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Rationale and design of GISSI OUTLIERS VAR Study in bicuspid aortic valve patients: Prospective longitudinal, multicenter study to investigate correlation between surgical, echo distinctive features, histologic and genetic findings in phenotypically homogeneous outlier cases☆,☆☆

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ABSTRACT

Background/objectives: Bicuspid aortic valve (BAV) is the most common congenital heart disorder, affecting up to 2% of the population. Involvement of aortic root and ascending aorta (aneurysm or, eventually, dissection) is frequent in patients with pathologic or normal functioning BAV. Unfortunately, there are no well-known correlations between valvular and vascular diseases. In VAR protocol, with a new strategy of research, we analyse multiple aspects of BAV disease through correlation between surgical, echo, histologic and genetic findings in phenotypically homogeneous outlier cases.
Methods: VAR protocol is a prospective, longitudinal, multicenter study. It observes 4 homogeneous small groups of BAV surgical patients (15 patients each): isolated aortic regurgitation, isolated ascending aortic aneurysm, aortic regurgitation associated with aortic aneurysm, isolated aortic stenosis in older patients (>60 years). Echo analysis is extended to first-degree relatives and, in case of BAV, genetic test is performed. Patients and relatives are enrolled in 10 cardiac surgery/cardiologic centers throughout Italy.
Conclusions: The aim of the study is to identify predictors of favorable or unfavorable evolution of BAV in terms of valvular dysfunction and/or aortic aneurysm. Correlations between different features could help in identification of various BAV risk groups, rationalizing follow-up and treatment.

1. Introduction

GISSI OUTLIERS studies are based on a completely new strategy of research. They are focused on small groups of patients with homogeneous outlier phenotypes. Outlier cases, with regard to population studies, are often buried by statistics because they are few and their behavior is different from the average. Focusing on a patient-targeted therapy, outlier protocols are designed to deeply investigate specific phenotypes starting from a single patient, "from bedside to bench".

Surgical heart valve disease, observed in a specific cardiac disorder with different clinical presentations, could offer many opportunities of investigation, from imaging to real view, from genetic blood tests to analysis of surgical tissue samples. Patients with bicuspid aortic valve

Abbreviations: BAV, bicuspid aortic valve; 2D, two-dimensional; 3D, three-dimensional; TOE, transesophageal echocardiography; TTE, transthoracic echocardiography.

☆ Authorship: The authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. All authors have read and approved the manuscript.

☆☆ The study was registered as a clinical trial on the Clinical Trial Registry, www.clinicaltrials.gov (identifier: NCT02283970).

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(BAV) could be an ideal setting to decline into practice this way of dealing with a disease.

BAV is the most common congenital heart valve disorder, affecting up to 2% of the population [1,2]. BAVs are likely to be the result of a complex developmental process during valvulogenesis, not simply the fusion of two normal cusps [3]. Only 20% of patients will maintain a normally functioning valve throughout life [4,5]. More than 30% of patients will develop serious morbidity: BAV disease is the major cause of pure aortic stenosis in most series; others, especially the young adults, may also develop pure regurgitation with or without infection (40–60% of severe aortic regurgitation in the BAV population is secondary to infective endocarditis) [5]. Generally, mid ascending aortic dilatation is the most common phenotypic pattern in older age and stenotic valve, whereas annulus and aortic root dilatations are correlated with younger age, male sex, and valve regurgitation. The main clinical implication is the frequency of BAV (up to 7–13%) in unselected cases of aortic dissection, regardless of valve function. Furthermore, taking into consideration all data from the literature, the most relevant problem still remains the unpredictable risk of aortic rupture.

Several family-based studies have shown that BAV disease, either alone or in combination with other cardiovascular malformations, can be inherited, and is therefore likely to have a genetic basis; most family studies suggest an autosomal dominant inheritance [6].

The aim of the study is to identify markers/predictors of favorable–unfavorable aortic wall evolution selecting homogeneous small groups of surgical patients with the same subtype of BAV and same aortic behavior in order to evaluate if there is a BAV phenotype more likely to be at high risk for aortic degeneration.

2. Study design and setting of the study

The research protocol is designed as prospective longitudinal study on 4 small groups of surgical patients affected by:

1. BAV with isolated regurgitation
2. BAV with normal valvular function but associated aorta dilatation
3. BAV with both valve regurgitation and aortic dilatation
4. BAV with isolated stenosis in over 60 year-old patients.

The first step will consist in enrolling patients with echocardiographic diagnosis of BAV and indication to surgery, according to current clinical guidelines [7] or best clinical practice. The inclusion and exclusion criteria are detailed in Table 1. From the echo data, we will assess aortic valve, as well as aortic root and ascending aorta morphology in order to classify patients in each phenotypic pattern.

Table 1
Inclusion and exclusion criteria of the GISSI VAR Study.

Inclusion
- Age between 18 and 60 years for groups 1, 2, 3
- Age > 60 years for group 4
- Echo diagnosis of BAV* and indication to surgery as described before
- Signed informed consent

Exclusion
- Patient with a previous cardiac or great vessel surgery
- Coexistent coarctation of the aorta
- Valve disease (other than aortic)
- Ischemic disease
- Congenital heart disease
- Marfan syndrome or other connective tissue disorders involving aortic valve and aortic wall disease (history of disease or clinical signs)
- Other conditions/circumstances likely to lead to poor study adherence (e.g. psychological or organizational reasons)
- Serious disease other than aortic, severely limiting life expectancy
- Patients who refuse to give informed consent

BAV = bicuspid aortic valve

* Definition of BAV: 2 clearly defined cusps or with the characteristic systolic fish mouth appearance of the aortic valve cusps and 2 of 3 supportive features of BAV, including systolic doming or diastolic prolapse of the aortic valve cusps and eccentric valve leaflet closure.

The second step will consider first-degree relatives of each enrolled patient. We will ask every first degree relative to perform a screening transthoracic echocardiography (TTE) to find out the presence of BAV and/or associated disease (aortic root or ascending aorta enlargement).

The third step will consist in collecting blood samples from each patient and from any of first-degree relatives with BAV diagnosis. BAV-specific genetic tests will be collected.

The fourth intraoperative step will consist in:

1. performing a three-dimensional (3D)-transoesophageal echocardiography (TOE) in order to study the geometry and the dynamic behavior of the aortic valve and root
2. confirming BAV from the direct vision of the valve
3. collecting surgical tissue samples: during operation there will be precisely described the anatomy of the valve and the aorta in detail; then, from the usual site of surgery, valve and aortic wall samples will be collected in order to perform histological, immunohistochemical and molecular investigations.

Blood and surgical samples will be stored in a bio-bank for future analysis related to this protocol.

2.1. Follow-up

Clinical and echo follow-up after 6 months and at 1, 2, and 3 years from surgery will be performed in order to evaluate changes/evolution of the disease in the valve/aorta not surgically treated. Clinical and echo

Table 2
Assessment schedule.

Patients						
	Time					
Visit	**1**	**2**	**3**	**4**	**5**	**6**
	Pre-cardiac surgery	**Cardiac surgery**	**Months**			
			6	**12**	**24**	**36**
Written informed consent	X					
Inclusion/exclusion criteria	X	X				
Medical history	X					
Clinical examination	X		X	X	X	X
Detailed family history (with family tree)	X					
Genetic profile only with regards to BAV	X					
Echocardiography	X		X	X	X	X

All first-degree relatives						
	Time					
Visit	**1**	**2**	**3**	**4**	**5**	**6**
	After patient'sinformed consent		**Months**			
			6	**12**	**24**	**36**
Written informed consent (for relative)	X					
Medical history	X					
Echocardiography	X					

Only first-degree relatives with BAV (after relative's BAV diagnosis)						
	Time					
Visit	**1**	**2**	**3**	**4**	**5**	**6**
	After BAV diagnosis		**Months**			
			6	**12**	**24**	**36**
Clinical examination	X		X	X	X	X
Genetic profile	X					
Echocardiography			X	X	X	X

BAV = bicuspid aortic valve

follow-up in relatives with BAV diagnosis will be performed at 6 months and at 1, 2, and 3 years. Table 2 summarizes the assessment schedule.

Among relatives, in case of BAV or vessel evolution eligible for surgical treatment, there will be enrollment in surgical and histological phases of the study.

3. Methods

3.1. Echocardiography [8–14]

Two-dimensional (2D) and 3D TTE or TOE will be performed using the iE33 ultrasound imaging system (Philips Medical Systems).

The preoperative 2D and 3D TTE or TOE (if necessary to confirm enrollment) and the intraoperatively 3D TOE will provide a comprehensive qualitative–quantitative evaluation, according to the current guidelines of:

- Aortic valve function
- Aortic valve morphology: leaflet orientation, presence or absence of a raphe; according to cusp anatomy BAV morphology will be classified as Type A (fusion of left coronary and right coronary cusps), Type B (fusion of right coronary and non-coronary cusps) or Type C (fusion of left coronary and non-coronary cusps)
- Aortic valve annulus, aortic root, sinotubular junction, ascending aorta, and aortic arch morphology (with diameter measurements)
- Aortic isthmus
- Left ventricular geometry and function.

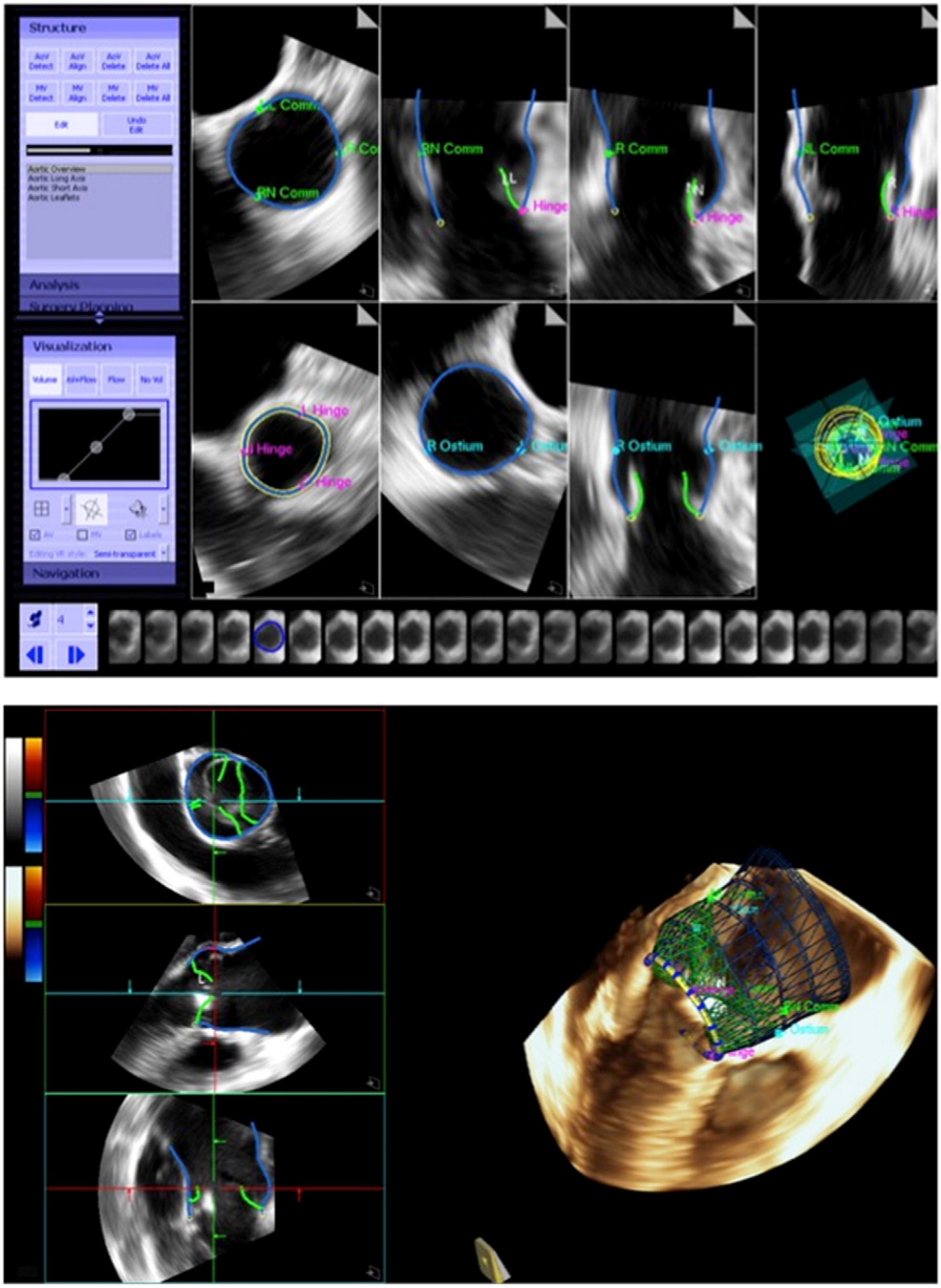

Fig. 1. Report from the off-line software (Auto Valve Analysis, Siemens, CA) for aortic root size and morphology.

The echo images will be sent to an echo core lab for analysis and all data will be recorded in a specific database. The 3D data sets will be analyzed quantitatively using an off-line customized software developed at University of California to study the geometry and dynamic behavior of the aortic valve and aortic root throughout the cardiac cycle (Fig. 1).

3.2. Genetic [6,15–18]

Total genomic DNA and total RNA will be extracted on the semi-automated platform Maxwell 16TM (Promega) according to the tissue type (blood:EDTA or fresh tissue) following the manufacturer's instructions.

According to current knowledge (MIM #109730, http://www.omim.org/entry/109730) the strongest causative gene associated with BAV is NOTCH1 (9q34.3). Its 34 coding exons will be sequenced to search for variants likely to be causative (Fig. 2). Amplification and sequencing reactions will be set up according to standard protocols on the ABI 3130Xl platform and analyzed by the Sequence Analysis software version 5.1. The different contigs will be assembled with the SeqScape software (applied Biosystems).

The presence of sequence variation will be confirmed using a different method, starting from a new amplification (RFLP or High Resolution Melting—HRM). The pathogeneticity of the mutation will be evaluated screening 500 DNA controls (1000 chromosomes) to estimate the alleles' frequencies (HRM; ABI Prism 7900HT). The evolutionary conservation of mutated residue will be estimated in public databases. Finally, a familial segregation analysis will be performed to verify the association with the disease.

In case the first molecular testing (direct sequencing) will give negative results, a Multiple Ligation Probe Assay (MLPA) will be performed on the *NOTCH1* gene and run on the ABI PRISM 3130 Platform.

In case of Insertion/Deletion, dedicated sets of primers and TaqMan probes will be dedicated to confirm the MPLA findings.

Our idea, based on preliminary data, suggests the searching for causative variants within the NOTCH1 signaling pathway (Panther ID P00045) upstream and downstream the NOTCH1 point of action.

3.3. Histology [19–25]

In all patients, at least 3 aortic surgical samples will be performed, one for histology (formalin 10%), one for electron microscopy (glutaraldehyde 2,5%), and one for molecular investigations (snap frozen −80°). In case of replacement of both sinusal and ascending portions of the aorta, the same tissue sampling will be repeated at both levels. In the case of isolated aortic valve replacement, 3 small samples will be obtained along the incision of aortotomy. Histological section will be stained with hematoxylin–eosin, trichrome staining, Alcian PAS and Weigert Van Gieson.

Stained slides will be viewed by a microscope to analyze the aortic wall degenerative changes, including intima, media, and adventitia. In detail, a semi-quantitative analysis will be performed on the tunica media adopting scores from 0 to 4 according to Larson and Edwards concerning the following parameters: medial necrosis, fibrosis, cystic degeneration, and elastic fragmentation. Moreover, the apoptotic index (TUNEL method) will be calculated in smooth muscle cells of the tunica media. The tunica intima will be evaluated for atherosclerosis and inflammation.

4. Statistical considerations and sample size

Descriptive summaries will be presented for all patients and for the 3 different groups. Statistical tests may be carried out for exploratory purposes, as appropriate. Differences between groups will be tested at baseline, according to the 4 groups previously described and according to genetic and histologic characteristics. Differences between baseline and follow-up co-variates will be tested, as appropriate. Being that the study is fully exploratory, a formal sample size was not calculated. Nevertheless, we expected 15 patients per group with 60 patients in total.

5. Ethical aspects

The last revision of the Helsinki Declaration provides the general framework for the ethical conduction of the study. The protocol, the proposed informed consent form and other information to subjects will be submitted to the Institutional Review Board/Independent Ethics Committee for approval. According to the Italian legislation (which complies with and implements European Union regulations),

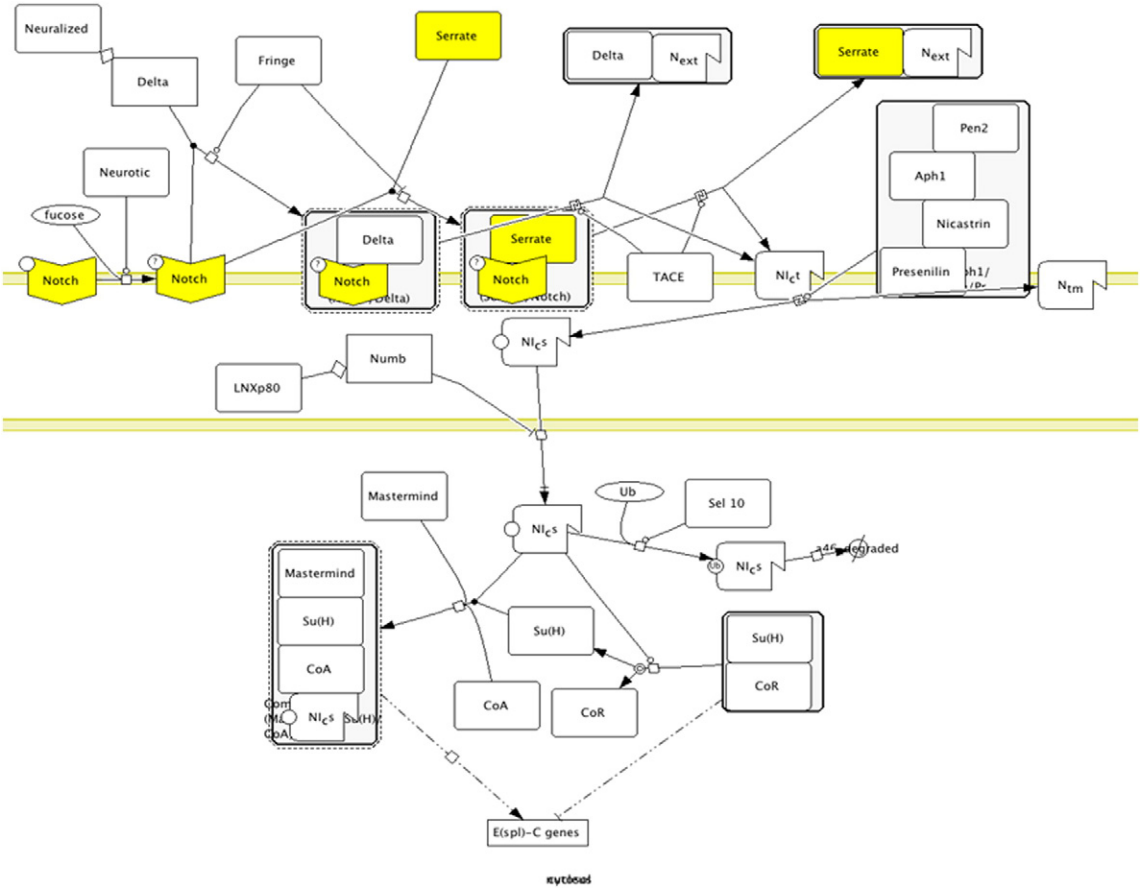

Fig. 2. NOTCH1 signaling pathway; yellow boxes indicate the NOTCH1 protein and its interactions within the signaling cascade.

participating patients must be duly informed, and give their explicit signed agreement, on the way their rights to the confidentiality of personal data are duly respected.

6. Discussion

The study will focus on multiple aspects of BAV disease. We will consider and combine the aortic valve, aortic root and ascending aorta morphology in order to obtain phenotypically homogeneous outlier cases on which we will collect surgical, echo, histologic, and genetic features. It would be useful to compare data within each group and then between different BAV groups.

Insights into the relation between BAV morpho-functional and genetic groups in terms of risk of dilatation and dissection are scarce. For surgical planning we can only rely on guidelines that have shown lights and shadows [26]. Several reports confirm the association between BAV and vascular disease without identifying why a certain patient with BAV will develop an aortic wall disease. Recently, some papers have hypothesized that the enlargement of the ascending aorta would be mainly due to hemodynamic alteration through the BAV. According to that, BAV would cause localized shear stress and a different compliance of aortic wall leading to an asymmetric aortic dilatation with a localized enlargement on the aortic convexity [27]. On the other hand, different authors observed that, after isolated BAV replacement, there could be an aortic dilatation more frequently than in tricuspid aortic valve replacement with a lower rate of freedom from cardiac event after surgery [28]. With regard to aorta disease, recent study explored the histological pattern of aortic wall in patients affected by BAV and ascending aorta aneurysm, realizing that there is a high rate of disruption of extracellular matrix and media cell apoptosis. They underlined the common origin from neural crest for both aortic wall and aortic valve, explaining these related diseases [29].

Other authors observed a connection between morphological patterns of BAV as a prognostic factor for ascending aorta and aortic root diseases. The different directions of commissures and the position of coronary ostia would be a good or poor prognostic factor in terms of vessel disease. There would be a statistically significant association between specific valve anatomy and a more severe degree of wall degeneration in the ascending aorta and dilatation of the aortic root at younger age compared with different valve morphologies [13,30].

The VAR study aims to find univocal correlations between valve morphology data, genetic patterns, and histological findings, building classes of risk in terms of aortic wall adverse evolution. Then it would be reasonable to think about reviewing follow-up schedule of BAV patients who are not yet eligible for surgery. Ideally, a closer follow-up for high risk patient and affected relatives could be considered; on the other hand, a relatively lighter follow-up for low risk BAV, those in which morphologic and genetic features of aortic wall implication are absent, could be depicted.

6.1. Organization

The GISSI VAR study is sponsored by the GISSI group who has the full responsibility not only for the formulation, but also for the overall conduct of the study, including onsite monitoring, and the utilization of the results.

Funding

The study is promoted by GISSI Group (ANMCO, Mario Negri Institute and Heart Care Foundation Onlus) which is also the owner of the database. The sponsor of the study is the Heart Care Foundation Onlus, a non-profit independent institution. The study is partially supported by Heart Care Foundation Onlus contributions collected in the year 2011, during the fundraising campaign "*Apri il tuo cuore alla ricerca*". ANMCO Research Centre of the Heart Care Foundation Onlus is responsible for database management, data quality control and data analyses. No fees are provided to cardiology centers, investigators and members of the study committees. The Steering Committee of the study takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

BM, BDC, APM, AMoreo, SP, GR, CFR, SR, LM, and AMaseri have no conflicts of interest to disclose.

Contributors

BM contributed in the conception and design of the research, acquisition of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content and supervision. BDC contributed in the drafting of the manuscript. APM made the analysis and interpretation of the data, obtaining funding, critical revision of the manuscript for important intellectual content. AMoreo contributed to the conception and design of the research. SP and SR made a critical revision of the manuscript for important intellectual content. GR contributed the analysis and interpretation of the data. CFR contributed in the conception and design of the research and acquisition of data. LM and AMaseri contributed to the conception and design of the research.

Appendix 1

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