ESCOLA BAHIANA DE MEDICINA E SAÚDE PÚBLICA

CURSO BIOMEDICINA

AMANDA ROSA ALVES

IFNGR1 and *IFNGR2* genes polymorphisms affecting the T2 high and T2 low asthma response

SALVADOR – BA

2024

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Trabalho de Conclusão de Curso apresentado à Escola Bahiana de Medicina e Saúde Pública, como parte dos requisitos para obtenção do título de Bacharel em Biomedicina.

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IFN-γR1 and _IFN-γR2_ genes polymorphisms affecting T2 high and T2 low asthma response

Este Trabalho de Conclusão de Curso foi julgado adequado à obtenção do grau de Bacharel em Biomedicina e aprovada em sua forma final pelo Curso de Biomedicina da Escola Bahiana de Medicina e Saúde Pública.

Salvador, 9 de novembro de 2024.

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PROGRAMA DE CONTROLE DE ASMA DA BAHIA (PROAR)

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RESUMO

Introdução:

A asma é uma doença heterogênea que pode ser classificada de acordo com o seu endótipo, em T2 low e T2 high. Neste contexto, os receptores de interferon gama (IFNGR1 e IFNGR2) são essenciais para a modulação da resposta imune, influenciando esses endótipos da asma. O objetivo deste estudo é analisar polimorfismos nesses genes em relação ao endótipo e severidade da asma.

Metodologia:

Trata-se de um estudo transversal baseado em uma análise de dados secundários. No total, foram analisados 600 pacientes com asma, dos quais 225 tinha asma T2 high e 375 T2 low. A genotipagem foi realizada usando o chip Illumina Multi-Ethnic Global Array e os dados extraídos foram submetidos ao equilíbrio de Hardy-Weinberg e à frequência do alelo menor (MAF) no Plink. Os modelos genéticos de codominância, dominância, log recessivo e aditivo, bem como outras análises estatísticas, foram conduzidos no software Rstudio, versão 4.2.1, considerando um nível de significância de 5%.

Resultados:

No total, dezesseis polimorfismos foram encontrados nos genes *IFNGR1 e IFNGR2*. No entanto, apenas dois foram associados ao *IFNGR2*. Em rs13052232 foi obtido um menor risco de desenvolver asma T2 high (OR = 6.15, IC 95% = 1.17-113.32, p = 0,029), para o rs145973257 foi observado um maior risco de ter endótipo T2 alto (OR = 2.58, 95% CI= 1,05 – 6,69; p = 0,038).

Conclusão:

Os polimorfismos rs13052232 e rs145973257 foram associados ao endótipo T2 high da asma. Contudo, nenhum polimorfismo foi relacionado com a severidade da doença nessa população.

Palavras-chave: Asma, polimorfismos, *IFNGR1, IFNGR2*

ABSTRACT

Introduction:

Asthma is a heterogeneous disease that can be classified according to its endotype, T2 low and T2 high. Interferon gamma receptors (IFNGR1 and IFNGR2) are essential for the modulation of the immune response, therefore impacting the T2 low and T2 high endotypes of asthma. The objective of this study is to analyze polymorphisms in these genes.

Methodology:

This is a cross-sectional study based on an analysis of secondary data. Genotyping was performed using the Illumina Multi-Ethnic Global Array chip and the extracted data were subjected to Hardy-Weinberg equilibrium and minor allele frequency (MAF) in Plink. The genetic models of codominance, dominance, recessive and additive log, as well as other statistical analyses, were conducted in the Rstudio software, version 4.2.1, considering a significance level of 5%.

Results:

Totally, sixteen polymorphisms were found in the *IFNGR1* and *IFNGR2* genes. However, only two were associated with *IFNGR2*. In rs13052232 a lower risk of developing high T2 asthma was obtained (OR = 6.15, 95% CI = 1.17-113.32, p = 0.029), for rs145973257 a higher risk of having high T2 endotype was observed (OR = 2.58, 95% CI = 1.05–6.69; p = 0.038).

Conclusion:

The rs13052232 and rs145973257 polymorphisms were associated with the T2 high endotype of asthma. However, neither polymorphism was associated with disease severity in this population.

Key-words: Asthma, polymorphisms, *IFNGR1, IFNGR2*.

SUMÁRIO

IFNGR1 and *IFNGR2* gene polymorphisms affecting T2 high T2 low asthma response

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ABSTRACT

Introduction: Asthma is a heterogeneous disease classified according to its endotype, T2 low and T2 high. Interferon gamma receptors (IFNGR1 and IFNGR2) are essential for modulating the immune response, therefore impacting the T2 low and T2 high endotypes of asthma. The objective of this study is to analyze polymorphisms in these genes.

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Conclusion: The rs13052232 and rs145973257 polymorphisms were associated with the T2 high endotype of asthma. However, neither polymorphism was associated with disease severity in this population.

Key-words: Asthma, polymorphisms, *IFNGR1, IFNGR2.*

1. Introduction

Asthma is the most common chronic non-communicable disease, becoming a global problem affecting approximately 262 million people [1]. It is a heterogeneous disease, typically characterized by chronic airways inflammation. Which is defined by symptoms, such as wheezing, chest tightness, coughing and shortness of breath, varying over time and intensity, together with limitation of expiratory flow [2]. It can be classified by endotype, T2 high and T2 low, which describes distinct pathophysiological mechanisms at the cellular and molecular level [3].

T2 high asthma has a classic T helper 2 (Th2) lymphocyte ignition and activation profile [4]. In this profile, the thymic stromal lymphopoietin protein activates dendritic cells, T cells, and B cells. The cytokines IL-25 and IL-33 activate type 2 innate lymphoid cells that induce IL-13 and IL-5, which stimulate the production, maturation, and recruitment of eosinophils. These cells have the capacity to synthesize and store a huge amount of inflammatory mediators, including IL-4, IL-5, and IL-13 [3,5]. On the other hand, T2 low asthma is vaguely described as the absence of T2 high asthma biomarkers. This endotype may exist concomitantly with the Th2 response, and may be a neutrophilic response in the airways or an activation of type 1 (T1) immune pathways, mediated by interferon (IFN) [4,6,7].

The interferon gamma (IFN-γ) receptor, has an alpha chain (IFN-γR1) and a beta chain (IFN-γR2), is essential for the modulation of the immune response [8]. Alpha chain receptor has high affinity and is the major ligand-binding subunit with interferon gamma, while IFN-γR2 is essential for forming a functional receptor complex with IFN-γR1 and IFN-γ. Also, beta chain enhances the receptor's ability to transduce IFN-γ signal to macrophages, dendritic cells or T cells [8,9]. This complex, while inhibiting the function

of Th2 cells, reducing the recruitment of eosinophils and lymphocytes, promotes general lung inflammation and the recruitment of neutrophils [10, 11].

While some studies revealed no association between polymorphisms in *IFNGR1* and *IFNGR2* genes and asthma [12,13]. Others have associated atopic asthma with three polymorphisms in *IFNGR2*: rs2834213 [14], rs2012075 [15] and rs1059293 [16]. Therefore, this study aims to analyze polymorphisms in *IFNGR1* and *IFNGR2* genes in relation to the T2 high and T2 low endotypes, also to identify association with the severity of asthma symptoms.

2. Methodology

2.1 Study design and location

This is a cross-sectional study that analyzes secondary data derived from the project entitled "Risk factors, biomarkers and endophenotype of severe asthma" carried out at Bahia Asthma Control Program (ProAR). The study conformed with the Ethics and Research Committee of the Climério de Oliveira Maternity Hospital, under opinion number 099/2009. All participants signed the free and informed consent form and good clinical practices were followed.

2.2 Population

All individuals were adults (age ≥ 18 years) evaluated by ProAR and were divided by asthma status as described by Cruz et al., 2020 [17]. This study included 600 participants, of which 318 had mild asthma and 282 had moderate/severe asthma by GINA classification [2].

The inclusion criteria were: participants from both sexes; residents of Salvador or the metropolitan region; and users of the Unified Health System. Furthermore, participants diagnosed with moderate/severe asthma had to be followed up at the ProAR outpatient clinic for the last six months. Meanwhile, the exclusion criteria were: participants who

did not have genotyping data and did not undergo eosinophil count; participants with blood relatives in the study; pregnant or lactating; and patients diagnosed with another chronic respiratory disease affecting the lower airways or lungs.

2.3 Clinical and laboratory data

Sociodemographic and clinical information were collected during an interview with the physician. The complete blood count examination (including eosinophils) were processed by the Cell-Dyn Ruby automated method. To assess atopy, the Skin Prick Test and specific IgE dosage were performed. It was tested *Dermatophagoides pteronyssinus, Dermatophagoides farina, Blomia tropicalis*, *Periplaneta Americana*, *Blatella germânica*, *Aspergillus fumigatus*, *Penicillium notatum, Alternaria alternata, Aspergillus flavus, Aspergillus niger*, *Cladosporium herbarum*, dog epithelium, cat epithelium as allergens in Skin Prick Test. Also, *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Blomia tropicalis, Periplaneta americana, Blatella germânica, Aspergillus fumigatus, Penicillium notatum*, dog epithelium, rat epithelium and rat urine was tested as allergens in specific IgE dosage.

Subsequently, pulmonary function testing was performed using a Koko® Spirometer (Ferraris Medical, USA) and participants underwent spirometry before and after inhalation of 400 mcg of salbutamol to determine Forced Expiratory Volume in 1 second (FEV1) and Forced Vital Capacity (FVC). All values used were predicted specifically for Brazilian adults [19] considering the percentage of pre-bronchodilator FEV1 as an indicator of airway obstruction and as an indicator of reversibility, an improvement of 12% and 200 mL of post-bronchodilator FEV1.

Genomic DNA was extracted from whole blood using the Qiagen Flexi Gene Kit (Qiagen, Hilden, Germany), followed by quantification by spectrophotometry using the Bioware DNA spectrophotometer.

2.4 Endotype classification

Individuals in the T2 high group were classified according to the blood eosinophil value (≥ 260 IU/mL) [20] and positive skin test (papules > 3mm) and/or specific IgE (> 0.70 kUA/L) for at least one allergen [18]. In contrast, individuals in the T2 low group were defined as the absence of T2 high.

2.5 Genotyping

The study of candidate genes (*IFNGR1* and *IFNGR2*) was performed using information extracted from the Illumina Multi-Ethnic Global Array (MEGA) genotyping chip. It used version GRCh37 of the genome available at the National Center for Biotechnology Information (NCBI).

2.6 *In silico* analysis

Data available at NCBI were used to investigate the function of single nucleotide variant (SNVs). The regulatory potential of SNVs was assessed using RegulomeDB, which assigns scores, and the closer it is to 7, the lower the impact of the variant on regulatory mechanisms [21]. The data extracted from the MEGA chip were submitted to PLINK 1.9 software as quality control and to identify genetic variants. It was established that SNVs with a p-value < 0.05 in Hardy-Weinberg equilibrium (HWE) or minor allele frequency (MAF) < 1% were excluded from the logistic regression analyses. Furthermore, SNVs and individuals with genotyping rates < 90% were also excluded.

2.7 Statistical methods

To analyze the distribution of genetic variants between the *IFNGR1* and *IFNGR2* genes about asthma endotypes and severity of asthma symptoms, Shapiro-Wilk normality test was performed to determine data normality in quantitative variables and then Student t-test, also chi-square association test was made for qualitative variables to define adjustment and covariates in the logistic regression. All statistical analyses were

performed using dominant, recessive, codominant and log additive genetic models. The statistical significance of differences was indicated by $p < 0.05$. These tests and the production of graphs and tables were performed using Rstudio software, version 4.2.1.

3. Results

Among 600 individuals, 375 (62.50%) were classified as having T2 low asthma, the majority of whom were female [315 (52.5%)], black and mixed [343 (57.17%)], and overweight [265 (44.3%)]. Similarly, patients for T2 high asthma 225 (37.50%), there was also a prevalence of female individuals [168 (28.00%)], black and mixed [208 (34.67%)], and overweight [140 (23.33%)]. For both groups, there was also a predominance of individuals with mild asthma [318 (53.0%)] (Table 1).

(Table 1 is here)

Regarding genetic data, 16 SNVs passed quality control, four in *IFNGR1* gene and 12 in *IFNGR2* gene. Nonetheless, only two had significant results (Table 2).

(Table 2 is here)

Analyzing association between the variants and T2 high and T2 low asthma, we found that rs13052232, in the recessive model, showed that individuals with AG + GG genotype (Graph 1) have 6.15 times higher odds of developing T2 high asthma compared to those with AA, with a 95% confidence interval (1.17-113.32; $p = 0.029$) (Table 3). This polymorphism has a score of 5 in RegulomeDB (Table 2).

Furthermore, codominant model analysis for rs145973257 showed a significant association between the AG genotype and T2 high asthma ($p = 0.038$). Individuals with AG genotype (Graph 1) were 2.58 times more likely to have T2 high asthma compared to those with the AA genotype, with a 95% confidence interval (1.05-6.69). RegulomeDB has a score of 4 for this polymorphism (Table 2).

(Table 3 is here)

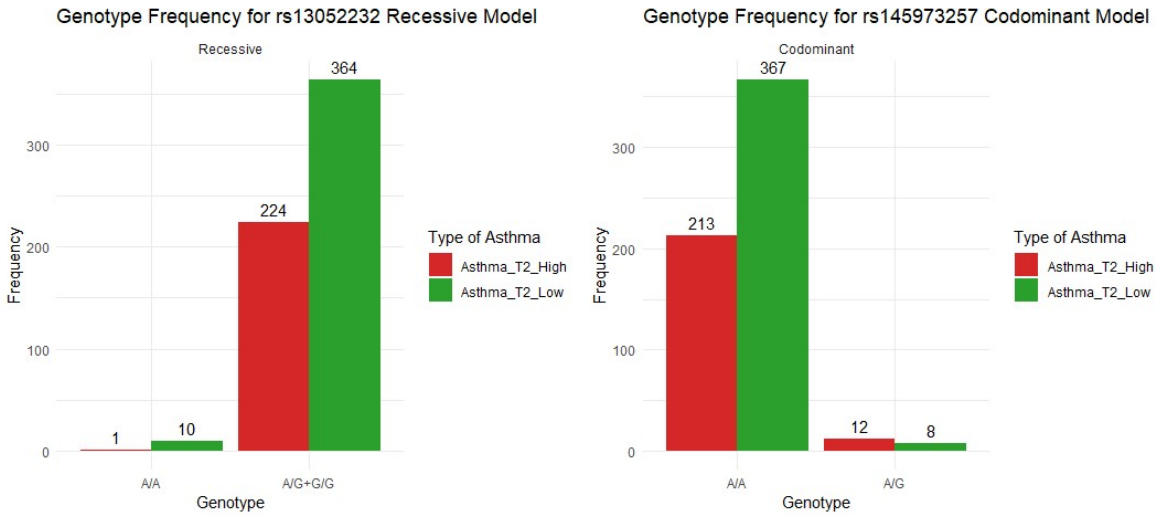

Figure 1: Genotype frequency for models recessive and codominant about T2 high and T2 low asthma.

All sixteen polymorphisms were tested for association with asthma severity. However, no significant results were found (results not shown).

4. Discussion

As a result of this study, it was observed that in T2 low and T2 high asthma there was a higher number of women, black and mixed people, overweight and mild asthma. Among the 16 polymorphisms found in the population, two SNVs showed a significant association with asthma. That information suggests the genetic factor may play a significant role in interferon gamma receptors thereby influencing asthma endotype.

This is the first study to evaluate the distribution of polymorphisms found in the *IFNGR2* gene in relation to asthma endotype. It was discovered that rs13052232 and rs145973257 are associated with T2 high asthma. On the other hand, rs1059293, rs2834213 and rs2012075 were not associated with atopic asthma in this population, as previously described in other studies from others populations [14,15,16].

Our data showed that rs13052232 and genotype A/G+G/G, in recessive model, increased the chance of T2 high asthma by 6.15 times ($p = 0.029$) thus acting as a risk factor. However, the wide confidence interval (1.17-113.32) reflects uncertainty in the estimate, likely due to the small number of individuals with the AA genotype.

On the other hand, rs145973257 and genotype AG were 2.58 times more likely to have T2 high asthma. This indicate that frequency of the G allele influences the asthma phenotype causing a higher risk to develop T2 high asthma.

According to RegulomeDB score, the SNVs found has less chances to have a regulatory function. Still, we are unable to know if these SNVs increase or decrease IFN- γ signal to macrophages, T cells or dendritic cells. More analyses are needed to determine what are the functions rs13052232 and rs145973257 play in asthma.

Additionally, none of the 16 SNVs were associated with the severity of asthma symptoms. However, among these 16 polymorphisms, rs1327475 [22,23] and rs9376267 [23,34] have been linked to pulmonary tuberculosis as protective factors. Nonetheless, there is no evidence to suggest that interferon gamma receptor genes influence asthma or other atopic respiratory diseases.

5. Conclusion

The polymorphisms rs13052232 and rs145973257 were associated with asthma for the first time. It was observed that rs13052232 and rs145973257 was related with higher chances to have T2 high. However, no association with asthma severity was observed. Although the specific mechanisms by which these SNVs influence asthma physiopathology were not identified. So more studies are needed to clarify how these polymorphisms act on IFN- γ signal transduction.

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Tables:

Table 1: Description of the sociodemographic and clinical information about PROAR population used in this study.

Variables	Total (n=600)	T2 low (n = 375)	T2 high (n = 225)	p value
Age (years)	42.43 ± 14.73	45.20 ± 14.90	37.80 ± 13.30	0.000[1]
Female Sex	483 (80.50%)	315 (52.50%)	168 (28.00%)	0.007[2]
Skin color				
Black and Mixed	551 (91.84%)	343 (57.17%)	208 (34.67%)	
White, Indigenous and Yellow	49 (8.16%)	32 (5.33%)	17 (2.83%)	0.797[1]
Individual with high BMI (≥24.99 Kg/m²)	405/599 (67.66%)	265/374 (44.33%)	140/225 (23.33%)	0.041[2]

Asthma Classification				
Mild	318 (53.00%)	192 (32.00%)	126 (21.00%)	
Moderate/Severe Asthma	282 (47.00%)	183 (30.50%)	99 (16.50%)	0.291[2]
Individuals with Atopy	243/391 (62.15%)	129/235 (32.99%)	114/156(29.16)	0.000[2]
% FEV1 pre bronchodilator	73.81 ± 17.82	74.40 ± 18.10	72.80 ± 17.30	0.298[1]
% FEV1 post bronchodilator	75.99 ± 64.87	73.90 ± 81.10	79.40 ± 16.40	0.847[1]
Reversibility to SABA	152/590 (25.77%)	70/222 (11.87%)	82/368 (13.90%)	0.017[1]
Eosinophils (cells/mL)	313.07 ± 260.83	199.00 ± 167.00	503.00 ± 277.00	0.000[1]

NOTE: SABA = short ß2-agonist bronchodilator; BMI = body mass index. Data are presented as: mean ± standard deviation; median (interquartile range 25-75) and absolute number (valid percentage); 1 - Student's t test; 2 - Chi-square test.

Table 2: Description of *IFNGR1* and *IFNGR2* variants.

Gene	SNV	Position	Alleles	MAF	EWH	Function	Rank Reg.DB
IFNGR1	rs1887415	137519238	C/A,T	0,017	1	Missense variant	5
	rs17175350	137519634	T/G	0,011	1	Missense variant	5
	rs9376267	137531031	C/T	0,173	1	Intron Variant	4
	rs1327475	137536455	G/A	0,099	0,253	Missense Variant	1f
IFNGR2	rs111519501	34776112	G/A	0,047	0,638	Intron Variant	2b
	rs2284553	34776695	A/G,T	0,22	0,812	Intron Variant	1f
	rs2268241	34781050	G/A	0,239	0,911	Intron Variant	1f
	rs201196496	34785326	G/A,C	0,082	0,048	Intron Variant	1f
	rs78607908	34787401	A/C,T	0,024	1	Intron Variant	4
	rs2834211	34789808	T/C	0,078	0,019	Intron Variant	1f
	rs13052232	34795343	G/A	0,138	1	Intron Variant	5
	rs145973257	34798334	A/G	0,017	1	Intron Variant	4
	rs8126514	34800149	A/C,T	0,359	0,328	Intron Variant	2b
	rs8128362	34801876	T/A,C	0,052	0,065	Intron Variant	1f
	rs17882619	34803377	C/T	0,114	0,55	Intron Variant	1f
	rs1059293	34809693	C/A,T	0,358	0,183	3 Prime UTR Variant	1f

NOTE: 1 - The first is the wild-type allele and the second is the polymorphic allele; 2 - minor allele frequency; 3 - Hardy-Weinberg equilibrium; 4 - NCBI (National Center for Biotechnology Information).

Table 3: Associations between polymorphisms from *IFNGR2* about T2 high and asthma.

Model	SNV	Genotype	OR[1]	95% CI[2]	p value[3]
Recessive	rs13052232	A/G + G/G A/A	6.15	1.17-113.32	0,029
Codominant	rs145973257	A/A A/G	2.6	1.05-6.69	0,038

NOTE: 1 - OR = Odds Ratio; 2 - CI = Confidence Interval; 3 - Likelihood ratio test.

ANEXO

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- Present/permanent address. If an author has moved since the work described in your article was carried out, or the author was visiting during that time, a "present address" (or "permanent address") can be indicated by a footnote to the author's name. The address where the author carried out the work must be retained as their main affiliation address. Use superscript Arabic numerals for such footnotes.

1.3 ABSTRACT

You are required to provide a concise and factual abstract which does not exceed 250 words. The abstract should briefly state the purpose of your research, principal results and major conclusions. Some guidelines:

- Abstracts must be able to stand alone as abstracts are often presented separately from the article.
- Avoid references. If any are essential to include, ensure that you cite the author(s) and year(s).
- Avoid non-standard or uncommon abbreviations. If any are essential to include, ensure they are defined within your abstract at first mention.

1.4 KEYWORDS

You are required to provide 1 to 7 keywords for indexing purposes. Keywords should be written in English. Please try to avoid keywords consisting of multiple words (using "and" or "of").

We recommend that you only use abbreviations in keywords if they are firmly established in the field.

1.5 HIGHLIGHTS

You are required to provide article highlights at submission.

Highlights are a short collection of bullet points that should capture the novel results of your research as well as any new methods used during your study. Highlights will help increase the discoverability of your article via search engines. Some guidelines:

- Submit highlights as a separate editable file in the online submission system with the word "highlights" included in the file name.
- Highlights should consist of 3 to 5 bullet points, each a maximum of 85 characters, including spaces.

We encourage you to view example article highlights and read about the benefits of their inclusion.

1.6 TABLES

Tables must be submitted as editable text, not as images. Some guidelines:

- Place tables next to the relevant text or on a separate page(s) at the end of your article.
- Cite all tables in the manuscript text.
- Number tables consecutively according to their appearance in the text.
- Please provide captions along with the tables.
- Place any table notes below the table body.
- Avoid vertical rules and shading within table cells.

We recommend that you use tables sparingly, ensuring that any data presented in tables is not duplicating results described elsewhere in the article.

1.7 FIGURES, IMAGES AND ARTWORK

Figures, images, artwork, diagrams and other graphical media must be supplied as separate files along with the manuscript. We recommend that you read our detailed artwork and media instructions. Some excerpts:

When submitting artwork:

- Cite all images in the manuscript text.
- Number images according to the sequence they appear within your article.
- Submit each image as a separate file using a logical naming convention for your files (for example, Figure_1, Figure_2 etc).
- Please provide captions along with the artwork.
- Text graphics may be embedded in the text at the appropriate position. If you are working with LaTeX, text graphics may also be embedded in the file.

1.8 ARTWORK FORMATS

When your artwork is finalized, "save as" or convert your electronic artwork to the formats listed below taking into account the given resolution requirements for line drawings, halftones, and line/halftone combinations:

- Vector drawings: Save as EPS or PDF files embedding the font or saving the text as "graphics."
- Color or grayscale photographs (halftones): Save as TIFF, JPG or PNG files using a minimum of 300 dpi (for single column: min. 1063 pixels, full page width: 2244 pixels).
- Bitmapped line drawings: Save as TIFF, JPG or PNG files using a minimum of 1000 dpi (for single column: min. 3543 pixels, full page width: 7480 pixels).
- Combinations bitmapped line/halftones (color or grayscale): Save as TIFF, JPG or PNG files using a minimum of 500 dpi (for single column: min. 1772 pixels, full page width: 3740 pixels).

Please do not submit:

- files that are too low in resolution (for example, files optimized for screen use such as GIF, BMP, PICT or WPG files).
- disproportionally large images compared to font size, as text may become unreadable.

1.9 FIGURE CAPTIONS

All images must have a caption. A caption should consist of a brief title (not displayed on the figure itself) and a description of the image. We advise you to keep the amount of text in any image to a minimum, though any symbols and abbreviations used should be explained.

Provide captions in a separate file.

1.10 RESEARCH DATA

We are committed to supporting the storage of, access to and discovery of research data, and our research data policy sets out the principles guiding how we work with the research community to support a more efficient and transparent research process.

Research data refers to the results of observations or experimentation that validate research findings, which may also include software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Please read our guidelines on sharing research data for more information on depositing, sharing and using research data and other relevant research materials.

For this journal, the following instructions from our research data guidelines apply.

Option C: Research data deposit, citation and linking

You are **required** to:

- Deposit your research data in a relevant data repository.
- Cite and link to this dataset in your article.
- If this is not possible, make a statement explaining why research data cannot be shared.

1.11 DATA STATEMENT

To foster transparency, you are required to state the availability of any data at submission.

Ensuring data is available may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you can state the reason why (e.g., your research data includes sensitive or confidential information such as patient data) during the submission process. This statement will appear with your published article on ScienceDirect.

Read more about the importance and benefits of providing a data statement.

1.12 RESEARCH ELEMENTS

This journal enables the publication of research objects (e.g. data, methods, protocols, software and hardware) related to original research in Elsevier's Research Elements journals.

Research Elements are peer-reviewed, open access journals which make research objects findable, accessible and reusable. By providing detailed descriptions of objects and their application with links to the original research article, your research objects can be placed into context within your article.

You will be alerted during submission to the opportunity to submit a manuscript to one of the Research Elements journals. Your Research Elements article can be prepared by you, or by one of your collaborators.

1.13 ARTICLE STRUCTURE

1.13.1.1.1 Article sections

- Divide your article into clearly defined and numbered sections. Number subsections 1.1 (then 1.1.1, 1.1.2, ...), then 1.2, etc.
- Use the numbering format when cross-referencing within your article. Do not just refer to "the text."
- You may give subsections a brief heading. Headings should appear on a separate line.

- Do not include the article abstract within section numbering.

1.13.1.1.2 Funding sources

Authors must disclose any funding sources who provided financial support for the conduct of the research and/or preparation of the article. The role of sponsors, if any, should be declared in relation to the study design, collection, analysis and interpretation of data, writing of the report and decision to submit the article for publication. If funding sources had no such involvement this should be stated in your submission.

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants, scholarships and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, it is recommended to include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

1.13.1.1.3 Appendices

We ask you to use the following format for appendices:

- Identify individual appendices within your article using the format: A, B, etc.
- Give separate numbering to formulae and equations within appendices using formats such as Eq. (A.1), Eq. (A.2), etc. and in subsequent appendices, Eq. (B.1), Eq. (B. 2) etc. In a similar way, give separate numbering to tables and figures using formats such as Table A.1; Fig. A.1, etc.

1.14 REFERENCES

1.14.1.1.1 References within text

Any references cited within your article should also be present in your reference list and vice versa. Some guidelines:

- References cited in your abstract must be given in full.
- We recommend that you do not include unpublished results and personal communications in your reference list, though you may mention them in the text of your article.
- Any unpublished results and personal communications included in your reference list must follow the standard reference style of the journal. In substitution of the publication date add "unpublished results" or "personal communication."
- References cited as "in press" imply that the item has been accepted for publication.

Linking to cited sources will increase the discoverability of your research.

Before submission, check that all data provided in your reference list are correct, including any references which have been copied. Providing correct reference data allows us to link to abstracting and indexing services such as Scopus, Crossref and PubMed. Any incorrect surnames, journal or book titles, publication years or pagination within your references may prevent link creation.

We encourage the use of Digital Object Identifiers (DOIs) as reference links as they provide a permanent link to the electronic article referenced. See the example below, though be aware that the format of such citations should be adapted to follow the style of other references in your paper.

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VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, https://doi.org/10.1029/2001JB000884.