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EDITORIAL

Prospects and advancements in C-reactive protein detection

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Abstract

C-reactive protein (CRP) is one of the earliest proteins that appear in the blood circulation in most systemic inflammatory conditions and this is the reason for its significance, even after identification of many organ specific inflammatory markers which appear relatively late during the course of disease. Earlier methods of CRP detection were based on the classical methods of antigen-antibody interaction through precipitation and agglutination reactions. Later on, CRP based enzymatic assays came into the picture which were further modified by integration of an antigen-antibody detection system with surface plasma spectroscopy. Then came the time for the development of electrochemical biosensors where nanomaterials were used to make a highly sensitive and portable detection system based on silicon nanowire, metal-oxide-semiconductor field-effect transistor/bipolar junction transistor, ZnS nanoparticle, aptamer, field emission transmitter, vertical flow immunoassay *etc*. This editorial attempts to summarize developments in the field of CRP detection, with a special emphasis on biosensor technology. This would help in translating the latest development in CRP detection in the clinical diagnosis of inflammatory conditions at an early onset of the diseases.

Key words: C-reactive protein; Inflammation; Diagnostic methods; Antibody; Biosensors

Core tip: Over time, C-reactive protein (CRP) has emerged as a versatile marker for the detection of systemic inflammatory conditions, providing preliminary information to clinicians for continuing with a more specific diagnostic methodology. Advancements in electroanalytical chemistry and knowledge of nanomaterials have helped modern age researchers to miniaturize detection systems with an enhanced level of specificity and sensitivity of CRP detection. Further research should be directed in this area to devise a better diagnostic platform that can detect the change in CRP level at a very early stage of the onset of inflammatory conditions.

Chandra P, Suman P, Airon H, Mukherjee M, Kumar P. Prospects and advancements in C-reactive protein detection. *World J Methodol* 2014; 4(1): 1-5 Available from: URL: http://www.wjgnet.com/2222-0682/full/v4/i1/1.htm DOI: http://dx.doi.org/10.5662/wjm.v4.i1.1

INTRODUCTION

In humans, there are many acute phase proteins whose level in blood plasma increases or decreases in response to inflammation (acute phase reaction). Some of the acute phase proteins are C-reactive protein (CRP), mannose binding protein, complement factors, serum amyloid A, fibrinogen, retinal binding protein, ceruloplasmin and antithrombin. Amongst them, CRP is the most important, sensitive and systemic marker of inflammation identified in the human body as its level rises rapidly in the blood plasma in response to a large number of foreign bodies, infections, tissue damage, renal and cardiovascular diseases[1]. It is secreted by hepatocytes

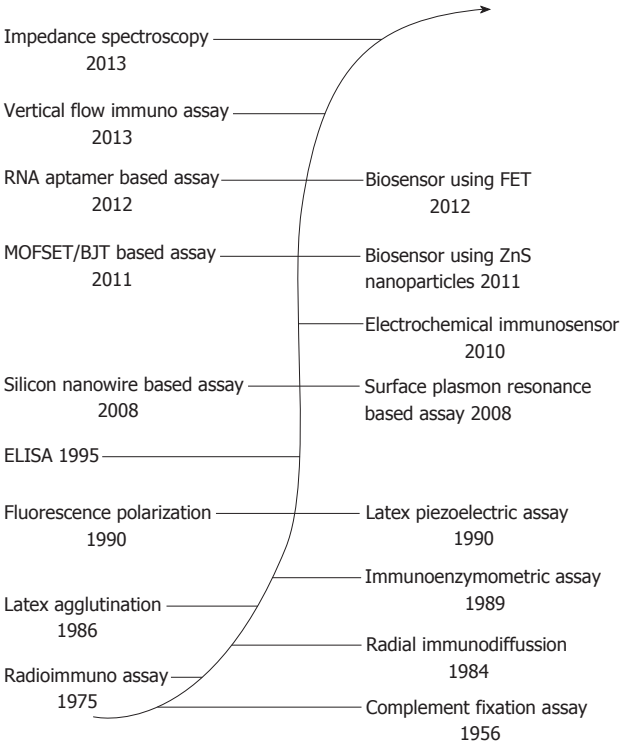

Figure 1 Diagrammatic representation of the advancement in C-reactive protein detection. MOFSET/BJT: Metal-oxide-semiconductor field-effect transistor/bipolar junction transistor; FET: Field effect transistor; ELISA: Enzyme-linked immunosorbent assay.

in response to cytokines, like interleukin 6, interleukin 1, tumor necrosis factor alpha *etc*[2]. CRP (M_r 115,135), a member of the pentraxin family of calcium dependent ligand binding plasma protein, is composed of 5 non-glycosylated polypeptide subunits, each of which is composed of 206 amino acid residues. Polypeptide units associate with each other through non-covalent bonding in an annular configuration forming cyclic pentameric symmetry. The ligand binding site of CRP comprises of loops with two calcium ions. During inflammation, phosphocholine present on necrotic or apoptotic cells binds at the active site of CRP, thereby activating the classical complement pathway essential for opsonization and induction of pro-inflammatory pathophysiological effects. Additionally, it activates the complement pathway but also increases a respiratory burst of neutrophils, encourages expression of adhesion molecules and synthesis of tissue factors. Based on this clinical importance of CRP, attempts have been made in this editorial to summarize the chronological development in the field of CRP detection. The physiological level of CRP in human plasma is 2 mg/L, whereas during inflammatory conditions, its concentration rises significantly in 6-8 h, even reaching up to 300 mg/L in the next 48 h. CRP level in patients with a cardiovascular disorder and/or myocardial infarction at the time of admission to the hospital have been observed to be above the physiological range (more than 3 mg/L)[3]. CRP deposits in the arterial walls during atherogenesis, thereby activating the complement pathway and augmenting the development of several cardiovascular disorders[4]. Abraham *et al*[5] observed a higher level of CRP (14.3 mg/L ± 11.2 mg/L) in patients before dialysis who were susceptible to chronic kidney disorder, renal failure or kidney malfunction. A higher concentration of CRP is also found during late pregnancy. People with obesity and high body mass index also have a higher level of CRP in blood plasma[6]. In a study by Lee *et al*[7], a raised level of high sensitivity CRP (hsCRP) was also correlated with the development of cancer. Hence, CRP is an important marker of clinical conditions like local and systemic inflammation, myocardial diseases, obesity *etc*. The prospect of developing a highly specific and sensitive method of detection of CRP at an early stage of these clinical conditions has been attempted by various research groups. The overall chronological development is elucidated in Figure 1.

Conventional methods of CRP detection rely on precipitation by C-polysaccharide of *Pneumococcus*, tube precipitation, complement fixation, latex agglutination, radioimmunoassay, radial immunodiffusion and fluorescence polarization. Detection of CRP by radial immunodiffusion uses radial immunodiffusion plates made of agarose containing 1% rabbit anti-human CRP. Sera samples are added into the wells punched on them and the diameter of the radial rings measured after a 48 h incubation period. The greater the diameter of the precipitation ring, the higher the CRP concentration in the serum. The time taken for the assay and its semi-quantitative nature are the major limitations of this detection system[8]. As an improvement of the previous technique, the latex agglutination method was developed which employs inert latex particles coated with anti-human CRP antibody. In the presence of CRP in the patient's serum, the agglutination reaction can be seen between anti-human CRP and CRP moieties. Unlike the precipitation reaction, it takes less time but still has the limitation of being semi-quantitative in nature[9]. In 1990, Kurosawa *et al*[10] developed a latex piezoelectric immunoassay using a piezoelectric quartz crystal which acts as the sensing element for the change in viscosity or density in the solution due to aggregation of latex particles. It negated the disadvantages of previous methods of detection of CRP using agglutination through the use of a latex bearing antibody with no film. Earlier piezoelectric assays employed the formation of an antibody coated thin film latex on a crystal by which the oscillating frequency of the crystal reduces. This approach removed the drawbacks of previous methods in terms of labeling reporter molecules and through improving the assay sensitivity. Furthermore, an immunoenzymometric assay for determination of CRP using two antibodies has been developed by Käpyaho *et al*[11]. It is a simple assay consisting of a single immunological reaction between CRP and peroxidase labeled antibody with another antibody attached to the wall of the test tube. The immune complex formed is determined by a colorimetric assay using a peroxidase substrate. The sensitivity of this technique is comparable to the turbidimetric method of CRP detection. However, concerns about enzyme stability, shelf life and time taken for detection raise the question of its practical applications and shelf

life of the diagnostic system[11]. An enzyme-linked immunosorbent assay (ELISA) kit for the detection of CRP (Cell Biolabs Inc., San Diego, CA, United States) has anti-CRP antibody coated onto the microtiter plate that reacts with the CRP antigens. An enzyme linked secondary antibody in the presence of specific substrate gives rise to a colorimetric reaction whose optical density can be measured to estimate the level of CRP. The detection limit of this is up to 0.1 ng/mL but high false positives due to non-specific binding limits the availability of this methodology. Other major disadvantages include the long detection time, lower sensitivity, low stability, cross reactivity with the serum proteins, lack of miniaturization and on-site analysis.

Thus, in recent years, various biosensor based detection systems have been attempted for quick, sensitive and on-site detection of CRP. A biosensor is an analytical device utilizing a biological reaction between receptor and target molecules, converting the biological response into readable and quantifiable signals using transducers[12-15]. Lee *et al*[16] developed a biosensor based on surface plasma resonance spectroscopy which involved measurement of molecular interactions at the gold/silver surface of the sensing element, thereby measuring reflectance of light with respect to the refractive index of the surface of biosensing element that changes when CRP molecular species react at the fabricated unit. This technique uses poly (3-(2-((N-succinimidyl)succinyloxy)ethyl)thiophene) (P3SET) which is a polythiophene with pendant N-hydroxysuccinimide (NHS) ester group as a biolinker between the anti-CRP (bioreceptor) and sensing surface. A self-assembled monolayer (SAM) of P3SET formed on the gold surface and anti-CRP was immobilized covalently. When CRP reacted with sensor, there was a shift in the refractive index of P3SET/anti-CRP due to the formation of P3SET/anti-CRP/CRP on the sensing surface and reflectance was deviated. Hence, the reaction between anti-CRP immobilized on gold surface and CRP can be monitored using surface plasma resonance with a high sensitivity[15].

With advancements in nanotechnology, nanobiosensors have become very popular in recent times. In this regard, Lee *et al*[7] attempted the silicon-nanowire based fabrication process which follows a top-down approach of fabrication using micro-machining technology. In a new study, Yuan *et al*[17] developed a method to adjust sensitivity using a gated lateral bipolar junction transistor (BJT) in the metal-oxide-semiconductor field-effect transistor-BJT hybrid mode which was fabricated using the complementary metal-oxide-semiconductor manufacturing system. Si_3N_4 was immobilized on the layer on gold which was then immobilized on a floating gate using an electron beam evaporator. A die chip consisting of gated lateral BJT was then embedded onto a printed circuit board which was further connected to the vertical collector, base and lateral collector, and emitter. Internal metal layers were also employed to enhance the rate of current flow. Monoclonal anti-CRP antibodies were linked to the gold layer using SAMs of 11-mercaptoundecanoic acid, N-Hydroxysuccinimide and N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride. On reaction with CRP species, capacitance between the liquid and floating gate changes is measured. This change in capacitance has been used to determine the concentration of CRP with high sensitivity and reliability. The advantages of such a system are the small size, ease of manufacturing, low noise, high transconductivity, good selectivity and reproducibility. It has also been claimed that the developed system can be used for other biomarkers by changing the corresponding antibody.

A biosensor integrated with a microfluidic device has been also developed for the detection of CRP. In a report, CRP along with other cardiac marker troponin c has been detected simultaneously using a microfluidic device. The device developed a chip that acted as a microreactor for the simultaneous detection of CRP and troponin c. Antibodies with bioconjugated CdTe and ZnSe were used in the system. These quantum dots release Zn^{2+} and Cd^{2+} ions that are detected by square-wave anodic stripping voltammetry to enable the quantification of the two biomarkers. This electrochemical immunosensor has a detection range of 0.5-200 μg/mL, with a detection limit of 307 attomole in 30 μL for CRP[18]. Another method of detection which uses Zn^{2+} ions for the detection of CRP was established by Cowles *et al*[19] where ZnS nanoparticles were used to transduce the signal *via* fluorescence spectroscopy. In this detection system, mouse anti-CRP coated magnetic microbeads were used. On addition of the serum sample containing CRP, the immune complex binds to these beads to which biotinylated mouse anti-CRP will fix. Neutravidin conjugated with ZnS nanoparticles will attach to this complex and in the presence of Flouzin3, a zinc ion selective fluorescence dye, generate a fluorescence signal. The bioassay possesses a detection limit of 10 pmol which makes it a highly sensitive method to detect CRP. In addition, it is also non-toxic and a less expensive system to fabricate. Another biosensor based on nanomaterial for the detection of CRP level was developed by Qureshi *et al*[20]. The detection system requires the use of specific interaction between CRP and its corresponding RNA aptamer. These CRP specific RNA aptamers are immobilized on carbon nanotubes activated gold interdigitated electrodes of capacitors *via* a physical adsorption. The selective binding of RNA aptamers with CRP is determined by measuring the capacitance after competitive binding between complementary RNA and CRP in pure forms and co-mixtures. It is a label-free method of detection based on affinity separation of target molecules with a limit of detection ranging from 1-8 μmol/L. Although the detection limit is very low, this method has merit in terms of a label-free approach and simple approach for detection of CRP. Kim *et al*[21] recently developed a biosensor using a field effect transistor in which silicon binding protein (SBP) is linked to surface protein A to simplify the tedious method of fabrication of the monolayer. SBP, an artificial protein,

Table 1 Various C-reactive protein detection techniques and their characteristics

No.	Technique employed	Features	Ref.
1	Radial Immunodiffusion	Qualitative analysis in less than 48 h	Harris *et al*[8], 1984
2	Latex agglutination	Time taken less than 24 h; qualitative analysis	Senju *et al*[9], 1986
3	Latex piezoelectric assay	Uses quartz crystal and latex bearing antibody; more sensitive than conventional methods; less time required.	Kurosawa *et al*[10], 1990
4	Immunoenzymometric Immunoassay	Single immunological reaction; sensitive; results comparable to turbidimetric detection	Käpyaho *et al*[11], 1990
5	Surface plasma resonance spectrophotometry	High sensitivity; on-site analysis; SAM usage	Kim *et al*[15], 2008
6	Silicon nanowire based assays	Micro-machining technology; higher detection limit	Lee *et al*[16], 2008
7	MOFSET/BJT based technique	High sensitivity, change in capacitance measurement; reliable; small size; ease of manufacturing; good selectivity; highly reproducible; high trans conductivity	Yuan *et al*[17], 2011
8	Electrochemical Immunosensor	Detection by square wave stripping voltammetry; quantitative analysis of 2 biomarkers; reproducible	Zhou *et al*[18], 2010
9	Nanotechnology using ZnS nanoparticles	Detection by fluorescence spectrophotometry; highly sensitive; non-toxic; low cast system; highly specific	Cowles *et al*[19], 2011
10	RNA aptamer based technology	Uses Carbon nanotube's interdigitated electrodes of capacitors; highly selective	Qureshi *et al*[20], 2012
11	Biosensor using FET	Involves SBP linked in protein A; point of care testing system; on-site analysis	Kim *et al*[21], 2013
12	Vertical flow Immunoassay	One-step assay; time taken 2 min; most rapid; employs gold nanoparticles	Oh *et al*[22], 2013
13	Electrochemical impedance spectroscopy	Most advanced technique; uses gold and diamond spray in fabrication; highly sensitive; reusable without sensitivity being lost; good detection limit	Bryan *et al*[23], 2013

FET: Field emission transmitter; MOFSET/BJT: Metal-oxide-semiconductor field-effect transistor/bipolar junction transistor; SAM: Self-assembled monolayer; SBP: Silicon binding protein.

can bind to the silicon surface with no bi-linker. A fabricated device is treated with hot piranha solution to maximize the affinity of SBP-protein A complex onto the sensing area. The SBP-protein A is then immobilized on the surface of sensing element and dipped into the solution containing anti-CRP. The anti-CRP is coated onto the fabrication unit where CRP forms the immune complex which is transduced in a detectable signal. This is the application of a biosensor point-of-care-testing system with a detection limit comparable to that of ELISA. Oh *et al*[22] has recently developed a one-step biosensor for hsCRP detection using a vertical flow immunoassay. It is composed of a sample pad, flow through films (FTH), conjugate pad and nitrocellulose membranes (onto which anti-hsCRP and secondary antibodies are immobilized below the holes) which are stacked upon one another. Anti-hsCRP conjugated with gold nanoparticles is encapsulated in the conjugate pad. This fabricated system detects hsCRP 0.01-10 μg/mL within 2 min and is the most rapid biosensor to date (Table 1).

Recently, an optimized biosensor for a label-free detection of CRP in a blood serum sample has been developed by Bryan *et al*[23], based on electrochemical impedance spectroscopy using gold electrodes. SAMs of polyethylene glycol (HS-C_{11}-$(EG)_3$-OCH_2-COOH) with the help of ethanol and nitrogen gas are made and dipped into piranha solution. NHS is used to activate the carboxylate group and monoclonal anti-CRP is linked to monolayers covalently. This device detects CRP in blood on the basis of difference in impedance when CRP species reacts with the monoclonal anti-CRP antibody bound to SAM. This system of detection has a very good selectivity and reusability with no loss of apparent sensitivity. This can be considered one of the latest methods of CRP detection where no specific labeling is required *i.e.*, a label free detection system even through the picomolar detection limit.

CONCLUSION

Our understanding of CRP detection systems has come a long way. Over the years, CRP has become a versatile inflammatory marker for the detection of systemic inflammatory conditions. In future, advancements in interdisciplinary approaches will be helpful for the quick, ultrasensitive analysis of these markers. Attempts should also be made to develop new CRP recognition molecules and new material to develop sensing platforms. While developing and implementing these concepts, care should be taken that these systems have promise for CRP analysis in body fluids.

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REVIEW

Off-pump coronary artery bypass grafting: Misperceptions and misconceptions

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Abstract

Coronary artery bypass grafting (CABG) continues to be one of the most commonly performed cardiac surgical procedures worldwide. Conventional CABG performed on cardiopulmonary bypass termed on-pump CABG is regarded as the gold standard. However, on-pump CABG results in several physiologic derangements including but not limited to thrombocytopenia, activation of complement factors, immune suppression, and inflammatory responses leading to organ dysfunction. Furthermore, manipulating an atherosclerotic ascending aorta during cannulation and cross-clamping can predispose to embolization and stroke risk. Recognition of these detrimental effects of on-pump CABG resulted in resurgence of off-pump CABG nearly two decades ago. Off-pump CABG since its resurgence has been a subject of intensive scrutiny and speculation. Despite numerous retrospective nonrandomized studies, prospective randomized trials, and meta-analyses validating the safety and efficacy of off-pump CABG, opponents of the technique have persistently demanded abandonment of off-pump CABG. Several misconceptions and misperceptions are used as an excuse for such demands. This review article examines published scientific evidence to evaluate these misperceptions and misconceptions about off-pump CABG.

Key words: Coronary artery bypass grafting; Cardiopulmonary bypass; Off-pump coronary artery bypass grafting; Surgical myocardial revascularization; Coronary artery surgery

Core tip: There is reluctance to adopt off-pump coronary artery bypass grafting owing to concerns about incomplete revascularization, poor graft patency, and long-term mortality. These concerns are the result of misperceptions and misconceptions rather than reality. This manuscript attempts to tackle these misperceptions and misconceptions.

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INTRODUCTION

Conventional coronary artery bypass grafting (CABG) is characterized by performance of delicate coronary anastomoses on cardiopulmonary bypass (CPB). However, the price of a still and bloodless operative field is ultimately paid by the patients in the form of negative effects of CPB including blood trauma, activation of a series of inflammatory responses, nonpulsatile flow, and possible embolization of air or debris-most particularly embolization of atherosclerotic debris from the aorta[1]. Off-pump CABG was rediscovered with the primary objective of avoiding these deleterious effects of CPB.

Since its resurgence nearly two decades ago off-pump

CABG has been extensively investigated and scrutinized. It has been compared with the gold standard on-pump CABG in numerous randomized controlled trials[2-10] as well as large retrospective observational studies[11-15]. Majority of the published evidence comparing on-pump and off-pump CABG has shown comparable outcomes for these two techniques. However, inability of small, prospective, randomized controlled trials that have lacked sufficient sample size to demonstrate differences in early and long-term outcomes coupled with misperceptions and misconceptions about incomplete revascularization, reduced long-term graft patency and increased need for repeat revascularization resulting in inferior long-term survival have prompted opponents of off-pump CABG to demand abandonment of this technique. Those who question the feasibility and utility of off-pump CABG completely ignore the fact that larger observational studies that are better powered to statistically compare outcomes have shown more favorable in-hospital outcomes and equivalent long-term outcomes with off-pump and on-ump CABG[11-16].

In the current era increasing number of patients with high-risk profile are being referred for CABG. The benefits of off-pump CABG are apparent for patients at high risk for complications associated with CPB and aortic manipulation. Recent studies have demonstrated improved outcomes in higher-risk patients undergoing off-pump CABG[6,17-19]. In view of changing patient profile it will be prudent to acknowledge that off-pump CABG is a valuable technique in the armamentarium of cardiac surgeons and is here to stay. Misleading the cardiac surgical community by using misperceptions and misconceptions and in the process denying patients, particularly those at high-risk for complications due to use of CPB, the opportunity to have safe and effective myocardial revascularization off-pump is not a wise move. This review article examines published scientific evidence to evaluate these misperceptions and misconceptions about off-pump CABG and attempts to allay unnecessary apprehension about the safety and efficacy of off-pump CABG.

Completeness of revascularization

The criticism regarding completeness of revascularization is no longer valid in the current era as technology to safely perform multivessel off-pump CABG has improved tremendously over the past decade. Grafting of vessels on the lateral and inferior aspects is no longer impossible. In fact, the majority of evidence from randomized trials suggests at least equivalent completeness of revascularization[2-7,9,10] (Table 1). Furthermore, it is equally important to understand that completeness of revascularization and number of grafts should not be used synonymously. A more logical way to address the issue of completeness of revascularization is to use the index of completeness of revascularization [number of grafts performed divided by the number of grafts needed (number of graftable vessels with angiographically significant stenoses)][20].

It is important to emphasize that the frequency of complete revascularization reported by various studies comparing off-pump and on-pump CABG is always influenced by relative experience with each technique of the reporting center(s) and surgeon(s). For example, centers where on-pump CABG is used for most cases, and off-pump CABG is used for only a few cases the rates of complete revascularization in the late *vs* early off-pump experience will remain the same highlighting the importance of learning curve as well as case load. Such centers can also have an impact on the final completeness of revascularization achieved by multicentre randomized trials. This fact is exemplified by the Veterans Affairs (VA) Randomized On/Off Bypass (ROOBY) trial[21]. Every year, approximately 4000 isolated CABG procedures are performed in the VA system at 42 cardiac surgery facilities[22]. During the recruitment period of the ROOBY trial only 7 of the 42 centers qualified as high-volume off-pump CABG centers performing at least 50 off-pump cases per year[23]. Since the recognized learning curve for off-pump CABG is between 50 and 75 cases[24] it is not surprising that the ROOBY trial reports incomplete revascularization with off-pump CABG.

Several additional caveats exist regarding reporting of incompleteness of revascularization with off-pump CABG. First, none of the trials or studies reporting incomplete revascularization provides an explanation for failure to completely revascularize the off-pump CABG cohort. Second, from these trials, it is difficult to determine the significance of the ungrafted territory for a

Table 1 Comparison of number of grafts performed and completeness of revacsularization off-pump and on-pump in randomized controlled trials

Ref.	No. of grafts off-pump CABG	No. of grafts on-pump CABG	*P*	Completeness of revascularization
Coronary trial, Lamy *et al*[2] 2012	3.0	3.2	< 0.001	Yes[1]
GOPCABE trial, Diegeler *et al*[3] 2013	2.7	2.8	< 0.001	No
ROOBY trial, Shroyer *et al*[4] 2009	2.9	3.0	0.002	No
DOORS trial, Houlind *et al*[5] 2012	2.9	3.1	0.007	Yes
On-off study, Lemma *et al*[6] 2012	3.0	3.3	0.001	Yes
The Best Bypass Surgery trial, Møller *et al*[7] 2010	3.2	3.3	0.11	Yes
SMART trial, Puskas *et al*[9] 2003	3.39	3.4	NS	Yes
BHACAS trial, Angelini *et al*[10] 2002	2.23	2.31	NS	Yes

[1]Rate of incomplete revascularization (as assessed by the surgeon at the time of surgery) was higher, though the *P* value for the difference was only marginally significant (11.8% *vs* 10.0%, *P* = 0.05). NS: Not significant; BHACAS: Beating Heart Against Cardioplegic Arrest Studies; GOPCABE: German Off-Pump Coronary Artery Bypass Grafting in Elderly Patients; ROOBY: Randomized On/Off Bypass; DOORS: Danish On-pump *vs* Off-pump Randomization Study; SMART: Surgical Management of Arterial Revascularization Therapies; CABG: Coronary artery bypass grafting.

number of reasons. Foremost, it is impossible to determine myocardial viability in the territory left ungrafted, because myocardial viability studies were not used in these trials; thus, the significance of a reduced number of grafts in the off-pump CABG cohort is impossible to predict. Likewise, none of the trials used a myocardium at risk score, which is a potentially valuable tool to aid in determining the true significance of the non revascularized territory because there is a recognized hierarchy of effect, depending on which vessels are left ungrafted and how much myocardium is at risk[25]. Synnergren *et al*[26] examined the effect of incomplete revascularization over a 5-year period in a nonrandomized cohort of 9408 patients. Leaving 1 diseased vascular segment without a bypass graft resulted in no increased risk of death. However, leaving 2 vascular segments ungrafted significantly increased the risk for mortality ($P = 0.01$). Finally, it is important to mention that majority of the trials reporting incomplete revascularization with off-pump CABG report similar early mortality and morbidity rates for the two cohorts[2-5].

Graft patency

Graft failure is one of the major determinants of clinical prognosis after CABG. There has been considerable concern among surgeons and cardiologists that the greater technical difficulty of off-pump coronary revascularization might translate into less precise anastomoses and subsequently diminished graft patency[27]. With conventional on-pump CABG, the 15-year patency rate is > 97%. This is the gold standard that any new revascularization method must compete against[28]. A steep learning curve, distractions caused by cardiac motion or pulmonary insufflations, and construction of anastomoses on a moving target have been implicated as factors responsible for inferior graft patency after off-pump CABG[28].

Interestingly, all concerns about suboptimal graft patency over the years have been predominantly attributed to 2 randomized controlled trials[4,29]. Shroyer *et al*[4] demonstrated that the patency rate of the off-pump arm was lower than that of the on-pump arm on 12-mo angiography, and the 1-year composite adverse outcome rate (death from any cause, nonfatal myocardial infarction, and any reintervention procedure) was higher for off-pump than for on-pump CABG. Such findings do not come as a surprise since the 53 participating surgeons enrolled on average only eight patients per year during the study period and had unacceptably high conversion rates to on-pump surgery (12%) and incomplete revascularization (18%). Moreover, in 60% of the cases a resident was the primary surgeon again raising concerns about the relative inexperience translating into poor graft patency. Another unrecognized confounder that contributed to poor graft patency in the ROOBY trial[4] was the concomitant use of endoscopic vein harvesting (EVH) in 1471 patients (on-pump = 907 and off-pump = 564). The incidence of a patient having 1 or more occluded saphenous vein grafts on follow-up angiography was 41.3% in the EVH group, compared with 28.0% in the open vein harvesting (OVH) group ($P < 0.0001$). Overall saphenous vein graft patency in the EVH group was 74.5%, which was significantly worse than the 85.2% rate in the OVH group ($P < 0.0001$)[30]. Since ROOBY trial was recruiting at a time when EVH was not being widely practiced the poor vein graft patency secondary to EVH can be attributed to learning curve and relative inexperience of the vein harvesters. Poor conduit quality, a consequence of the learning curve for EVH, has been shown to be a predictor of early graft failure, blunted positive remodeling, and greater negative remodeling[31].

The other frequently cited randomized trial supporting the argument of poor graft patency after off-pump CABG is the trial by Khan *et al*[29] reporting decreased patency at 3 mo in the off-pump group. However, closer analysis of this reveals that limited experience of the operating surgeons, consisting of only 98 off-pump procedures, which require a different skill set, during the two years before the study (an average of 25 procedures per surgeon per year) coupled with the relatively low dose of intraoperative heparin, the absence of aggressive antiplatelet therapy with clopidogrel postoperatively, and the failure to use new suction devices to optimize exposure were perhaps some of the confounding factors for poor graft patency[32,33].

Long-term survival

The negative impact of incomplete revascularization and lower graft patency on late mortality rates is well-recognized[34]. Takagi *et al*[35] recently published a meta-analysis of 11 randomized trials demonstrating a statistically significant increase in ⩾ 1 year all-cause mortality by a factor of 1.37 with off-pump relative to on-pump CABG (RR = 1.373; 95%CI: 1.043-1.808). It is extremely important to highlight that the sensitivity analysis in this meta-analysis revealed that the ROOBY trial[4] strongly contributed to the pooled estimate. The aforementioned criticisms of this trial provide an explanation for the inferior survival of off-pump cohort. Furthermore, majority of the recently conducted trials reporting 30-d mortality[2,3,5,6] have not yet reported outcomes for long-term follow-up.

It is expected that once longer follow-up data is available for recently conducted randomized trials, that utilized newer technology for stabilization and exposure and had similar index of completeness of revascularization for off-pump and on-pump CABG, this controversy will be resolved.

CONCLUSION

Although there are numerous clinical studies attesting to the benefits of off-pump CABG[36-38], skepticism, fuelled by misperceptions and misconceptions, persists regarding the safety, efficacy, and equivalence of revascularization with off-pump CABG compared with on-pump CABG[39]. It is extremely important to highlight that off-

pump CABG is a technically demanding strategy and central to all the concerns associated with this technique is the issue of learning curve[1]. The learning curve in off-pump surgery can be safely negotiated with appropriate patient selection, individualized grafting strategy, peer-to-peer training of the entire team, and graded clinical experience (preoperative planning, adequate exposure, proximal anastomoses to the aorta, and distal anastomoses initially to anterior wall vessels, followed by inferior wall vessels and then lateral wall vessels)[40].

Contrary to the proponents and opponents of off-pump CABG, the authors' view is that both on-pump and off-pump CABG have their place in the field of myocardial revascularization. Present day cardiac surgeons must adopt off-pump CABG rather than condemn and castigate it. The rational for this view is the changing profile of patients that are being referred for surgical revascularization. At the same time, technical precision, anastomotic quality, and completeness of revascularization should not be compromised in an attempt to avoid the deleterious effects of CPB unless these short-term risks outweigh any potential long-term benefit.

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REVIEW

Impact of HLA-G analysis in prevention, diagnosis and treatment of pathological conditions

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Abstract

Human leukocyte antigen-G (HLA-G) is a non-classical HLA class I molecule that differs from classical HLA class I molecules by low polymorphism and tissue distribution. HLA-G is a tolerogenic molecule with an immune-modulatory and anti-inflammatory function on both innate and adaptative immunity. This peculiar characteristic of HLA-G has led to investigations of its role in pathological conditions in order to define possible uses in diagnosis, prevention and treatment. In recent years, HLA-G has been shown to have an important implication in different inflammatory and autoimmune diseases, pregnancy complications, tumor development and aggressiveness, and susceptibility to viral infections. In fact, HLA-G molecules have been reported to alternate at both genetic and protein level in different disease situations, supporting its crucial role in pathological conditions. Specific pathologies show altered levels of soluble (s)HLA-G and different *HLA-G* gene polymorphisms seem to correlate with disease. This review aims to update scientific knowledge on the contribution of HLA-G in managing pathological conditions.

Key words: Human leukocyte antigen-G; Pathology; Prognosis; Diagnosis; Treatment; Marker

Core tip: Human leukocyte antigen-G (HLA-G) is a tolerogenic molecule. HLA-G has been shown to have important implications in different pathological conditions where it is reported to alternate at both protein and genetic level. The peculiar immunoregulatory function of HLA-G and its dysregulation in different diseases have led to investigation of its role in pathological conditions in order to define possible uses in diagnosis, prevention and treatment. This review aims to update scientific knowledge on the contribution of HLA-G in managing pathological conditions.

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INTRODUCTION

Diagnosis and prevention of diseases is mainly based on the identification of specific biological markers and drug targets. In view of this, the possibility of easy and fast identification of molecules, for example in biological fluids, seems to be even more necessary.

In recent years, different studies have demonstrated that human leukocyte antigen-G (HLA-G), a non-classical class I molecule, could fulfil this necessity[1-3]. In fact, HLA-G expression and levels in biological fluids, cells and tissues in different pathological conditions have been shown. Several authors reported that the level of soluble

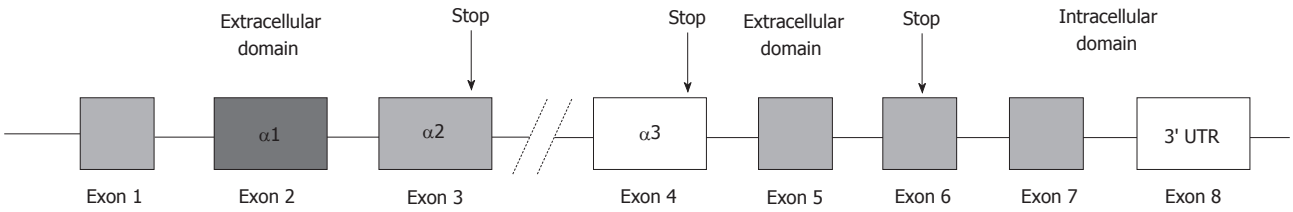

Figure 1 Human leukocyte antigen-G gene. UTR: Untranslated region.

HLA-G and gene polymorphisms correlate with disease outcome and the therapeutic success of treatment[4-6].

HLA-G MOLECULE

HLA-G is a major histocompatibility complex class I antigen encoded by a gene on chromosome 6p21. It differs from classical HLA class I molecules by its restricted tissue distribution and limited polymorphism in the coding region. To date, 50 alleles (IMGT HLA database, August 2013) and 16 proteins are known. The gene structure of HLA-G is homologous to other HLA class I (Ia) genes consisting of 7 introns and 8 exons coding the heavy chain of the molecule. Exon 1 encodes the peptide signal, while exons 2, 3 and 4 encode the extracellular α1, α2 and α3 domains, respectively. Exons 5 and 6 encode the transmembrane and cytoplasmic domains of the heavy chain. Exon 7 is always absent from mature mRNA due to the stop codon in exon 6; exon 8 is not translated (Figure 1). Seven HLA-G isoforms exist due to mRNA alternative splicing and differential association with β2-microglobulin; two of these are found on the cell surface and in biological fluids: Membrane-bound G1 and soluble G5, which lacks the trans-membrane and intracellular domains of membrane-bound G1 (Figure 1)[7]. HLA-G possesses an unpaired cysteine residue at position 42 on an external loop of the peptide binding groove that enables the dimerisation[8,9]. HLA-G monomers are recognized by the inhibitory receptors LILRB1 and LILRB2 and by KIR2DL4[10]. LILR receptors have a greater affinity for the dimeric form that increases the signaling transduction, especially in natural killer (NK) cells[11,12]. The interaction of HLA-G molecules with inhibitory receptors induces apoptosis of activated Crohn's disease ($CD8^+$) T cells[11], modulates the activity of NK cells[13,14] and of dendritic cells (DC)[15,16], blocks allo-cytotoxic T lymphocyte response[17] and induces expansion of suppressor T cell populations, such as $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells[18,19]. Moreover, HLA-G is expressed at high levels on DC-10 cells, human DCs with tolerogenic activity and an outstanding ability to produce interleukin (IL)-10[16]. Interestingly, the expression of membrane-bound HLA-G1 and that of its receptors is up-regulated by IL-10 on DC-10 and the expression of high levels of membrane-bound HLA-G1, ILT4 and IL-10 by DC-10 is critical to the generation of allergen-specific Tr1 cells by DC-10[16].

The HLA-G production is controlled by several polymorphisms, both in the promoter and in the 3' untranslated region (3' UTR), modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors, respectively[20].

Twenty-nine single nucleotide polymorphisms (SNPs) have been identified in the HLA-G promoter region which may be involved in the regulation of HLA-G expression, considering that many of these polymorphisms are within or close to known or putative regulatory elements. The HLA-G 5' upstream regulatory region (URR) is unique among the *HLA* genes[21] and is unresponsive to NF-κB[22] and interferon (IFN)-γ[23] due to the presence of a modified enhancer A and a deleted interferon-stimulated response element (ISRE). A locus control region located -1.2 kb from exon 1 exhibits a binding site for CREB1 factor, which also binds to two additional cAMP response elements at -934 and -770 positions from the ATG. In addition, a binding site ISRE for interferon response factor-1 is located at the -744 base pair (bp) position[24] and is involved in HLA-G transactivation following IFN-β treatment[24]. The HLA-G promoter also contains a heat shock element at the -459/-454 position that binds heat shock factor-1[25] and a progesterone receptor binding site at -37 bp from ATG[26]. Several promoter region polymorphisms coincide with or are close to known or putative regulatory elements and thus may affect the binding of HLA-G regulatory factors[27]. The -725 C > G/T SNP is very close to ISRE, in which the -725 G allele is associated with a significantly higher expression level compared with the others[28]. The polymorphic sites at the 5' URR are frequently in linkage disequilibrium with the polymorphic sites identified at the 3' UTR, some of them influencing alternative splicing and mRNA stability.

A 14 bp insertion/deletion (INS/DEL) polymorphism (rs66554220) in exon 8 involves mRNA stability and expression[29,30]. In particular, the DEL allele stabilizes the mRNA with a consequent higher HLA-G expression[30,31]. The presence of an adenine at position +3187 modifies an AU-rich motif in the HLA-G mRNA and decreases its stability[32]. One SNP C > G at the +3142 bp position (rs1063320) affects the expression of the HLA-G locus by increasing the affinity of this region for the microRNAs (miR)-148a, miR-148b and miR-152, therefore decreasing the mRNA availability by mRNA degradation and translation suppression[33]. The influence of the +3142G allele has been demonstrated by a functional study in which HLA-G high-expressing JEG-3 choriocarcinoma-derived cells have been transfected with miR-148a, decreasing soluble HLA-G levels. The

discordant results obtained by Manaster *et al*[34], who have reported the lack of +3142 C > G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression. Other SNPs are identified as implicated in miRNA interaction. In particular, +3003, +3010, +3027 and +3035 SNPs are targets for miR-513a-5p, miR-518c*, miR-1262 and miR-92a-1*, miR-92a-2*, miR-661, miR-1224-5p and miR-433 miRNAs[35]. The miR-2110, miR-93, miR-508-5p, miR-331-5p, miR-616, miR-513b, and miR-589* miRNAs target the 14bp INS/DEL fragment region and miR-148a, miR-19a*, miR-152, mir-148b, and miR-218-2 target the +3142 C/G polymorphism.

HLA-G is a stress-inducible gene; heat shock, hypoxia and arsenite increase different HLA-G alternative transcripts[25,36,37]. The indoleamine 2,3-dioxygenase, an enzyme which metabolizes tryptophan, induces HLA-G expression during monocyte differentiation into DCs[38]. The anti-inflammatory and immunosuppressive IL-10 has been correlated with concomitant HLA-G expression[30,39]. Transactivation of HLA-G transcription has also been demonstrated by leukemia inhibitory factor[40], progesterone[26] and methotrexate[41] cell exposure. Furthermore, IFN-α, -β and -γ enhance HLA-G cell-surface expression by tumors or monocytes[42,43]. HLA-G expression could be acquired by trogocytosis, where a "donor" cell that expresses membrane HLA-G exchanges membrane parts containing HLA-G with a "recipient" cell that is not expressing HLA-G molecules. In this particular situation, "recipient" cells will acquire and make use of membrane HLA-G molecules from a "donor" HLA-G positive cell without the activation of HLA-G gene. Trogocytosis of antigen presenting cell HLA-G1 by T cells in humans makes T cells unresponsive[44]. It has been shown that HLA-G1 can be acquired by NK cells from tumor cells. NK cells that acquire HLA-G1 stop proliferating, are no longer cytotoxic and behave like suppressor cells capable of inhibiting other NK-cell functions[14].

HLA-G's role in immune-tolerance was discovered studying its expression in trophoblast cells at the fetus-maternal interface[45]. The importance of HLA-G production by placental trophoblasts is evident in pre-eclampsia and unexplained recurrent spontaneous abortion (RSA). Several studies have found an aberrant or reduced expression of both HLA-G mRNA and protein in pathological compared with control placentas[46-48], with a possible implication in fetal protection and vascular events.

HLA-G expression has been documented in a few tissues during physiological conditions, such as cornea, thymus, erythroid and endothelial precursors[49-51], and in a variable percentage of serum/plasma samples from healthy subjects[52] where the main producers are activated CD14 positive monocytes[53]. A modified expression of HLA-G molecules has been observed during "no-physiological" conditions, such as viral infection[54-57], cancer[58,59], transplantation[60-64], inflammatory and autoimmune diseases[65,66].

Thus, a growing body of evidence has indicated HLA-G as a suitable key factor in different pathologies. In fact, the immune-modulation by HLA-G may exhibit two distinct effects in pathological conditions: It could be protective in inflammatory and autoimmune diseases[2,65-67], or on the other hand it could be dangerous, for example in tumors or infectious diseases[54-56,58,59]. Based on this evidence, the role of HLA-G in inflammatory and autoimmune diseases has gained considerable clinical interest for the possibility of exploiting it as a molecular biomarker and a therapeutic target.

HLA-G AND PATHOLOGICAL CONDITIONS: PERSPECTIVES IN PREVENTION, DIAGNOSIS AND TREATMENT

Given the immunomodulatory nature of HLA-G molecule, it could be considered a good reference parameter for prevention, diagnosis and treatment in autoimmune and inflammatory diseases.

HLA-G has been analyzed in different pathologies. In this review, we focus on the importance of HLA-G analysis in common and debilitating pathologies characterized by a dysregulation in host immune system in which HLA-G plays a central role.

HLA-G impact in rheumatic disease

Rheumatic disease is a general term used to describe numerous conditions that affect the joints [rheumatoid arthritis (RA)], connective tissues [scleroderma, systemic lupus erythematosus (SLE)] and vessels (vasculitis). Rheumatic diseases are inflammatory and autoimmune diseases, the second most common cause of disability after musculoskeletal injuries. RA (OMIM, #180300) is caused by the immune system attacking synovial cells and treatments include disease modifying anti-rheumatic drugs (DMARDs) and, more recently, biological agents. An important goal of RA therapy has shifted to initiate treatment early and aggressively to achieve remission or low disease activity as quickly as possible. This "treat-to-target" concept has been shown to maximize long-term healthy life[68,69].

Interestingly, RA patients present with an abnormal regulatory network in the immune response, which includes *HLA-G* gene[70]. Serum sHLA-G protein concentration is significantly lower in RA[71] patients than in controls. The decreased sHLA-G concentrations may lead to a chronic activation of inflammatory cells and contribute to the development of the disease. The evaluation of sHLA-G molecules at the specific inflammation site of the synovia reported higher levels of sHLA-G in RA[72] patients. The release of HLA-G in the inflamed synovium may be related to the recruitment of activated HLA-G positive immune cells and the local production by activated synovial fibroblasts[73] that could interact with immune inhibitory receptors and maintain a chronic inflammatory response. These data suggest that there is

a different production of HLA-G molecules on the basis of the local and systemic environments, characterized by different molecular factors and cell types. Interestingly, a recent work confirmed the role of HLA-G molecules in RA. The authors used an intracutaneous treatment of HLA-G monomer or dimer molecules in collagen-induced arthritis model mice. These molecules produced excellent anti-inflammatory effects with a single, local administration[74]. Notably, the dimer exhibited higher immunosuppressive effects than the monomer due to the higher dimer affinity for PIR-B, the mouse homolog of the LILRBs. The HLA-G 14 bp INS/DEL polymorphism has been evaluated as a pharmacogenetic marker of MTX therapy[41]. The authors showed an increase of the 14 bp DEL/DEL genotype in the responder group, characterized by a reduction in disease activity score (DAS28) measured before and after six months of treatment with MTX. In contrast to this study, there are two researches with negative results: (1) 130 RA patients responsive to MTX did not show a significant difference in 14 bp DEL/INS allelic and genotypic distribution (DAS28 < 3.2)[75]; and (2) 186 RA patients, previously untreated with MTX, were prospectively followed up and considered as responders with a DAS28 of up to 2.4 after six months of treatment[76]. No significant association between HLA-G 14 bp INS/DEL and MTX efficacy was observed. Comparing these studies, the discordant results may reflect population differences in gene expression that could influence the power of association studies and lead to different levels of association. In addition, the different doses of MTX and the different cut-off used for RA therapy response definition could affect the results obtained.

Rizzo *et al*[2] evaluated the possible role of HLA-G molecules as biomarkers for RA treatment in a follow-up study. Twenty-three early RA (ERA) patients were analyzed during a 12 mo follow-up disease treatment for sHLA-G levels in plasma samples, mHLA-G and ILT2 expression on peripheral blood CD14 positive cells, and typed HLA-G 14 bp DEL/INS polymorphism. Interestingly, the authors observed that ERA patients with low sHLA-G and membrane HLA-G expression suffered a more severe disease. In fact, sHLA-G levels inversely correlated with DAS28 and ultrasonographic power Doppler scores, used to define the severity and progression of the disease. Interestingly, sHLA-G up-modulation is evident after 3 mo of DMARDs therapy, while a significant reduction in tumor necrosis factor-α levels is evident after 9 mo therapy when a clear amelioration of the disease is evident, with a high specificity for HLA-G detection in EA condition. Moreover, the implication of the HLA-G 14 bp INS/DEL polymorphism is confirmed as the presence of the DEL allele characterizes the patients with a significant improvement in disease status.

SLE (OMIM, #601744) is a systemic autoimmune disease of the connective tissue that can affect any part of the body. Rosado *et al*[77] and Chen *et al*[78] showed higher sHLA-G and IL-10 levels in SLE patients in comparison with healthy controls, while Rizzo *et al*[66] observed lower sHLA-G concentrations in SLE patients. The differences in sHLA-G levels in these two papers could be due to the difference in the analyzed samples (serum or plasma) since it is known that the highest sHLA-G levels are recovered from plasma samples compared with serum collected from the same subjects because of a trapping phenomenon during clot formation that could subtract sHLA-G from the serum[79]. As a proof, Monsiváis-Urenda *et al*[80] evidenced a diminished expression of HLA-G in monocytes and in mature CD83 positive DCs from SLE patients compared with healthy controls. In addition, monocytes from SLE patients showed a decreased induction of HLA-G expression in response to IL-10. Finally, lymphocytes from SLE patients displayed a lower acquisition of HLA-G (by trogocytosis) from autologous monocytes compared to controls. Interestingly, ILT-2 receptor expression is increased on lymphocytes from SLE patients, in particular, in CD3 positive cells, CD19 positive cells, CD56 positive cells and related to IL-10 and anti-DNA antibodies[78]. These results confirm the presence of a HLA-G impaired expression in patients with SLE and a possible role in the pathogenesis. Using a SNP mapping approach, HLA-G gene is reported to be a novel independent locus with SLE interaction[81]. In particular, HLA-G 14 bp INS/DEL polymorphism and HLA-G +3142 C > G SNP were analyzed in a SLE population. SLE patients showed a higher frequency of 14 bp INS allele and 14 bp INS/INS genotype[66]. Moreover, 14 bp INS/INS patients presented the highest disease activity[82]. On the contrary, the evaluation of HLA-G 14 bp INS/DEL polymorphism in a SLE Brazilian population failed to present an association[83], while the +3142 G allele was found to be associated with SLE susceptibility[84]. The +3142 G allele and the +3142 GG genotype frequencies are increased among SLE patients compared with controls[85]. These data support the role of HLA-G molecules in the control of the SLE condition and in particular several results sustain the lower HLA-G expression as a risk factor for SLE development.

HLA-G impact in central nervous system inflammatory diseases

Multiple sclerosis (MS) (OMIM, #126200) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) with unknown etiology that is widely considered to be autoimmune in nature[86]. The presence in CSF of detectable sHLA-G levels in relapsing-remitting MS (RRMS) patients and, occasionally, in other inflammatory neurological disorders and non-inflammatory neurological disorders was reported for the first time by Fainardi and coauthors[87]. In addition, sHLA-G levels in CSF are higher in RRMS than in controls and increased, in association with IL-10 values, in RRMS patients without than in those with magnetic resonance imaging (MRI) evidence of disease activity[88]. The importance of sHLA-G level evaluation as a biomarker for MS is confirmed[89]. Of note, in RRMS

patients, CSF concentrations of sHLA-G and IL-10 are positively correlated with inactive MRI disease and CSF IL-10 titers are more elevated in patients with than in those without CSF measurable levels of sHLA-G. These data suggest that CSF sHLA-G levels may modulate MS disease activity acting as anti-inflammatory molecules under the control of IL-10 CSF levels which may enhance sHLA-G production together with the influence due to HLA-G polymorphisms[67]. The existence of high CSF concentrations of sHLA-G in MS patients and their association with clinical and MRI stable disease have been repeatedly confirmed in subsequent investigations in which: (1) An intrathecal production of sHLA-G is more frequent in MS than in inflammatory and non-inflammatory controls and predominated in clinically and MRI inactive compared to clinically and MRI active MS[88]; (2) sHLA-G concentrations reciprocally fluctuate in CSF and serum of MS patients because they are decreased in the serum of clinically stable MS and increased in CSF of MRI inactive MS[65]; (3) CSF levels of HLA-G5 and not those of sHLA-G1 isoforms are increased in MS compared to controls and in MS patients without MRI appearance of disease activity than in those with MRI Gd-enhancing lesions[90]; and (4) CSF values of sHLA-G and antiapoptotic sFas molecules are inversely correlated in MS patients with no evidence of MRI disease activity since CSF concentrations of sFas are lower in MS than in controls and in MRI inactive than in MRI active MS[90]. Interestingly, HLA-G and its inhibitory receptors (ILT-2 and ILT-4) are strongly up-regulated within and around MS lesions where microglia, macrophages and endothelial cells are recognized as the cellular sources[91]. Furthermore, protein HLA-G expression is higher on cultured human MS microglial cells after activation with Th1 proinflammatory cytokines and a novel subpopulation of naturally occurring CD4 positive and CD8 positive Treg cells expressing HLA-G (HLA-Gpos Treg) has been recently described in peripheral blood of MS patients with relapse[92].

Further studies demonstrated that IL-10 contributes to mediating the suppressive activity of CD4 positive HLA-G^{pos} T_{reg}[93] which are highly represented in CSF and inflammatory brain lesions of MS patients as activated central memory T cells capable of migrating from the periphery to intrathecal compartment due to the expression of CCR5[94]. These results strengthen the assumption of an association between HLA-G antigens and MS.

Collectively, these observations provide evidence that HLA-G antigens are likely to be involved in the resolution of MS autoimmunity acting as anti-inflammatory molecules and suggest that HLA-G positive Treg could play a role in the development of a CNS immunosuppressive microenvironment at the sites of inflammation in MS.

HLA-G impact in other inflammatory and autoimmune diseases

HLA-G proves to also be an important biological marker in other pathologies, for example, gastrointestinal and allergic diseases and diabetes.

Inflammatory bowel disease (OMIM, #266600) is the general term for CD and ulcerative colitis (UC), two chronic inflammatory disorders of the intestine which have different clinical, morphological and immunological characteristics.

Torres *et al*[95] studied intestinal samples of UC and CD patients and, by using an immunohistochemistry technique, demonstrated that while UC intestinal cells presented with HLA-G on their surface, CD intestinal biopsies did not. This result combined with high levels of IL-10 found in the lamina propria of the colon of UC patients suggested that HLA-G can regulate the mucosal immune responses in UC. The distribution of the 14 bp INS/DEL polymorphism in UC and CD was investigated by Glas *et al*[96]. They observed an increase of both 14 bp DEL/INS and 14 bp INS/INS genotypes and a consequent decrease of the high producer genotype (14 bp DEL/DEL) in UC subjects in comparison with CD patients. Also, Rizzo *et al*[97] found a different HLA-G expression in UC and CD patients. Non activated peripheral blood mononuclear cells from CD patients spontaneously secrete sHLA-G, while those from UC patients and healthy donors do not. Furthermore, after stimulation with LPS, both cells from CD and healthy subjects show sHLA-G production, while this does not happen in UC patients. This defective production in UC patients seems to be due to an altered secretion of IL-10 in response to inflammation. The different HLA-G expression profiles in UC and CD patients sustain the different etiopathogenesis at the origin of these two diseases. This hypothesis is sustained by the different modulation of HLA-G observed in the two pathologies after therapy[98]. On the basis of this evidence, it is possible to propose sHLA-G and IL-10 levels as diagnostic parameters to facilitate the diagnosis of UC and CD patients.

Asthma (OMIM, #600807) is a chronic disease affecting approximately 300 million people worldwide, with 180000 deaths resulting annually from severe asthma attacks. Asthma is characterized by chronic inflammation in the airway, which consequently narrows more easily in response to a variety of triggers than the airway of a healthy individual. Nicolae *et al*[99] suggested the role of HLA-G as a potential asthma and bronchial hyperresponsiveness (BHR) susceptibility gene. In particular, susceptibility varies depending on whether the mother has asthma or BHR. A G/G genotype at SNP -964G/A in the promoter region was associated with asthma in the offspring of mothers with either asthma or BHR, whereas the A/A genotype was associated with asthma in the offspring of asthma- and BHR-free mothers. Tan *et al*[33] discovered an association between +3142 C > G (rs1063320) and asthma. HLA-G5 is expressed by airway epithelium and is present in the bronchoalveolar lavage fluid from asthmatic patients[100,101]. In addition to the local presence in airways, sHLA-G may also be found in asthmatic subjects outside the lung. The plasma sHLA-G

levels are higher in atopic asthmatic children than in both non-atopic, asthmatic and non-atopic, non-asthmatic children[101]. The 14 bp INS/DEL polymorphism has no impact on plasma sHLA-G levels in the atopic, asthmatic children. Thus, circulating HLA-G may be important as a biomarker and could potentially modulate immune function more broadly, while the local abundance in airways may have a more direct relationship with immune modulation in the mucosa. There is also *in vitro* evidence that the presence of HLA-G may be different in an asthma condition in comparison with physiological status. sHLA-G expression by peripheral blood mononuclear cells is reduced in asthmatic patients[102] while it is increased in asthma induced by isocyanates[103]. This different behavior may represent differences in biological roles in different disease contexts. A loss of HLA-G could reduce immunosuppression and perpetuate inflammation, whereas increased HLA-G in asthma could be an attempt to reassert immunosuppression. Interestingly, HLA-G is differentially expressed during the lung development[104], suggesting a potential role in lung inflammation induction and chronicization.

Allergic rhinitis (AR) (OMIM, #607154) is characterized by a Th2 polarized immune response. sHLA-G molecules are increased in sera of patients with pollen-induced AR studied outside the pollen season[105], during the pollen season[106] and in perennial AR patients[107]. Interestingly, sublingual immunotherapy (SLIT) for AR is able to reduce sHLA-G serum levels in pollen allergic patients[108,109], suggesting a clinical implication as a biomarker of response to SLIT. Interestingly, children with AR have significantly higher levels of sHLA-G molecules than normal controls or children with allergic asthma[110].

HLA-G impact in pathological pregnancies

During human pregnancy, the maternal immune system recognizes and eliminates alloantigens derived from bacteria or virus, but it tolerates genetically different fetal cells, especially extravillous trophoblast cells invading the maternal decidua or entering the spiral arteries. The expression of HLA-G antigens by trophoblasts is of major importance in protecting the fetus from the semiallogeneic response of the mother[111].

The lack of an established immunological tolerance in pregnancy results in an immune response against paternal antigens expressed by the fetus at the placenta, causing severe health problems for both the fetus and the mother. Complications during pregnancy may affect the woman, the fetus, or both. Miscarriage, RSA and pre-eclampsia account for the most frequent pregnancy complications[112] and the dysregulation of the immunological control at the fetal-maternal interface seems to play a role in these pregnancy complications.

Interestingly, there is a reduced expression of both HLA-G mRNA and protein in pathological compared with control placentas[46-48,113]. In pregnant women, there is a peak of sHLA-G levels in plasma samples in the first trimester that is not evidenced in complicated pregnancies[114,115]. In particular, pregnant women with low sHLA-G plasma levels are characterized by a relative risk of 7.12 of developing placental abruption[116].

The lower secretion of HLA-G by maternal immune cells seems to be in part influenced by HLA-G gene polymorphisms, affecting mRNA stability. In particular, the HLA-G 14 bp ins allele decreases mRNA stability[29,117] and protein production[30,39,118-120]. The HLA-G 14 bp INS/DEL polymorphism seems to affect the fetal HLA-G expression as independent studies have reported fetuses carrying the homozygous genotype for the 14 bp INS allele with a significantly increased risk of pre-eclampsia[121-124]. In addition, the 5' URR seems to be implicated in pathological pregnancies[125]. The confirmed role of HLA-G molecules during pregnancy suggests a potential use in clinical practice. Most pregnancy complications are controversial in terms of diagnosis and treatment. As an example, pre-eclampsia can mimic and be confused with many other diseases and none of the signs are specific. The lower levels of sHLA-G detected in maternal plasma and the HLA-G polymorphism association could assist clinicians in an accurate and reliable diagnosis. Moreover, the HLA-G genetic background of the mother could be an a priori sign of an increased risk of complication during pregnancy. These women could be identified and proposed for a stricter follow-up. It is noteworthy that with an appropriate and timely treatment, the success rate is approximately 80%. Therefore, the use of HLA-G as a biological and genetic marker could improve the management of pregnant women. Moreover, the ability to control HLA-G expression in pathological pregnancies and in women with a high risk of pregnancy complications and infertility could be a tool to cure and prevent these conditions with a deep impact, not only for the individual but also for society.

Until now, more than 15000 embryo culture supernatants have been evaluated for sHLA-G expression, with a positive correlation with embryo implantation rate and pregnancy outcome[126]. However further research is needed to investigate HLA-G in assisted reproductive technologies, but recent studies suggest that sHLA-G is a good candidate as a valuable non-invasive embryo marker to improve pregnancy outcome[127]. Three aspects should be taken into consideration: (1) The recognition of a common sHLA-G detection protocol; (2) The necessity to identify a standardized range for positivity; and (3) The comprehension of the factors involved in the differential expression of sHLA-G between equal stage embryos originating from the same woman.

HLA-G impact in tumors

A high frequency of HLA-G surface expression and increased sHLA-G serum levels has been detected in both hematological and solid tumors. HLA-G and sHLA-G expression correlates with a poor clinical outcome in tumor patients, suggesting a role in the immune escape mechanism of tumors. The frequency of HLA-G expression varies between different types of cancer and even between

different studies in the same type of tumor, probably due to the criteria of patient selection and the methodology used. In hematological malignancies, HLA-G expression was documented with a higher frequency in acute myeloid leukemia cases[128], B and T acute lymphoid leukemia and chronic B lymphocyte leukemia[1,129].

HLA-G expression is frequent in choriocarcinoma[45,130,131], breast[132-135], endometrial[136], and ovarian cancers[137]. In digestive tumors, HLA-G expression was described in esophageal squamous cell carcinoma[138], colorectal cancer[139,140], gastric cancer[19], and liver cancer[141]. In relation to increased membrane HLA-G expression in cancer, higher circulating sHLA-G concentrations were described in patients suffering from different types of cancer[142,143].

These data suggest that HLA-G levels might be used as a diagnostic tool to distinguish between malignant and benign tumors and during disease follow-up. Moreover, HLA-G might serve as a possible marker for tumor sensitivity to chemotherapy and as a prognostic marker for advanced disease stage and clinical outcome. HLA-G assay, either in biological fluids or in biopsies, may have a clinical value in diagnosis, staging, or prognosis of cancer, but prospective validation studies should be conducted in order to use it as a biomarker.

Indeed, it would be important to suppress its immune-suppressive expression in cancer. HLA-G blockade in those tumors that express it remains an attractive therapeutic strategy against cancer. Targeting HLA-G-expressing cancer cells would be also important for maximize the efficacy of anticancer therapies. An experimental approach to target HLA-G-expressing cells in a renal cell carcinoma model was the use of HLA-G- derived peptides based on the binding motif to the HLA-A24[144]. HLA-G peptides induced a cytotoxic attack against HLA-G-expressing HLA-A24 tumor cells, suggesting that HLA-G-mediated suppression can be overcome using peptide-derived immunotherapy.

HLA-G impact in viral infections

Host immune defence mechanisms are efficient at eliminating most viral infections. However, some viruses have developed multiple strategies for subverting host immune defences, thus facilitating their spread in the host[145]. Virus-infected cells are protected against attack by NK cells by HLA-G, providing a long-term immunosuppression function. It may be, therefore, that the diminished immune function induced by HLA-G in the host sometimes leads to an advantage for virus progression by helping viruses subvert the host's antiviral defences[146].

Human immunodeficiency virus type 1 (HIV-1) infection is associated with severe and progressive loss of the immune function in infected persons. It is known that HIV-1 protects infected cells from T lymphocytes and NK cell recognition and lyses by classical HLA-A and B down-regulation and non-classical HLA-G molecule up-regulation, respectively. Since the immunoregulatory ability of HLA-G has become known, the involvement of this molecule in the progression of HIV-1 infection has been widely examined. Studies have focused on the expression of HLA-G in monocytes, which are relevant as reservoirs of HIV-1, and in lymphocytes, which are more susceptible to be infected by HIV-1. Monocytes obtained from HIV-1 seropositive patients expressed HLA-G, although only a small proportion of healthy individuals express this molecule[147]. This might be a consequence of highly active antiretroviral therapy (HAART) since a greater proportion of monocytes expressing HLA-G was observed in patients undergoing HAART compared to untreated[148]. T cells obtained from HIV-1 seropositive individuals were found to express HLA-G at a higher proportion[149] and behave as HLA-G$^+$ Treg.

Human cytomegalovirus (HCMV) is a herpes virus causing widespread, persistent human infection in a delicate balance between the progression of the virus and the defences of the host[150]. HCMV has evolved a number of independent strategies to evade the immune system. HLA-G is produced during viral reactivation in macrophages and astrocytoma cells[56] and the percentage of HLA-G-positive monocytes and sHLA-G levels in patients with active HCMV infection were both dramatically higher than in healthy individuals[151]. The up-regulation observed in HLA-G is probably related to a virus-encoded homologue of human IL-10 (cmvIL-10)[151], which prevents NK cell recognition of infected cells.

Evidence also supports a role of HLA-G in human papilloma virus (HPV) infections. In fact, HLA-G may play a role in mediating HPV infection risk[152] and facilitate cervical cancer development[153].

The ability of specific neurotropic viruses to induce the formation of HLA-G in infected neurons, thus conferring protection against NK cells, was demonstrated. For example, herpes simplex virus-1 and Rhabdovirus[154], trigger the expression and up-regulation of membrane and soluble HLA-G molecules in actively infected neurons.

There is also some evidence that HCV and HBV viruses use HLA-G as a strategy to evade the immune response[155-158].

In summary, one of the main mechanisms of virus evasion is the induction of changes in levels of the classical HLA-G proteins. This enables the virus to prevent infected cells from being recognized and attacked by CTL and NK cells. The main challenge would be to block HLA-G up-modulation by viral infection in order to allow the recognition by immune cells.

CONCLUSION

This review has underlined the importance of HLA-G molecules in pathological conditions.

The literature data suggest that HLA-G could be implicated in both risk and disease chronicization where this antigen is characterized by an impaired expression depending on the different disease environment.

In fact, HLA-G proteins seem to be involved in the

Table 1 Summary of the main studies on human leukocyte antigen G and pathological conditions

Topics	HLA-G genetics and polymorphism	Protein	Ref.
Autoimmune and inflammatory pathologies			
Rheumatoid arthritis		Lower plasma sHLA-G levels than in controls	71
		Higher sHLA-G levels in the synovia	73
		Plasma level of sHLA-G correlates with disease activity parameters	2
	Increase in 14 bp DEL/DEL genotype frequency in responsive patients to MTX treatment		41
	Increase in 14 bp DEL allele frequency in patients with improved disease status		2
Systemic lupus erythematosus		Higher level of sHLA-G and IL-10 in plasma that in controls	77,78
	Higher frequency of 14 bp INS allele and 14 bp INS/INS genotype than in controls	Lower concentration of sHLA-G in serum than in controls	66
		Decrease in HLA-G expression in monocytes and DCs	80
	14 bp INS/INS genotype is associated to the highest disease activity		82
	+3142 G allele and +3142 GG genotype are more frequent in SLE and associated to SLE susceptibility		84,85
Multiple sclerosis		sHLA-G levels in MS CSF are higher than in controls	87,88
	sHLA-G levels in MS could be influenced by HLA-G 14 bp and +3142 C < G polymorphisms		67
		sHLA-G level are increased in serum of CFS of MRI inactive MS	65,90
		HLA-G expression in monocytes is lower than in controls	91
		Presence of HLA-Gpos Treg cells in peripherial blood	92
Inflammatory bowel disease Crohn's disease and ulcerative colitis		HLA-G is present on UC intestinal cells but not in CD biopsies	95
	14 bp INS/DEL and 14 bp INS/INS are increased in UC in comparison with CD patients		96
		PBMCs from CD patients secrete spontaneously sHLA-G	97
		Different modulation of HLA-G by therapy in UC and CD	98
Asthma	-964 G < A and +3142 C < G SNPs are associated with asthma	Expression of HLA-G in airway epithelium and airway system	99,100
		sHLA-G plasma levels are higher in atopic asthmatic children	101
		sHLA-G secretion is increased in asthma induced by isocyanates	103
Allergic rhinitis		Higher sHLA-G serum levels than controls	106-110
Pathological pregnancy		Decreased HLA-G expression in placenta than uncomplicated pregnancies	46-48,112,113
Pre-eclampsia	Increased 14 bp INS/INS genotype frequency than uncomplicated pregnancies		120-125
Tumors		Increased HLA-G expression in tumor cells	1,45,128-143
		Higher sHLA-G serum levels than controls	142
Viral infection		Increased HLA-G expression in viral infected cells	145
HIV-1		Increased HLA-G expression in infected monocytes and T cells	147
HCMV		Increased HLA-G expression in infected monocytes	56
		Increased sHLA-G serum levels than controls	151

sHLA-G: Soluble human leukocyte antigen G; IL-10: Interleukine-10; SNPs: Single nucleotide polymorphisms; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; MS: Multiple sclerosis; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; AR: Allergic rhinitis; HCMV: Human cytomegalovirus; HIV-1: Human immunodeficiency virus-1; MRI: Magnetic resonance imaging; HLA-G: Human leukocyte antigen G; INS/DEL: Insertion/deletion; CFS: Chronic fatigue syndrome.

regulation of the immune system during autoimmune and allergic conditions, such as gastrointestinal, skin, neurological, rheumatic diseases, in pathological pregnancies and in the immune escape mechanisms during viral infections and tumor transformation. In particular, in these disorders, HLA-G proteins could directly interact with immune cells or control the balance between Th1 and Th2 cytokines. In fact, a disequilibrium in this setting would maintain an inflammatory and immune-deregulated condition.

The comprehension of the specific role and mechanisms of action of HLA-G antigens in the development and progression of inflammatory and autoimmune disorders could justify the use of HLA-G molecules as a marker of inflammation and drug treatment and open up new therapeutic perspectives. Moreover, the definition of the role of HLA-G genetic polymorphisms as risk and pharmacogenetic markers could sustain the clinical relevance of HLA-G typing in the laboratory routine. In particular, the possibility to use simple, non-invasive and standardized tools for HLA-G analysis makes it quickly transferable to the health care system practice. These could help in pathology outcome prediction and support treatment decisions.

As reported in Table 1, there are still contrasting results that need to be taken into consideration. The present challenge is to confirm whether HLA-G molecules have a potential role in prevention and diagnosis of pathological conditions. The perspective to identify pharmacological strategies to control the HLA-G production would represent a concrete possibility to improve the control of inflammation and to guide the therapeutic approach. In fact, the possible use of HLA-G as a therapeutic target is of extreme interest.

The ability to modulate HLA-G molecules on the cell surface and to administer HLA-G molecules[74] seems to be at the basis of these cell therapies, suggesting the importance of further studies on HLA-G role in pathological conditions and the possibility of having a controlled modification of the HLA-G level according to disease status and pregnancy complications.

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MINIREVIEWS

Molecular biomarkers for grass pollen immunotherapy

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Abstract

Grass pollen allergy represents a significant cause of allergic morbidity worldwide. Component-resolved diagnosis biomarkers are increasingly used in allergy practice in order to evaluate the sensitization to grass pollen allergens, allowing the clinician to confirm genuine sensitization to the corresponding allergen plant sources and supporting an accurate prescription of allergy immunotherapy (AIT), an important approach in many regions of the world with great plant biodiversity and/or where pollen seasons may overlap. The search for candidate predictive biomarkers for grass pollen immunotherapy (tolerogenic dendritic cells and regulatory T cells biomarkers, serum blocking antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers) opens new opportunities for the early detection of clinical responders for AIT, for the follow-up of these patients and for the development of new allergy vaccines.

Key words: Biomarkers; Molecular allergy; Grass pollen immunotherapy

Core tip: A concomitant approach of the component-resolved diagnosis biomarkers used to guide prescription of grass pollen immunotherapy, particularly important in regions of the world where grass pollen seasons temporal overlap with other types of pollen, together with candidate predictive biomarkers of clinical efficacy for this type of immunotherapy, classified as tolerogenic dendritic cells and regulatory T cells biomarkers, antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers, represents a methodological original presentation with an important educational role in the field molecular allergy considered imperative for clinical practice.

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INTRODUCTION

Molecular biomarkers are indicators of biological or pathogenic processes, or reponses to therapeutic interventions, which possess properties that allow their objective (reliable and accurate) measurements in biological samples, and include nucleic acid-based biomarkers, gene expression products, metabolites, polysaccharides and other molecules. These non-imaging markers, with an important role in the development of personalized medicine, can be classified (Table 1) into disease-related and therapy-related biomarkers[1-11]. Such biomarkers can be exploratory, probably valid or valid, according to differences in their scientific proposals, consensus in the medical community and acceptance by regulatory agencies.

The role of biomarkers has become increasingly important in molecular diagnostics and in guiding decisions related to drug development, clinical trials and modern

Table 1 General classification of molecular biomarkers[1-11]

Biomarkers	Definitions, comments
Disease-related genomic and proteomic biomarkers	
Disease risk biomarkers	Biomarkers associated with the risk of a disease
Diagnostic biomarkers	Indicators of the presence of a disease in an individual, including molecular diagnosis, early disease detection and screening biomarkers
Disease staging biomarkers	Biomarkers for assessing disease severity
Disease prognostic biomarkers	Indicators of the likely course/outcome of a disease for an individual; originally defined as markers that indicate the likely natural course of a disease in an untreated individual, also used to define the baseline risk that suggest the likely outcome of a disease independent of treatment
Drug-related biomarkers (provide information about a patient's response to a therapeutic intervention)	
Pharmacogenomic biomarkers	Defining a DNA or RNA characteristic that is indicator of a response to a therapeutic intervention, facilitate the combination of therapeutics with diagnostics through pharmacogenetics (the study of genetic influence on drug response) and pharmacogenomics (the study of how genomic variation influences drug response)
Proof-of-mechanism biomarkers	Assess, in clinical trials, whether a drug has impacted its target
Drug activity biomarkers	Track the effect of a therapeutic intervention in accordance with its mechanism of action
Pharmacodynamic biomarkers	Measure the effect of a drug on the disease and determine the most effective dose for the patient, as efficacy biomarkers
Toxicity biomarkers	Determine the underlying susceptibility of a patient for a particular side effect or group of side effects
Surrogate biomarkers	Intended to substitute a clinical endpoint in clinical trials and expected to predict clinical benefit
Integral biomarkers	Used in clinical trials for eligibility, stratification, or treatment assignment
Integrated biomarkers	Intended to be used in clinical trials for hypothesis generation or testing, without impact on the treatment
Predictive biomarkers	Pretreatment or baseline measurements used to predict the patient response to a particular treatment

personalized therapy. Significant progress has been made in the scientific research of oncology and neurological biomarkers, and also in the field of inflammatory and immunological biomarkers[12-14].

Allergen-driven inflammation is the key pathogenic mechanism in respiratory allergies. Standard treatments, such as receptor agonists (glucocorticosteroids, beta$_2$-agonists), inverse agonists or antagonists (nonsedating H_1 antihistamines, $CysLT_1$ leukotriene receptor antagonists) are used to treat symptoms, without eliminating the cause of allergy. Because conventional pharmacotherapy fails to restore dysregulated immune responses and, in some patients, to totally control clinical manifestations of allergy, there is a need for new treatment strategies. Although therapeutic tools for manipulation of gene expression in allergic diseases has received increased attention in the emerging era of functional genomics[15], only allergy immunotherapy (AIT) that aims to induce immune tolerance to allergens has reached a good level of robustness as an evidence-based therapy and is currently the only treatment with long-lasting clinical effects with the potential to modify the natural course of the disease. For allergic rhinitis and asthma, AIT is effective in reducing symptom scores and medication use, improving quality of life, and inducing favorable changes in specific immunological markers[16]. The diagnosis of respiratory allergy is usually based on skin prick tests and/or the measurement of allergen-specific IgE in serum. Currently, two types of AIT are in clinical practice: subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT). SLIT is a valid non-invasive and better tolerated alternative to SCIT. Special indications of this local type of immunotherapy exist in patients uncontrolled with optimal pharmacotherapy, in whom pharmacotherapy induces undesirable side effects, those who do not want to be on long-term pharmacotherapy or refusing injections[17,18]. SLIT tablets, with the convenience of self-administration, fulfill the requirements from the regulatory agencies that make mandatory pharmaceutical quality and are authorized as drugs available for grass pollen allergy[19]. Intranasal and intrabronchial immunotherapies are not commonly used because of administration-associated local symptoms[16]. New routes for grass AIT are under evaluation in clinical trials (intralymphatic into inguinal lymph node under ultrasound control, epicutaneous *via* patch type epidermal delivery system)[20,21]. Second generation AIT vaccines based upon recombinant allergens (combined with mucoadhesive vector systems in sublingual products) are being developed as an alternative to conventional allergen extracts[22]. A mixture of different wild-type recombinant grass-specific allergen components of Timothy grass, adsorbed onto aluminium hydroxide, was studied as SCIT in grass pollen allergy, some of them being strong candidates for use as therapeutic vaccines[23,24]. Recombinant allergens for AIT aim to overcome the problems of natural extracts as they can be produced in unlimited amounts with exact physicochemical and immunological properties[25].

Currently, molecular diagnostic biomarkers can be used to guide AIT in the frame of component-resolved management of allergic diseases[26]. Identification and validation of biomarkers that are predictive of AIT clinical response are still unmet needs[16]. Recent advances in molecular biotechnology are destined to revolutionize immunotherapy treatments[27].

The major global health problem represented by respiratory allergies is due to their high prevalence, significant influence on quality of life and strong impact on work and school performance, productivity and economic burden. Allergic rhinitis is estimated to affect some 1.4 billion people globally and asthma is estimated to affect 300 million individuals worldwide. Respiratory allergies

affect all age groups and frequently coexist in the same subjects[28-31].

Pollen allergy is a public health threat of pandemic proportions. The most common outdoor allergens responsible for respiratory allergies are the pollen grains of anemophilous plants (wind-pollinated plants), such as of grasses, trees and weeds, each with specific seasons. Exposure to pollen grains depends of the plant type, wild spreading or cultivation, geographic area, altitude, air currents, temperature, precipitation and other weather events. Grass pollen is an important cause of pollinosis with a remarkable clinical impact all over the world. Its frequency differs regionally, but in many parts of the world, grass-induced respiratory allergy is the most common pollen allergy[27,32,33].

In the search for genomic biomarkers, some researchers tried to identify genetic variants associated with pollen sensitization. In studies performed more than a decade ago, susceptibility to grass allergy was associated with an increased frequency of HLA-DQB1*0301 when compared with the control population[34], while by both non-parametric and parametric statistical methods, scientists found significant associations between specific IgE to ryegrass group 1 and 2 allergens with HLA-DR3[35] and specific IgE to ryegrass group 3 allergens with HLA-DR3 and DR5[36]. A recent genome-wide meta-analysis revealed genetic variants associated with grass pollen sensitization in European adults. The HLA variant rs7775228 (6p21.32), which *cis*-regulates HLA-DRB4, was strongly associated with grass sensitization ($p_{grass} = 1.6 \times 10^{-9}$). Single nucleotide polymorphism (SNP) rs2155219, located at 11q13.5, upstream of chromosome 11 open reading frame 30 and downstream of leucine-rich repeat containing 32, was also strongly and consistently associated ($p_{grass} = 9.4 \times 10^{-9}$). The third-strongest association ($p_{grass} = 1.2 \times 10^{-8}$) was for rs17513503 located at the 5q22.1 locus near transmembrane protein 232 and solute carrier family 25, member 46. SNP rs1898671 from thymic stromal lymphopoietin gene showed weak association with grass sensitization ($p_{grass} = 9 \times 10^{-3}$)[37]. In a Japanese study on matrix metalloproteinase 9 gene SNPs and pollen allergy in children, a haplotype associated with -1590T and 668Q revealed a significant association with cedar pollinosis and orchard grass pollinosis (corrected $p = 0.0012$ and $p = 0.0059$, respectively)[38]. Although findings from such studies could enhance the understanding of immunological mechanisms involved in the pathogenesis of pollen allergy, with possible implications for prevention and treatment, additional scientific data are needed to evaluate genetic determinants, not only for IgE sensitization, but also for potential circulating biomarkers.

Currently, component-resolved diagnosis (CRD) biomarkers can be used to evaluate sensitization to grass pollen allergens. In patients with multi-sensitization, sensitization to cross-reactive panallergen biomarkers, specific IgE to profilins and/or polcalcins, may reduce the anticipated response to pollen AIT. In patients with mono-/oligo-sensitization profiles, major species-specific non-glycosylated allergen biomarkers, specific IgE to *Poaceae*- and *Pooideae*-specific molecules, suggest suitability for AIT[26].

A better understanding of the AIT mechanisms of action to induce peripheral tolerance to allergens is useful to identify proper candidate predictive biomarkers for AIT efficacy: biomarkers of tolerogenic dendritic cells (DCs), T cell biomarkers, antibody biomarkers, immune activation and immune tolerance soluble biomarkers, and apoptosis biomarkers[39-44].

Molecular biomarkers for grass pollen immunotherapy are summarized in Table 2.

COMPONENT-RESOLVED DIAGNOSTIC BIOMARKERS

Used to guide prescription of grass pollen immunotherapy

Recognition of disease-causing allergen components involved in pollen allergy, using the specific IgE against recombinant allergen components as molecular biomarkers, is of utmost importance, especially in patients with multiple sensitizations to different pollen types from plants, with total or partial, temporal and spatial overlap of significant airborne pollen concentration periods. This is particularly imperative in patients with a clinical suboptimally informative history, in regions of the world with great anemophilous plant biodiversity and/or areas where unrelated plants have pollination seasons which are at least partially concomitant in some months of the year[45]. Retrospective symptom assessment is not a reliable method as grass pollen symptoms interfere with the recollection of symptoms induced by other pollen[46].

There is general consensus that AIT should be indicated in patients presenting with established clinical relevance for an allergen source. When seasonal symptoms point to grass pollen allergy, *in vivo* and/or *in vitro* testing typically confirm the presence of specific IgE to this type of pollen. In cases of IgE-sensitization to more than one pollen source from grasses, trees or weeds, it is essential to identify the clinically significant pollen types and exclude any source that may appear involved due to cross-reactivity, thus misrecognizing the primary sensitizing source, and compromising the expected immunological responses to AIT[26].

Grasses are universally distributed. Grass pollen grains are produced by wild or cultivated herbaceous plants (Table 3) belonging to *Liliopsida* class, *Poales* order, *Poaceae* family (*Gramineae*).

The most abundant allergenic grass pollen in many temperate regions originates from tall grasses (up to 1.4 m tall), such as *Phleum pratense*, *Dactylis glomerata* and *Arrhenatherum elatius*. Cultivated rye also has a remarkably high pollen production. Allergenic cross-reactivity between the members of the *Pooideae* subfamily grasses of temperate regions (*Lolium perenne, Phleum pratense, Poa pratensis*) is extensive, but it is limited with other tropical or

Table 2 Molecular biomarkers summarized for grass pollen allergy immunotherapy[26,39-44]

Biomarkers	Description, comments
CRD biomarkers used to guide AIT prescription	
Serum specific IgE antibodies to rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6	Molecular specific biomarkers of genuine sensitization to *Poaceae* grass pollen
Serum specific IgE antibodies to nCyn d 1	Molecular specific biomarkers of genuine sensitization to *Chloridoideae* grass pollen
Serum specific IgE antibodies to CCDs	Molecular biomarkers of sensitization to CCDs involved in specific IgE assays cross-reactivity
Serum specific IgE antibodies to rPhl p 7	Molecular biomarkers of sensitization to pollen polcalcin panallergens cross-reactive with pollen from most plants
Serum specific IgE antibodies to rPhl p 12	Molecular biomarkers of sensitization to pollen profilin panallergens cross-reactive with pollen, some plant-derived foods and latex
Predictive candidate biomarkers of AIT clinical efficacy	
Stabilin-1 (intracellular scavenger receptor), C1Q complement component expression	Intracellular biomarkers of tolerogenic dendritic cells
Coregulatory PD-L1 (B7-H1, CD274) expression	Surface cell biomarker of tolerogenic antigen presenting cells
Peripheral IL-10$^+$Foxp3$^+$ cells proportion among CD25$^+$ CD4$^+$ leukocytes	Regulatory T cell biomarker
Serum allergen-specific IgE to total IgE ratio	Allergen-specific antibodies biomarkers
Serum allergen-specific IgG_4, IgG_1 and IgA_2	
Inhibition of CD23-dependent IgE-FAB to B cells, serum specific IgE-BF competing with IgE for allergen binding	Functional biomarkers of serum IgG-associated inhibitory activity
Serum neopterin and kynurenine-tryptophan ratio	Molecular biomarkers of T cell mediated immune activation
Serum sHLA-G	Non-classical MHC class I immune tolerance molecular biomarker
Serum sTRAIL	TRAIL biomarker

CRD: Component-resolved diagnostic; CCDs: Carbohydrate cross-reactive determinants; IgE-FAB: IgE-facilitated allergen binding; IgE-BF: IgE-blocking factor; sHLA-G: Soluble HLA-G; sTRAIL: Soluble tumor necrosis factor-related apoptosis-inducing ligand; AIT: Allergy immunotherapy; PD-L1: Programmed death ligand-1; MHC: Major histocompatibility complex.

Table 3 Grasses (*Poaceae* family) which are sources of the most allergenic pollen grains[31,32,47]

Subfamily	Tribe	Species (common names)
Pooideae	*Poeae*	*Phleum pratense* (Timothy grass, Herd's grass, meadow cat's-tail)
		Lolium perenne (perennial ryegrass)
		Dactylis glomerata (orchard grass or cock's foot grass)
		Poa pratensis (smooth meadow grass or bluegrass)
		Anthoxanthum odoratum (sweet vernal grass)
		Festuca pratensis syn. *F. elatior* (meadow fescue)
		Holcus lanatus (velvet grass or Yorkshire fog)
		Agrostis capillaris syn. *A. tenuis or A. vulgaris* (bent grass)
		Arrhenatherum elatius syn. *Helictotrichon elatius* (tall oat grass)
		Avena fatua (wild oat)
		Avena sativa (cultivated oat)
	Triticeae	*Hordeum vulgare* (barley)
		Secale cereale (rye)
		Triticum aestivum ssp. *vulgare* (cultivated bread wheat)
	Bromeae	*Bromus inermis* (smooth brome)
Chloridoideae	*Cynodonteae*	*Cynodon dactylon* (Bermuda grass, Bahama grass, Devil grass)
Panicoideae	*Andropogoneae*	*Sorghum halepense* (Johnson grass)
	Paniceae	*Paspalum notatum* (Bahia grass)

subtropical grasses, such as *Cynodon dactylon* and *Paspalum notatum*[32,47,48].

Monitoring pollen in the air, carried out by various gravimetric, impaction and suction sampling devices, may be used for the management of pollen allergy, and for biomedical and biological research. The Hirst trap and later modified Burkard or Lanzoni traps are widely used samplers. Counting and identifying pollen grains is performed by optical microscopy. Pollen calendars are created based on differences in airborne pollen recorded in time[49]. Although pollen is routinely monitored, it is unknown whether pollen counts represent allergen exposure because pollen grains can vary substantially in allergen release, even although they are morphologically identical. There is a switch of importance from pollen count to pollen potency in the modern molecular era of aerobiology[50,51]. Phenological studies reveal that airborne grass pollen results from both local and distant sources, although the pollen airborne concentration peaks usually appear when such local herbaceous plants are shedding the greatest amounts of pollen. Although there is an association between flowering phenology and airborne pollen records for some of the tree and weed pollen types, for *Poaceae* the flowering and airborne pollen peaks usually do not coincide, with up to one week difference in phase[52]. Moreover, diurnal variations, climate and weather changes impact pollen exposure. Meteorological factors (temperature, wind speed, humidity, rain, thunderstorms) along with climatological regimes (warm or cold anomalies, dryer and wetter periods) influence pollen distribution. Human activities increase atmospheric greenhouse gases, such as carbon dioxide, and induce changes in global climate. Over the last decades, high

Table 4 Grass pollen seasons timing and temporal overlap in Europe[32,55-59]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
Northern, Western, Central and Eastern Europe	Grass flowering period starts at the beginning of May, finishes at the end of July Some grass plants are in flower even in August, even September (*e.g.*, Poland) Air concentration of grass pollen usually peaks in June; pollination occurs about two-three weeks earlier at sea level and thereafter in the mountain regions Birch (*Betula* spp): Western Europe flowering period starts at the end of March, Central and eastern Europe from early April until May (2-8 wk), Northern Europe from late April to late May Ash tree (*Fraxinus* spp): In Central and South-Eastern Europe flowering period may partially overlap (April to May) with grass pollen season *Asteraceae* weeds, such as ragweed *Ambrosia artemisiifolia* var. *elatior* and mugwort *Artemisia vulgaris*, pollen season in Central and Eastern Europe may last from July to August-September Plantain *Plantago* spp: Pollen season from May to September in Eastern Europe
Mediterranean regions of Europe	Different grasses are flowering between April and August Olive (*Olea europaea*): Pollen season lasts from April to June, in regions of Greece, Spain, and southern Italy, overlapping grass pollen season Plane (*Platanus* spp): Pollen season partially overlapping with grass season, from April to May, in Southern France or Spain Pellitory *Parietaria* spp: Pollen has a long persistence in the atmosphere in the Mediterranean region, from April to October, even longer (perennial) *Asteraceae* weeds: Pollinate from August to October, similar to *Chenopodiaceae*/*Amaranthaceae* pollen from salt-tolerant weeds significant also for semi-arid areas Plantain *Plantago lanceolata*: Pollen season from April to July in Northern Spain
European islands with special climate characteristics	Iceland (cold-temperate oceanic country): Some grass species and sorrel (*Rumex* spp) flower in June, both with peaks in July; a second peak of grass-pollen is possible in some years in August; pollen season tails off in September; birch pollen season is short, starting in the second part of May until the beginning of June Canary Islands (Spanish archipelago with subtropical climate): Long-range transport of *Poaceae* and *Amarantaceae*/*Chenopodiaceae* pollen from southern Iberian Peninsula and Morocco (mixed with *Oleaceae* tree pollen) and from the African Saharan sector and Sahel

Table 5 Grass pollen seasons timing and temporal overlap in Africa[60-65]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
Extremely variable pollen seasons exist due to great differences in plant distribution	
Morocco (North Africa)	Mediterranean region: grass pollen season starts in April and has highest air pollen concentration in May and June, overlapping olive pollen season Date palm (*Phoenix dactylifera*) from *Arecaceae* family is distributed not only in the Mediterranean areas of Morroco, but also Middle East and central Africa
Nigeria (West Africa)	Southwest region: at the end of the dry season, in March, airborne pollen grains of *Chenopodiaceae*/*Amaranthaceae* and *Poaceae* coexist in significant concentrations
South Africa	Grass pollinating season peaks from October to February (longer grass flowering); in subtropical regions, grasses of the subfamily *Panicoideae* are predominant: *Cenchrus clandestinum* (kikuyu) and *Stenotaphrum secundatum* (buffalo grass) South African *Cupressaceae* (cypress) trees start flowering in June, followed by *Quercus robur* (oak) in late July, *Platanus* (plane) in September and *Olea europaea* subsp. *africana* (olive) in January, and because their pollen season duration lasts three to four months it overlaps the grass pollen season *Plantago lanceolata* (English plantain) and *Chenopodiaceae* (goosefoot) are most important weeds with pollen seasons in late summer and autumn, overlapping with the grass pollen season

temperatures and atmospheric carbon dioxide concentration have impacted plant and pollen distribution and induced changes in quantitative production and dispersion of pollen, pollen seasons and allergen content of pollen grains, which are region and species-specific[32,53,54].

Grass pollen seasons timing and temporal overlap with other types of pollen must be discussed for different regions in the world (Tables 4-8). Diagnostic molecular biomarkers represented by specific IgE against recombinant allergen components, are especially important in patients with multiple sensitizations to different pollen types within this context.

As is inferred from the presented data, grass species produce the only allergenic pollen with ubiquitous representation and clinical significance across the globe. In many regions, grass pollen seasons overlap other pollination periods of other anemophilous plants (trees and weeds); therefore, commercially marketed CRD assays for inhalant sources include grass pollen allergens[26].

Serum levels of specific IgE to recombinant and native allergen components (specific and cross-reactive pollen allergen components) can be measured *in vitro* using two types of tests. Singleplex diagnostic tests (one result for a single serum specimen) are the same immunoassays as those used for the IgE determinations for allergenic extracts, the difference being that the antigen is a highly purified molecule, either natural or recombinant. Multiplex diagnostic tests (several results for a single speci-

Table 6 Grass pollen seasons timing and temporal overlap in Asia[32,66-84]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
Western Asia, Middle East	Most Middle East countries: significant grass and weed pollen seasons during April to May and September to October Desert and semi-desert countries: *Chenopodiaceae* pollen season overlapping with grass pollen season, such as for *Cynodon dactylon* Indigenous trees/shrubs, such as mesquite (*Prosopis juliflora*) and date palm (*Phoenix dactylifera*) pollen seasons from March to May, also in Egypt Israel: *Cupressaceae* (cypress family) and *Poaceae* pollen seasons throughout the year, grasses especially in spring (March-May), cypresses February and April; *Olea europaea* flowering begins in late March till July-August; *Parietaria judaica* pollen highly allergenic in northern Israel Turkey Mediterranean coastal area (Antalya): grass pollen frequently detected between April-May and October-November and found in the atmosphere in high concentrations during May to July; *Pinaceae* pollen airborne between March and June; *Cupressaceae* pollen, in high levels in February, usually present until May; *Chenopodiaceae*/*Amaranthaceae* pollen grains found in air during June to October, all overlapping with the grass pollen season
South Asia	Grass pollen season overlaps with seasons of many other anemophilous plants Pakistan: paper mulberry (*Broussonetia papyrifera*, family *Moraceae*) pollen season from March to April overlaps with the grass pollen season India: spring (February-April), autumn (September-October), winter (November-January) pollen seasons include periods of flowering of grasses (*Cynodon dactylon, Paspalum distichum, Sorghum vulgare, Poa annua*), of weeds *Cannabaceae* (*Cannabis sativa*), *Asteraceae* (*Parthenium, Artemisia* spp), and *Chenopodiaceae* (*Amaranthus*, *Chenopodium* spp), and of trees (*Prosopis juliflora*, *Cocos* and *Eucalyptus* spp)
East Asia	Beijing and different provinces of the People's Republic of China, such as Guangdong, Yunnan and Hebei: pollen season lasting from August to October is due to weed pollen from different plant families, *Chenopodiaceae*, *Asteraceae* (mugwort *Artemisia* spp), *Cannabaceae* (hop *Humulus* spp), but also to grass pollen South Korea: grass pollen airborne between end of April and November, especially Korean lawn grass, Timothy grass, Bermuda grass, and orchard grass; Pollen seasons of trees (pine, birch, oak) and weeds (mugwort, ragweed, Japanese hop) overlap with grass pollination period in the first, respectively last part of it Japan: pollen season for Japanese cypress/hinoki (*Chamaecyparis obtusa*) lasts from March to May, while for orchard grass (*Dactylis glomerata*), in May-June to August, and for weed yomogi *Artemisia*, from August to October Olive pollen from May to June overlaps with orchard grass season in Shodoshima *Plantago lanceolata* pollen dispersed from mid-May to early September, in Sapporo

Table 7 Grass pollen seasons timing and temporal overlap in America[28,85-93]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
United States of America and Canada (North America)	Temperate regions: tree pollen predominates in spring, grasses in late spring and early summer, and weeds from summer until fall, with variable overlap periods Ragweed (*Ambrosia* spp) pollen season starts in July and peaks between August and October, warming by latitude being associated with increased length of pollen season in central North America Mountain cedar (*Juniperus ashei*) pollen season, in Oklahoma, Arkansas, central Texas, lasts from December to February, not overlapping with grass pollen period Southern Texas, on the western Gulf Coast: airborne grass pollen concentrations have two peaks, one in May (due to cool temperate grass species) and one in September and October (due to temperate and subtropical species), long distance dispersal of grass pollen is possible also out of season Subtropical regions, such as Southern California, Florida: grass pollen season dispersed all year overlaps with *Cupressaceae* trees (December through May), *Asteraceae*, *Chenopodiaceae* weeds pollen seasons (usually May through December)
Mexico, Central and South America (Latin America)	Subtropical regions in Mexico: similar to Southern California and Florida Mesquite (*Prosopis* sp) pollen in Northern Mexico and Southwestern United States Tropical regions: grass pollen grains airborne throughout the year, overlapping with the pollination periods of trees, such as *Anacardiaceae*, *Cupressaceae*, and weeds, such as *Amaranthaceae*, *Asteraceae* and *Euphorbiaceae* spp Maule region of Chile: *Platanus acerifolia*, *Olea europaea*, *Cupressus* spp pollen and grass pollen detected in August through November until end of January; *Plantago* spp, *Rumex* and *Chenopodium* spp pollen present from October to April

Table 8 Grass pollen seasons timing and temporal overlap in Australasia[48,94-98]

Regions	Grass pollen seasons timing and temporal overlap with other types of pollen
Australia	Subtropical northern regions (Brisbane, Queensland): grass pollen season, such as for *Paspalum notatum*, *Sorghum halepense* and *Cynodon dactylon*, from summer to autumn months, December to April, overlaps with the pollen season of groundsel bush (*Baccharis halimifolia*, *Asteraceae* family) Oceanic southern regions (Melbourne, Victoria): temperate grasses pollinate especially in spring, from September to November, overlapping with trees *Cupressus* and *Betula* spp pollen season
New Zealand	Temperate grasses form the major component of atmospheric pollen levels during spring and summer (October to February) and *Plantago* spp pollen season overlap

men) are immuno solid-phase allergen chip based on multiplex microarray-based technology, multiparameter immunoblot test system based on single purified allergen components, and a multiplex flow cytometry allergenic molecule-based micro-bead array system[45,99-103].

In contrast to traditional specific IgE biomarkers, CRD in allergy does not rely upon whole extract preparations from native allergen sources, but on quantification of specific IgE antibodies to single protein components, purified from natural sources (native allergen components) or obtained using recombinant techniques (recombinant allergen components). These modern diagnostic biomarkers are useful for a detailed CRD of the sensitization and cross-reactivity profiles, discriminating between clinically significant and irrelevant specific IgE, reduce the need for provocation testing and improve the prescription and specificity of AIT[26,45,104,105].

Molecular specific biomarkers of genuine sensitization to grass pollen

Molecular and biochemical characterization of grass pollen reveals several important specific allergen components. Timothy grass (*Phleum pratense*), also known as Herd's grass, meadow cat's-tail or common cat's tail, belongs to the *Pooideae* subfamily and it is one of the most significant source of grass pollen allergens in temperate regions. Bermuda grass (*Cynodon dactylon*), also known as Scutch grass, Bahama grass, Devil grass, belongs to the *Chloridoideae* subfamily, and it is an important grass which typically grows in warm temperate, subtropical and tropical climates areas of the world.

Specific IgE antibodies to recombinant temperate grass-specific pollen allergen components, rPhl p 1, rPhl p 2, rPhl p 5 and rPhl p 6, are biomarkers of genuine sensitization to *Poaceae* pollen. From references[26,33,106-124], these specific components and correspondent antibody biomarkers are discussed below.

Phl p 1 belongs to the group 1 grass pollen allergens, acidic glycoproteins with molecular mass of 31-35 kDa, a family of major allergens present in all grass species (*Poaceae* family-specific marker). More than 90%-95% of grass pollen allergic patients, adults or children, have specific IgE to group 1 grass pollen allergens. Group 1 grass pollen allergens are glycosylated proteins that show 60%-70% sequence identity to beta-expansin family of cell wall-loosening proteins with a role in pollen tube penetration into the style and pollen tube growth. A major IgE-reactive domain of Phl p 1 exhibits significant sequence identity of 43% with the family of immunoglobulin domain-like group 2/3 grass pollen allergens. Recombinant Phl p 1, rPhl p 1 (27 kDa) is not glycosylated and resembles native Phl p 1 (nPhl p 1) closely binding to IgE in about 90% of patients with grass pollen allergy, revealing that rPhl p 1 shares many of the IgE epitopes with natural grass allergens of the group 1. Sensitization to rPhl 1 seems to appear earlier in life in comparison with other allergen components. Group 1 grass pollen allergens with great sequence identities and homologies include, besides Phl p 1, other important allergen components from important grass pollen grains: *Anthoxanthum odoratum* (Ant o 1), *Dactylis glomerata* (Dac g 1), *Holcus lanatus* (Hol l 1), *Lolium perenne* (Lol p 1), *Poa pratensis* (Poa p 1). There is a partial cross-reactivity between Phl p 1 and Cyn d 1, the group 1 major allergen in Bermuda grass *(Cynodon dactylon)*, thus Phl p 1 is only partially specific for the *Pooideae* grass subfamily.

Phl p 5 is another major allergen from Timothy grass pollen and is one of the most reactive of the group 5 allergens, ribonucleases generally restricted to the *Pooideae* subfamily of grass pollen. Between 65%-90% of grass pollen allergic patients in temperate climate areas are sensitized against group 5 grass pollen allergens components. Grass pollen grains in ambient air is not quantitatively correlated with the airborne Phl p 5 concentration. Rainfall contributes to an increase in respirable particles containing group 5 allergens, which bursts the pollen grains. Moreover, exposure of pollen to gaseous pollutants induces a decrease in Phl p 5 detection in pollen extracts due to a mechanical loss of allergens from the altered pollen grains and/or post-translational modifications, such as ozone acidification. Phl p 5b, a smaller isoform (32 kDa), contains at least one more IgE antibody binding epitope than Phl p 5a isoform. rPhl p 5 is very similar to nPhl p 5 and reacts with serum IgE antibodies in a great part of grass pollen-allergic patients. rPhl p 5 is cross-reactive with similar group 5 allergen components: Dac g 5, Lol p 5, Poa p 5, Ant o 5. Because group 5 allergens are restricted to the *Pooideae* subfamily, there is a limited cross-reactivity between the pollen of temperate-type *Pooideae* subfamily grasses and pollen from warm temperate/subtropical-type grasses belonging to *Chloridoideae* (*Cynodon dactylon*) and *Panicoideae* (*Paspalum notatum*) subfamilies. Common reed (*Phragmites communis*), a grass from the *Arundinoideae* subfamily with a low phylogenetic affinity to *Pooideae* plants, produces pollen in late summer to autumn with a very low degree of cross-reactivity to group 5 allergens. There is a dissociation of the major IgE and T-cell-reactive peptide domains in Phl p 5. Specific IgE antibodies against Phl p 1 and Phl p 5 might be used as a reliable biomarker of allergy to *Poaceae* pollen. These major allergen components are defined on the basis of both frequency (prevalence of specific IgE antibodies) and potency (average level of specific IgE antibodies). Mono-sensitization to rPhl p 1 seems important in patients with lower IgE against Timothy grass pollen extract levels, while sensitization to rPhl p 5 is rarely found as the only sensitizing allergen.

Other grass-specific pollen allergen components must be discussed. IgE to rPhl p 2 (13 kDa) may also be regarded as a fairly specific biomarker for patients sensitized to grass species of the *Pooideae* subfamily. Immunologically significant group 5 and group 2 allergens seem to be absent in non-*Pooideae* grass pollen grains. Phl p 6 (a group 6 acidic, nonglycosylated protein of 15 kDa, for which N-terminal sequencing reveals homology to an internal region of group 5 allergens), along with Phl p 5,

do not exhibit significant serological cross-reactivity to pollen allergens outside the *Pooideae* subfamily. rPhl p 6, with the same reactivity with serum IgE antibodies as the native molecule, can be used for *in vitro* diagnosis of grass pollen allergy.

In conclusion, specific IgE against rPhl p 1 is a *Poaceae* family-specific biomarker for genuine sensitization to grass pollen and specific IgE antibodies against rPhl p 2, rPhl p5 and rPhl p 6 are *Pooideae* subfamily-specific biomarkers for true sensitization to temperate grass pollen. rPhl p 1, rPhl p 5 and natural Timothy extract are used to identify grass pollen allergy. Mono/oligo-sensitized patients with specific IgE to non-glycosylated major species-specific allergen markers (Phl p 1, Phl p 5) are suitable for *Pooideae* grass-specific AIT[26,33,117,123].

Specific IgE antibodies to nCyn d 1, a warm climate grass-specific native pollen allergen component, represent biomarkers of genuine sensitization to *Chloridoideae* subfamily grass pollen, as discussed below[26,125-129]. Cyn d 1 is a major allergen most abundant in Bermuda grass pollen, representing 15% of the whole-pollen extract. The frequency of sensitization to Cyn d 1 in Bermuda grass-allergic individuals is between 76% and 100%. Cyn d 1 belongs to Group 1 grass pollen allergens, including highly cross-reactive pollen allergens from other *Chloridoideae* subfamily grasses, such as Bou g 1 from the pollen of the North American Grama grass (*Bouteloua gracilis*). Cyn d 1 is to some extent immunologically distinct from Phl p 1 from Timothy grass and therefore a suitable marker for sensitization to *Cynodon dactylon*. Partial cross-reactivity between Phl p 1 and Cyn d 1 may impede the identification of the sensitizing allergenic source. When testing for rPhl p 5 as a *Pooideae*-specific molecular biomarker is negative, relatively higher levels of IgE specific to nCyn d 1 than to rPhl p 1 have been suggested to be indicative of primary sensitization to Bermuda grass pollen, an AIT extract containing *Cynodon dactylon* pollen might be suitable. If testing for IgE, anti-rPhl p 5 is positive and specific IgE against nCyn d 1 higher than to rPhl p 1, there is a true double sensitization. Finally, if antibodies against *Pooideae*-specific molecules, such as rPhl p 5, are positive and specific IgE levels against rPhl p 1 have higher levels than those to nCyn d 1, the case is most probably primary sensitization to *Pooideae* grasses and *Cynodon dactylon* pollen representation can be omitted from the AIT regimen.

Specific IgE antibodies to recombinant and native specific allergen components from tree and weed pollen are important to differentiate the true sensitization profile in patients with multiple sensitizations, including grasses, as described below[26,47,129-139]. When testing for these specific pollen components is negative and testing for IgE against specific and cross-reactive grass allergen components, then IgE sensitization is to grass pollen. If testing for IgE against recombinant specific grass pollen components is positive and specific IgE against specific tree or weed components are also significant, the condition is a true double or multiple sensitization.

Tree pollen-specific allergen components are described for the anemophilous plants belonging to the *Betulaceae* family: rBet v 1, a 17 kDa pathogenesis-related protein PR-10 with ribonuclease activity from the pollen of silver birch *Betula pendula* or *Betula verrucosa*, cross-reactive with other *Betulaceae* pollen PR-10 components with about 70% identity to Bet v 1 (black alder *Alnus glutinosa* rAln g 1, hazel *Corylus avellana* rCor a 1.0101); *Oleaceae* family: nOle e 1 and rOle e 1, a 19-20 kDa trypsin inhibitor from the pollen of olive *Olea europaea*; *Platanaceae* family: rPla a 1, a 18 kDa invertase inhibitor, and nPla a 2, a 43 kDa polygalacturonase, from the pollen of plane tree *Platanus acerifolia*; *Cupressaceae* family: nCup a 1, 43 kDa pectate lyase from the pollen of Arizona cypress *Cupressus arizonica*, cross-reactive with other *Cupressaceae* pollen pectate lyase components (Japanese cedar *Cryptomeria japonica* nCry j).

Major native or recombinant weed pollen-specific allergen components are described for herbaceous weeds belonging to the *Asteraceae* (*Compositae*) family: nArt v 1, a 28 kDa defensin from the pollen of mugwort *Artemisia vulgaris* and nAmb a 1, a 38 kDa pectate lyase from the pollen of short ragweed *Ambrosia artemisiifolia* var. *elatior*; family *Plantaginaceae*: rPla l 1, a 17 kDa Ole e 1-like trypsin inhibitor from the pollen of plantain *Plantago lanceolata*; family *Urticaceae*: rPar j 2, a 14 kDa lipid transfer protein, member of the PR-14 protein family, from the pollen of wall pellitory *Parietaria judaica*; family *Amaranthaceae/Chenopodiaceae*: rChe a 1, a 24 kDa trypsin inhibitor from the pollen of goosefoot *Chenopodium album* and nSal k 1, a 43 kDa protein belonging to the pectin methylesterase family from the pollen of saltwort *Salsola kali*.

Molecular biomarkers of sensitization to carbohydrate cross-reactive determinants

Carbohydrate cross-reactive determinants (CCDs) are carbohydrate moieties of glycoproteins that induce the production of highly cross-reactive IgE, as discussed below[26,140-143]. Many allergens are glycoproteins containing carbohydrate moieties called N-glycans or O-glycans, according to their site of attachment to the protein. N-glycans containing beta1,2-xylose and alpha1,3-fucose in many glycoproteins are more extensively studied. Markers of sensitization to CCDs are bromelain (nAna c 2) and MUXF3 (Ana c 2.0101) carbohydrate epitope, the purified N-glycan from *Ananas comosus* bromelain, able to detect IgE to N-glycans in most pollen sources. Anti-CCD IgE biomarkers indicate the presence in serum of IgE directed against carbohydrate epitopes. CCDs rarely cause allergic reactions, but may produce positive *in vitro* test results to CCD-containing allergens from pollen, plant foods, insects and venoms. Patients sensitized to grass pollen develop anti-CCD IgE that also binds to CCD monovalent peanut allergens, but does not induce any clinical symptoms. Approximately 20% of patients with multiple pollen allergies have IgE antibodies to pollen allergens with molecular masses higher than 30 kDa and a great part of their IgE-binding is dependent on CCDs,

a major cause of cross-reactivity for *in vitro* specific IgE assays. If testing for IgE against a specific native allergen component, such as nCyn d 1, is positive, because native components are CCD-containing natural purified glycoproteins, it is necessary to assess the epitope protein nature in multi-sensitized patients. In cases of positive *in vitro* results to a natural allergen component, negative IgE to CCD markers reveal the protein nature of IgE epitopes. Positive IgE to CCD markers should optimally be accompanied by assessment of biological activity, such as positive skin prick testing or nasal/conjunctival challenge with the allergen, important aspects in the AIT decision process.

Molecular biomarkers of sensitization to cross-reactive pollen panallergens

Panallergens, usually classified as minor allergens, are defined as homologous and structurally related proteins belonging to different biological sources and causing IgE cross-reactivity between evolutionary unrelated species. Among panallergen families, only profilins are distributed ubiquitously throughout the plant kingdom and are responsible for allergic reactions to a multitude of evolutionary unrelated pollen and food allergen sources. Occurring exclusively in pollen grains of plants, polcalcins are not involved in pollinosis-associated plant food allergies. Bet v 1 homologues represent major allergens in pollen of trees *Fagales* (including the *Betulaceae* and *Fagaceae* families) but can also be found in many allergenic foods belonging to the botanical families of *Rosaceae* (PR-10 proteins with 50%-60% identity to Bet v 1: apricot Pru ar 1, plum Pru c 1, peach Pru p 1, cherry Pru av 1, apple Mal d 1, pear Pyr c 1), *Betulaceae* (hazelnut Cor a 1.0101 with 50% identity to Bet v 1) and *Apiaceae* (PR-10 proteins with 40%-50% identity to Bet v 1: carrot Dau c 1, celery Api g 1), giving rise to many birch pollinosis-associated food allergies. Bet v 1-like allergens are not normally present in the pollen of grasses or weeds[132,144,145]. Although AIT with the recombinant major birch pollen allergen Bet v 1 proved as efficient as purified native Bet v 1 or birch pollen extract[22,146], the presence of IgE-sensitization to minor allergen components acting as panallergens, profilins and/or polcalcins, would be expected to decrease the efficacy of pollen AIT, at least to some extent, especially in the absence of IgE to species-specific allergen components. Sensitization to both profilin and/or polcalcin typically follows previous cosensitization to other molecular allergens from the same pollen source, being recognized at a later stage, and it is associated with a longer duration of allergic disease and with resulting cosensitization to a larger number of species-specific allergen molecules. When molecular multi-sensitization is present, sometimes it is associated with the practical inability to administer a more appropriate, allergen-matching AIT extract. Even if the content in various pollen AIT extracts, at least for profilin, is remarkably low, if specific IgE antibodies against major allergens are present, AIT with extracts containing these allergens can be administered, especially as the clinical relevance of profilins and polcalcins is still arguable[26].

Only a limited number of pollen panallergens are available for routine use (grass profilin, rPhl p 12, and birch profilin, rBet v 2; grass polcalcin, rPhl p 7 and birch polcalcin, rBet v 4), but due to marked structural homology among allergenic species, these serve as efficient markers of IgE-mediated hypersensitivity to the entire group of homologous proteins, with the possible exception of profilins from pollen of wall pellitory *Parietaria judaica* (Par j 3) and cypress *Cupressus sempervirens* (Cup s 8), the latter being cross-reactive with the goosefoot *Chenopodium album* profilin, Che a 2. The molecular biomarkers of sensitization to cross-reactive grass pollen panallergens are discussed below[26,117,140,145,147,148].

rPhl p 7, a 9 kDa calcium-binding protein, is used as a polcalcin marker. Phl p 7 is a minor allergen of Timothy grass pollen, recognizing serum IgE antibodies in 10%-15% of grass pollen-sensitized subjects. Phl p 7 is a polcalcin cross-reactive with other polcalcins contained in pollen grains of non-*Pooideae* Bermuda grass (Cyn d 7), trees, such as birch (Bet v 3), alder (Aln g 4), olive (Ole e 3), juniper (Jun o 4), and weeds, such as goosefoot (Che a 3). Unlike Bet v 3 which contains three typical calcium-binding motifs, Bet v 4 is a polcalcin which contains only two calcium-binding domains. rBet v 4, a 8 kDa calcium-binding protein, is also used as a polcalcin marker. Other weed pollen polcalcins are from *Asteraceae* family (Art v 5, Amb a 10). Polcalcin rPhl p 7 is therefore likely to cross-react with pollen proteins from most plants, in particular with other grass species, several weeds and trees.

rPhl p 12, a 14 kDa actin-binding protein, is used as a profilin marker. This acidic protein is involved in cytoskeleton dynamics by binding to actin. Phl p 12 is a minor allergen of Timothy grass pollen, binding IgE antibodies from approximately 15%-30% of grass pollen-allergic subjects with varying degrees in different geographical regions. Phl p 12 has more than 75% sequence identity with profilins from pollen, various plant-derived foods and latex. It is cross-reactive with pollen profilins from many plants, such as birch (Bet v 2), olive tree (Ole e 2), date palm (Pho d 2), Bermuda grass (Cyn d 12) and sunflower (Hel a 2). rBet v 2, a 15 kDa profilin, is also used as a cross-reactive marker. Other pollen profilins are those from ragweed (Amb a 8) and mugwort (Art v 4). Cross-reactivity between profilins of mugwort pollen (Art v 4) and *Apiaceae* foods, such as celery (Api g 4), carrot (Dau c 4) and spices, are involved in the pathogenesis of the celery-mugwort-spice syndrome. Cross-reactivity between profilins of ragweed pollen (Amb a 8) and fruits, such as melon (Cuc m 2) and banana (Mus xp 1), are involved in the pathogenesis of the ragweed-melon-banana association.

Molecular diagnosis biomarkers, together with clinical history data, can help clinicians make a better selection of the most appropriate patients and allergens for AIT[140]. Moreover, application of the component-resolved diagnosis biomarkers may change the diagnosis and the

choice of AIT in some patients[149].

Taken together, the CRD biomarkers are used to guide prescription of grass pollen AIT after an initial basic diagnostic discrimination between mono/oligo- and multi-sensitization, based on skin prick testing results and/or values of *in vitro* evaluation of specific IgE using common pollen extracts. The use of a panel of species-specific allergen molecular markers, representing the most common allergenic species in the region, along with the panallergen screening molecules from grass pollen (polcalcin rPhl p 7 and profilin rPhl p 12), may facilitate the selection of those AIT candidates with an increased probability of benefiting from this type of treatment.

PREDICTIVE BIOMARKERS OF CLINICAL EFFICACY

In grass pollen immunotherapy

Because very complex immunological mechanisms of action, both cellular and humoral, are involved in the AIT efficacy, its long-lasting effect and the way it changes the course of IgE-mediated allergic disease, candidate biomarkers of clinical efficacy or biomarker combinations remain to be validated in order to clearly distinguish between strong and weak or early and late AIT responders[42].

The AIT mechanisms of action to induce peripheral tolerance to grass allergens may be useful to classify some candidate *predictive biomarkers* for AIT efficacy, especially those derived from the antigen presenting cell (APC)-regulatory T cell (T_{reg})-IgG_4 antibody immunoregulatory loop[150]. These candidate biomarkers can be classified as tolerogenic DCs biomarkers, regulatory T cell biomarkers, serum blocking antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers[39-42,44].

Biomarkers of tolerogenic DCs

Oral APCs are key players in SLIT. Langerhans cells, $CD207^+$ cells (Langerin or CD207 being a C-type lectin receptor localized in Birbek granules) located in the mucosa itself, with a $Fc_{epsilon}RI$ expression greater compared with similar cells in the skin[151], and a predominant subpopulation of myeloid DCs located along the *lamina propia*, $CD11b^+CD11c^-$ monocyte-derived DCs (moDC), are critical in capturing allergen and processing it as small peptides presented in association with major histocompatibility complex (MHC) class I and class Ⅱ molecules at the cell surface. DCs loaded with allergen-derived peptides migrate to the cervical lymph nodes within 12-24 h, where they interact with naive $CD4^+$ T cells to support the differentiation of T_{reg} cells within 2-5 d. These $CD4^+$ T cells subsequently migrate through blood back to mucosal tissues, resulting in allergen tolerance associated with downregulation of Th_2 responses[152,153].

Intracellular and surface biomarkers of tolerogenic DCs are important to be presented.

Biomarkers of tolerogenic DCs (DC_{reg}) are biomarkers of DCs driving differentiation of T_{reg} cells, evidenced by differential gel electrophoresis and mass spectrometry[154]. Two such biomarkers must be discussed. Stabilin-1 (STAB1) is an intracellular scavenger receptor expressed by DCs and macrophages. Complement component 1 (C1Q) is the first component of complement which may be associated with arrest of moDC differentiation and may induce tolerogenic properties in developing DCs[154-156]. Tolerogenic moDCs are the most prominent source of *C1Q* and *STAB1* gene expression in the blood and are generated *in vitro* from peripheral blood mononuclear cells (PBMCs). Induction of DC_{reg} biomarkers (DCs *in vitro* treatment with dexamethasone) in PBMCs (containing < 0.5%-1% DCs) of patients with grass pollen allergy treated four months with SLIT is indicative of clinical tolerance induced by AIT (short-term efficacy)[154].

Regarding surface biomarkers of tolerogenic DCs, SLIT downregulates APC functions by modulating the expression of costimulatory molecules. There is a recent role revealed for the programmed death-1 receptor (PD-1) and PD-1 ligand (PD-L1) pathway in regulating lymphocyte activation and promotion of T_{reg} cell development and function[157]. PD-L1 (B7-H1, CD274), the programmed death ligand-1, is a coregulatory molecule critical for T_{reg} generation with important expression on tolerogenic APCs (upregulated by TLR4 ligand monophosphoryl lipid A). PD-L1 may play an important role in induction of T regulatory cells by SLIT[158]. Pollen SLIT reduces the expression of CD86 on B cells ($CD19^+$) and the expression of CD80 on monocytes ($CD14^+$), and increases the expression of PD-L1 on APCs ($CD14^+$, $CD19^+$) evaluated by flow cytometry analysis. PD-L1 may be a major target of pre-seasonal pollen SLIT and that modulation of its expression could be used as a clinical efficacy marker[150].

Regulatory T cell biomarkers

These biomarkers may also be important because multiple mechanisms are related to T_{reg} cells in AIT. T_{reg} cells directly and indirectly control the activity of effector cells of allergic inflammation, such as eosinophils, basophils and mast cells. AIT-induced T_{reg} cells inhibit the $Fc_{epsilon}RI$-dependent mast cell degranulation, OX40-OX40 ligand interaction playing an important role, decrease the thresholds for mast cell and basophil activation and reduce IgE-mediated histamine release[159-163]. Both main subsets, naturally occurring forkhead box P3 (FoxP3) expressing $CD4^+CD25^+$ regulatory T cells and inducible IL-10-producing T regulatory type 1 (Tr_1) cells, are decisive for the development of immune tolerance to allergens under AIT[163]. Mucosal T_{reg} cell induction in SLIT was revealed by immunofluorescence microscopy, $FoxP3^+$ cells being increased in the oral epithelium of grass pollen SLIT[164]. The induced T_{reg} cell level defined as the proportion of $IL\text{-}10^+FoxP3^+$ cells among $CD25^+CD4^+$ leukocytes, analyzed in the peripheral blood by flow cytometry, may be a potential therapeutic biomarker for SLIT, as revealed in a preliminary report in Japanese cedar (*Cryptomeria japonica*) pollinosis[165]. Allergen-specific $CD4^+$ T cell responses in

peripheral blood do not predict the early onset of clinical efficacy during grass pollen SLIT, as revealed in a more recent study in which these peripheral allergen-specific $CD4^+$ T cells were assessed using pMHCII-tetramers or flow cytometry surface phenotyping, as $CTLA\text{-}4^+IL\text{-}10^+$ or $CD25^+CD127^-FoxP3^+$ T_{reg} cells. Moreover, transcription factors (GATA-3, FoxP3) and cytokines (TGF-beta) gene expression assessed by quantitative reverse transcriptase polymerase chain reaction in allergen-stimulated peripheral cells do not predict clinical efficacy in SLIT, and the downregulation of *IL-4* or *IL-10* gene expression, as well as IL-10 secretion, by allergen-stimulated T cells seems to be unrelated to clinical benefit[166].

Antibodies biomarkers

The candidate antibodies biomarkers for the prediction of efficacy and monitoring of grass AIT must be discussed correlated with the allergen-specific IgE and IgG_4 responses during AIT.

Serum allergen-specific IgE antibodies

Although AIT rapidly induces peripheral T-cell tolerance, B-cell changes seem to appear at a relatively later phase. Serum allergen-specific IgE values are not generally considered appropriate biomarkers to assess SIT efficacy. Sometimes they transiently increase early in SCIT, and then gradually decrease over months or years of continued treatment. In pollen-sensitive patients who have undergone AIT and become desensitized, these values do not increase during the pollen season. There is a blunting of seasonal increases in specific IgE antibodies by AIT. Very late in the course and after termination of AIT, a decrease of allergen-specific IgE values is possible, occurring one to three years after starting therapy. Changes in IgE levels cannot account for reduced responsiveness to specific allergens after AIT because the decrease in serum IgE levels is late, relatively small and poorly correlated with efficacy. The reason for the persistence of serum IgE despite clinical improvement may relate to long-lived bone-marrow-resident IgE producing plasma cells[16,40,163,167,168].

The ratio of allergen-specific IgE to total IgE (sIgE/tIgE) was proposed as a candidate prognostic biomarker for SLIT. Symptom-medication score in patients treated with pollen SLIT seems to be correlated with the sIgE/tIgE ratio before treatment, being significantly improved in patients with a low sIgE/tIgE ratio compared to that in patients with a high sIgE/tIgE ratio. The grass-specific IgE to total IgE ratio seems significantly higher in responders than in nonresponders following four years of pollen SLIT. Further validation studies are needed before this biomarker can be considered in the clinical management of SLIT[158,169].

Serum allergen-specific IgG_4 antibodies

IgG_4 blocking antibodies prevent allergen-induced IgE-mediated release of inflammatory mediators from basophils and mast cells, directly compete with IgE on mast cells and APCs, inhibit IgE-facilitated allergen presentation to T cells and allergen-induced IgE production during allergen exposure. There is also an IgG_4-dependent blocking of IgE binding to B cells. IgG_4 production is confined to human IL-10-producing regulatory B (BR1 cells or $CD73^-CD25^+CD71^+$ B cells)[40,163,170,171].

Regarding serum allergen-specific IgG_4 antibodies as biomarkers, only specific IgG_4 antibodies with high affinity and avidity are functionally relevant. Pollen specific IgG_4 may be evaluated by fluoro-enzyme immunoassay. Serum allergen-specific IgG_4 levels significant increase relatively early in SIT (weeks to months after AIT start), in an allergen-dose dependent manner (10-100-fold increase) and persist for up to two years after AIT discontinuation. Although this indicates a good immunological response to AIT, there are contradictory correlations with clinical improvement, there is no correlation with clinical outcomes (after up-dosing) and there is no common cut-off value for specific IgG_4 antibodies[163,170,172].

Basophil activation evaluation may be used to detect IgG blocking activity in AIT. Allergen-IgG_4 complexes bind to $Fc_{gamma}RIIb$ (low affinity IgG receptor) containing a cytoplasmic immunotyrosine inhibitory motif that counters immunoreceptor tyrosine-based activation motif signals from $Fc_{epsilon}RI$ (high-affinity IgE receptor). Phosphorylated $Fc_{gamma}RIIb$ mediates inhibition of $Fc_{epsilon}RI$ signaling, coaggregation of $Fc_{epsilon}RI$ with $Fc_{gamma}RIIB$ inhibits degranulation, although there is a controversial role of $Fc_{gamma}RIIb$ in mediated post-AIT serum inhibitory activity[173,174]. Basophil activation test by flow cytometry evaluating CD203c expression, an ecto-nucleotide enzyme associated with basophil activation and piecemeal degranulation, may be a candidate biomarker for AIT monitoring, as suggested by a Japanese cedar pollen allergy study revealing a reduction in CD203c expression post-AIT[175].

Functional biomarkers of serum IgG-associated inhibitory activity in AIT may be more useful surrogates of clinical response than serum IgG_4 levels.

The inhibition of CD23-dependent IgE-Facilitated Allergen Binding (IgE-FAB) to B cells assay evaluates the serum inhibitory activity for binding of allergen-IgE complexes on to B cells. It is performed incubating allergen-IgE complexes with an EBV-transformed B-cell line, complexes bound to CD23 on the surface of cells being detected by flow cytometry. Addition of serum from patients who have received AIT inhibits allergen-IgE complex binding to CD23 on B cells. The following formula may be used to calculate the percentage relative B cell binding: % relative allergen-IgE complex binding to B cells = (% IgE-FAB using indicator and immunotherapy serum/% IgE-FAB using indicator serum only) × 100. Pollen SCIT induces in grass allergic rhinitis patients time- and dose-dependent increases in antibody-associated serum inhibitory activity for IgE-FAB and increases in IgE-blocking factor (IgE-BF)[168].

Serum specific IgE-BF competing with IgE for allergen binding is determined using a wash assay, IgE measurement with a chemiluminescent immunoassay, and

no-wash assay, allowing non-IgE antibodies to interact with biotinylated allergens in competition with the solid-phase absorbed IgE antibodies. The (IgE binding in competition with non-IgE)/(IgE binding with remaining Igs washed away) ratio varies from 0 to 1 (no blocking antibodies induced). Successful grass pollen SCIT is associated with significant reduced allergen-IgE binding (IgE-FAB) and increased IgE-BF[168].

Whether such functional assays of inhibitory IgG_4 and IgE-BF will be validated as predictive biomarkers of clinical AIT efficacy in individual patients requires further detailed investigation.

Serum allergen-specific IgA_2 antibodies

Regarding IgA subclasses, IgA_1 is found in serum and produced by bone marrow B cells, while IgA_2 is made by B cells located in the mucosa. The development of mucosal immune tolerance is associated with the expression of immunoregulatory cytokines (IL-10, TGF-*beta*) and protective antibody subclasses (IgG_4 and IgA_2)[164]. Long-term grass pollen AIT seems to induce a selective IgA_2 subclass systemic response, which may reflect a local mucosal response. Serum Phl p 5-specific IgA_2 response to AIT is associated with nasal TGF-*beta* expression. Allergen-specific IgA_2 concentrations can be determined by sandwich enzyme-linked immunosorbent assay (ELISA) and the systemic specific IgA_2 response might also be surrogate biomarker of the clinical response to AIT[176].

All of these studies approaching various humoral immunological pathways involved in AIT efficacy may create a framework regarding the usefulness of *antibodies biomarkers*, but the mechanisms of grass pollen-specific IgG_4 and also IgG_1 antibody subclasses in AIT are not very well understood. IgG_4 antibodies act as blocking antibodies (better than IgG_1), but IgG_4 production may be also an epiphenomenon, its production reflecting conditions favorable for immune tolerance such as activation of T_{reg} cells, while regulatory B cells may produce IL-10 that promote IgG_4 production[177]. Very recent data complicate the opinion on the proven utility of such humoral biomarkers. In a randomized, double-blind placebo-controlled study using an allergen challenge chamber and quantitative, qualitative and functional analyses of allergen-specific IgE, IgG_{1-4} and IgA responses, clinical responders to grass pollen SLIT include both immunoreactive patients who exhibited strong increases in titers, affinity and/or blocking activity of grass-pollen-specific IgGs, as well as patients with no detectable antibody responses. Seric IgG responses may contribute to SLIT-induced clinical tolerance in some subjects, but additional immune mechanisms are involved in most patients[178]. Therefore, at the current level of knowledge, it is difficult to support the fact that antibody responses can be used as reliable biomarkers of AIT efficacy at an individual patient level.

Immune activation and immune tolerance soluble biomarkers

The immunopathogenesis of pollen respiratory allergy includes a preponderance of Th_2-type responses and the biochemical pathways triggered by Th_1-type cytokine interferon-*gamma*, such as tryptophan degradation by indoleamine 2,3-dioxygenase and neopterin production, might be altered[179]. Neopterin is a low molecular weight soluble biomarker of immune activation, synthesized from guanosine-triphosphate and produced preferentially by human monocytes/macrophages. Neopterin production and tryptophan catabolism through the kynurenine pathway, measured by the kynurenine-tryptophan ratio, are induced by interferon *gamma* (IFN-*gamma*), thus both are considered markers of T cell mediated immune activation. Serum neopterin concentrations can be determined by an enzyme immunoassay technique. SLIT may reduce serum neopterin levels, this phenomenon being possible due to the T_{reg} response able to induce IL-10 production, that may inhibit neopterin production. Thus, serum neopterin could be a serum biomarker of achieved immune tolerance toward the causal allergen in allergic patients successfully treated with SLIT[44,180]. Tryptophan and kynurenine serum concentrations seem to be higher in allergic rhinitis patients, especially out of pollen season. Simultaneous measurement of serum tryptophan and kynurenine may be performed by high performance liquid chromatography. Some authors suggested that non-responders to SCIT seem to have significantly higher tryptophan concentrations, higher tryptophan levels being a result of lower indoleamine 2,3-dioxygenase activity[179], and others revealed that serum tryptophan and kynurenine concentrations decrease after pollen SCIT, and a correlation between changes in tryptophan metabolism and neopterin concentrations was also possible after AIT[181].

The non-classical MHC class I molecule HLA-G plays important immunomodulatory activities. The differentiation of Tr_1 cells by tolerogenic IL-10-producing human DCs requires the IL-10-dependent ILT4/HLA-G pathway[182]. Leukocyte immunoglobulin-like receptor B2 (LILRB2) or ILT 4 (CD85d) is a human inhibitory immune receptor that recognizes HLA-G with a higher affinity[183]. Soluble HLA-G (sHLA-G) has increased serum values in patients with pollen allergic rhinitis studied outside the pollen season[184]. These can be determined by ELISA, while cell production of IFN-*gamma* is possible to be evaluated by enzyme-linked immunoabsorbent spot assay[185]. sHLA-G serum levels are reduced by pollen SLIT in allergic rhinitis patients and lowering of these levels and the increased IFN-*gamma* production after SLIT in pollen allergic rhinitis are significantly related phenomena. Thus, sHLA-G might be considered as a candidate biomarker of response to SLIT[43].

Apoptosis biomarkers

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)/Apo2L is a type Ⅱ transmembrane protein that was identified and cloned based on its sequence homology with members of the TNF ligand family. TRAIL-induced initiator caspase-8 and executioner caspase-3 cleavage is enhanced by IgE-dependent activation

of mast cells, which increases the expression of anti-apoptotic molecules FLIP (Fas-associated death domain-like IL-1 beta-converting enzyme-like inhibitory protease) and myeloid cell leukemia 1 (MCL-1 belonging to the bcl-2 family proteins), and a pro-apoptotic molecule Bcl-2 interacting mediator (BIM of cell death), thus fine modulating mast cell apoptosis[186]. Apoptosis of mast cells may be also regulated by some IgG receptors, such as $Fc_{gamma}RIIB$[187]. TRAIL is also present in cells, eosinophils, fibroblasts and airway epithelial cells. The soluble TRAIL (sTRAIL) is an apoptosis biomarker which can be measured in the serum by a sandwich enzyme-linked immunosorbent assay. sTRAIL levels may decrease after SCIT to healthy levels and may be of use as a marker of efficacy of immunotherapy in allergic rhinoconjunctivitis patients[41]. The role of sTRAIL in AIT is poorly understood and this makes the evaluation of the value of this biomarker difficult.

CONCLUSION

CRD biomarkers have proven utility in the assessment of sensitization to grass pollen allergens, allow the clinician to confirm genuine sensitization to the corresponding allergen plant sources and guide an accurate prescription of AIT, important in many regions of the world with great plant biodiversity and/or where pollen seasons may overlap. These disease-related molecular biomarkers, important tools for the future in allergy diagnostics, are hitherto available for the most important grass pollen allergens, although they have not currently replaced the classical existing methods of *in vivo*/*in vitro* allergy testing. Molecular diagnostic algorithms to guide pollen immunotherapy in some European regions are already designed[26].

It is difficult to estimate which of the presented candidate predictive biomarkers for grass pollen AIT will be validated in clinical practice, but those related to tolerogenic regulatory cellular responses are most promising. Some answers to questions regarding the upcoming guidelines for the use of predictive biomarkers for AIT and the possible role of combined application of biomarkers are not known and should be addressed as potential issues in future research. The search for candidate predictive biomarkers in AIT opens new opportunities for the early detection of clinical responders during AIT, for the follow-up of AIT patients and for the development of new allergy vaccines.

Molecular allergy biomarkers represent a complex area providing novel and relevant information for allergists and educational programs on their use in clinical practice are imperative[188].

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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