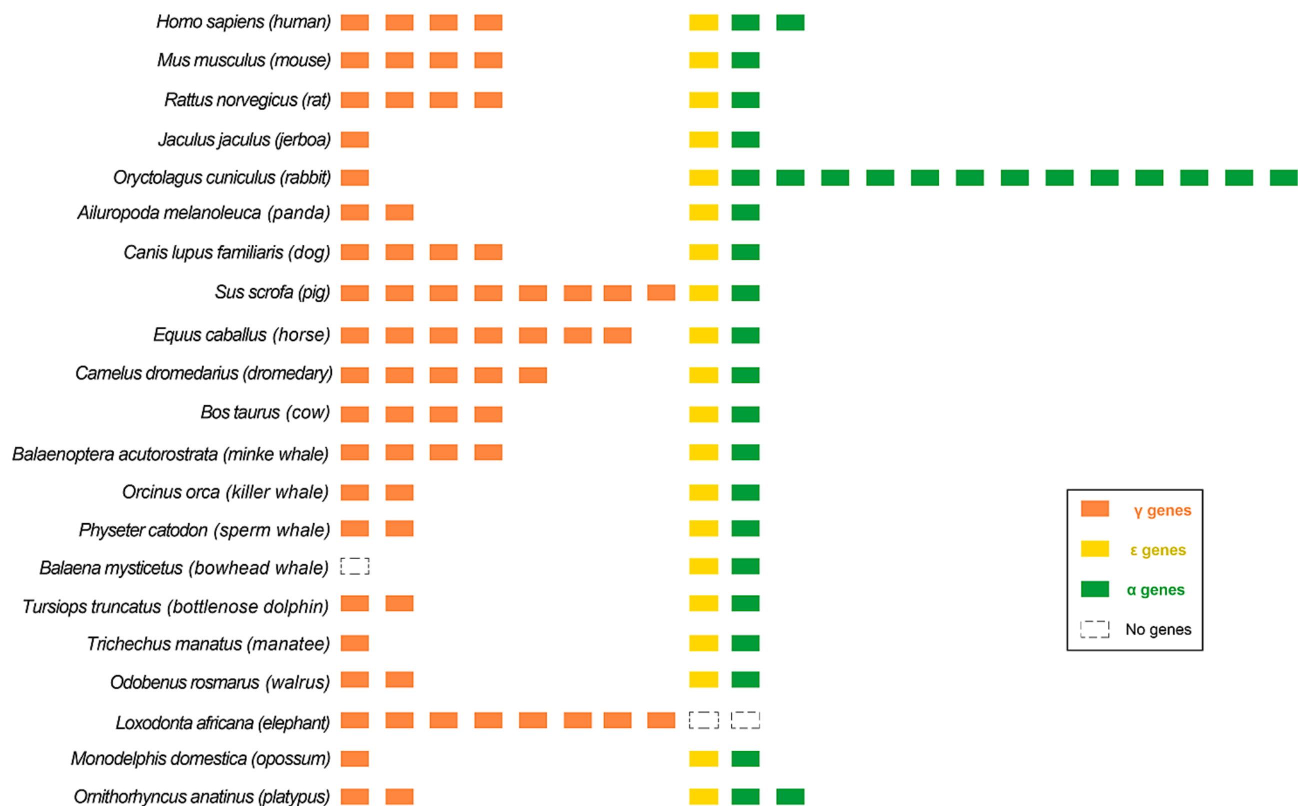


Fig. 5. Igα, Igε and Igγ gene copies detected in mammalian genomes. Genomes of 21 mammalian species showed to contain variable numbers of Ig of the α and γ isotypes, while seems constrained to contain no more than one functional copy of the isotype ε. Orange boxes are Igγ genes, yellow Igε and green Igα. A white dotted box indicates that no gene of the missing isotypes was found in that genome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

knowledge several copies of IgA and IgG were eventually detected, while so far, no more than a single functional copy of the IgE has ever been found in mammalian genomes (e.g. in human the second copy is just a pseudogene) (Fig. 5) (Li et al., 2018). Multiple alleles and duplicates of the 3′RR lead to hypothesize a putative effect on the expression of all the Ig class, IgE included. We checked the hypothesis of a linkage between 3′RR alleles and IgE levels. The switch from IgM to IgE goes through IgG1; the second switch from IgG1 to IgE is therefore needed to achieve final IgE production. During this second switch, the 3′RR1 is deleted and this further supports our findings, given the lack of a correlation between IgE production and the enhancer hs1.2 alleles of the 3′RR1. In mature cells with a rearranged chromosome, the ε constant gene region is near the 3′RR2, which harbours allele 4 in the 98% of human population and therefore is very uniform (Giambra et al., 2006). Consequently, the IgE level cannot be modulated neither by the 3′RR2 hs1.2 enhancer, almost monomorphic. We hypothesize that the production of IgE is modulated by an independent mechanism, different from that regulating the IgG subclasses levels and switch.

5. Conclusion

The 3′RR1 could influence both the commitment to the switch and the subsequent number of plasma cells and potentially drive the serum quantities of the four IgG subclasses by a positional cis-effect on the IgG subclasses genes. Our data suggest that, differently from what occurs in a wide range of autoimmune diseases, the copies of the human hs1.2 enhancer in the 3′RR1 and 3′RR2 are not implicated in allergic asthma that is characterized by a higher and dysregulated production of IgE.

CRediT authorship contribution statement

Rossella Cianci: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Giorgio Mancino:** Methodology, Investigation, Resources, Data curation. **Elena Galli:** Methodology, Investigation, Resources, Data curation. **Eliseo Serone:** Methodology, Investigation, Resources, Data curation. **Renato Massoud:** Methodology, Investigation, Resources, Data curation. **Pietro D'Addabbo:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Andrea Poscia:** Formal analysis, Data curation. **Alberto Borghetti:** Formal analysis, Data curation. **Ottavia Porzio:** Methodology, Investigation, Resources, Data curation. **Riccardo Marmo:** Formal analysis, Data curation. **Giovanni Gambassi:** Data curation, Writing – review & editing. **Domenico Frezza:** Conceptualization, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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