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Autoantibodies targeting G protein-coupled receptors: An evolving history in autoimmunity. Report of the 4th international symposium

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ABSTRACT

G protein-coupled receptors (GPCR) are involved in various physiological and pathophysiological processes. Functional autoantibodies targeting GPCRs have been associated with multiple disease manifestations in this context. Here we summarize and discuss the relevant findings and concepts presented in the biennial International Meeting on autoantibodies targeting GPCRs (the 4th Symposium), held in Lübeck, Germany, 15–16 September 2022. The symposium focused on the current knowledge of these autoantibodies' role in various diseases, such as cardiovascular, renal, infectious (COVID-19), and autoimmune diseases (e.g., systemic sclerosis and systemic lupus erythematosus). Beyond their association with disease phenotypes, intense research related to the mechanistic action of these autoantibodies on immune regulation and pathogenesis has been developed, underscoring the role of autoantibodies targeting GPCRs on disease outcomes and etiopathogenesis. The observation repeatedly highlighted that autoantibodies targeting GPCRs could also be present in healthy individuals, suggesting that anti-GPCR autoantibodies play a physiologic role in modeling the course of diseases. Since numerous therapies targeting GPCRs have been developed, including small molecules and monoclonal antibodies designed for treating cancer, infections, metabolic disorders, or inflammatory conditions, anti-GPCR autoantibodies themselves can serve as therapeutic targets to reduce patients' morbidity and mortality, representing a new area for the development of novel therapeutic interventions.

1. Introduction

The concept of autoimmunity has evolved significantly over the years. In 1900, Paul Ehrlich developed the concept of horror autotoxicus, according to which the immune system can produce antibodies only against non-self-antigens [1], leading to the interpretation that "autoimmunity cannot happen" [2]. Due to this paradigmatic view and despite first reports of the existence of autoantibodies as early as 1904 [3], the concept of autoimmunity remained unaccepted [4] until 1946 when Boorman et al. [5] demonstrated that autoantibodies caused acquired hemolytic anemia. These observations paved the way for the idea of autoimmune diseases as a consequence of destructive immunological processes against self-antigens [2,6]. Frank Macfarlane Burnet reinforced the pathological effect of autoantibodies and the concept of autoimmunity when, in 1959, he proposed the clonal selection theory based on the clonal deletion of antibody-forming lymphocytes. This process is essential for the development of a highly diversified antibody repertoire [7]. It would follow that autoimmune diseases result from the emergence of "forbidden clones" [8], producing pathogenic autoantibodies. This concept has defined the historical basis of autoimmune diseases [9].

Our understanding of the different mechanisms by which autoantibodies initiate pathologies has significantly expanded and is reviewed in detail elsewhere [10]. Currently, it is well-known that autoimmune diseases have a multifactorial background (Fig. 1), such as genetic, epigenetic, immunological, neurological, endocrinological behavior (e.g., smoking and diet), comorbidities (e.g., diabetes and hypertension), and environment [11]. Hence, these various factors might influence the production of autoantibodies, which are central players in the development of autoimmune diseases. Countless autoantibodies have been associated with systemic and organ-specific autoimmune diseases. Among them are autoantibodies targeting the largest superfamily of integral membrane proteins, called G protein-coupled receptors (GPCRs) [12]. The first discovered functional autoantibodies targeting GPCRs (considered regulatory autoantibodies [RAB] when their function had been confirmed) were those against the β2-adrenergic receptor, implicated in developing rhinitis and asthma [13]. Following this first demonstration of the pathological relevance of anti-GPCR autoantibodies, these molecules have been shown to play an essential role in the outcome of autoimmune diseases [14].

2. The 4th RAB symposium in Lübeck

This article reviews and discusses the most recent findings and concepts presented during the fourth biennial symposium on autoantibodies targeting GPCRs. So far, four international meetings on functional autoantibodies targeting GPCRs have been organized, all held in Lübeck, Germany. The first symposium (October 7-9, 2016 [15]) was followed by three other biannual conferences, as follows: the second was also an in-person international meeting on September 28-30, 2018 [16]; the third symposium coincided with the coronavirus disease 2019 (COVID-19) pandemic, occurring in online mode on September 24-25, 2020 [17]; the fourth, and most recent meeting, was a hybrid symposium, called "the RAB-Symposium - Regulatory Autoantibodies Targeting GPCRs," held on September 15–16, 2022 [18].

These meetings aimed to combine current knowledge about the role of GPCRs in different pathologies, their mode of action, and state-of-the-art research techniques to identify common fundamental pathways that can be transferred to other disease entities with similar manifestations. The two-day meeting of the most recent RAB Symposium was marked by great talks, fruitful discussions, and poster presentations by speakers with research expertise in autoantibodies targeting GPCR. The topics discussed in the scientific sections were developed as follows.

3. Anti-GPCR autoantibodies linked to different pathological conditions

Dysregulation of anti-GPCR autoantibody production has been demonstrated in various diseases, as illustrated in Fig. 2 and discussed below in detail.

3.1. Rheumatic diseases

We have recently reviewed the role of several autoantibodies targeting GPCRs in different rheumatic diseases [14] (e.g., systemic lupus erythematosus [SLE], rheumatoid arthritis [RA], systemic sclerosis [SSc]), a topic intensively discussed during the 4th RAB Symposium in Lübeck. For example, recent advances in elucidating the role stimulating autoantibodies against angiotensin and endothelin receptors play in the pathogenesis of SSc, a severe and heterogeneous autoimmune disease hallmarked by dysregulated immunity, vasculopathy, and fibrosis [19].

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High levels of autoantibodies directed against the angiotensin II type 1 receptor (AT1R) and endothelin-1 type A receptor (ETAR) promote severe disease complications [20]. Notably, Bankamp and coworkers from the University of Tuebingen, Germany, communicated that functional anti-AT1R and anti-ETAR autoantibodies of SSc patients influence autologous stem cell transplantation (aSCT) and correlate with clinical outcomes [21]. SSc patients ($n = 43$) were tested for the presence of anti-AT1R autoantibodies before aSCT and at different time points after aSCT (4-217 months, median 28 months) using a commercially available ELISA (enzyme-linked immunosorbent assay; CellTrend, Luckenwalde, Germany) and an in-house luminometric cell-based assay. In the ELISA, the expected values given by the manufacturer were confirmed with sera from healthy donors ($n = 36$). In the luminometric assay, the normal range was determined using sera from healthy controls (HC) and used to define inhibitory and stimulatory anti-AT1R autoantibodies. The ELISA measurements indicated that 51% of the SSc patients had high levels of anti-AT1R autoantibodies before aSCT. Anti-AT1R autoantibodies prevalence and reactivity decreased ($p < 0.01$) between time point 1 (1-4 months after aSCT) and time point 4 (18-24 months after aSCT). Anti-AT1R autoantibodies did not correlate with the outcome of aSCT. Using the luminometric assay to measure functionally active anti-AT1R autoantibodies, 40% of SSc patients had stimulatory and 12% inhibitory anti-AT1R autoantibodies before aSCT. While the prevalence and reactivity of anti-AT1R autoantibodies were not influenced by aSCT, the presence of stimulatory anti-AT1R autoantibodies before aSCT was associated with a favorable outcome of aSCT.

Van Oostveen and coworkers presented their unpublished work on anti-AT1R and anti-ETAR autoantibodies' role in endothelial cell activation and pro-fibrotic responses. IgG derived from SSc patients induced a significant upregulation of EC activation markers, such as monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and *E*-selectin, in an AT1R- and ETAR-dependent manner, when compared to the effect of HC-IgG. Moreover, SSc-IgG induced AT1R- and ETAR-mediated expression of interleukin (IL)-6, IL-8, and transforming growth factor beta (TGF-β), while HC-IgG did not.

3.2. Acute COVID-19

The involvement of anti-GPCR autoantibodies in the development of severe Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [22], was also covered in the 4th RAB Symposium. Some COVID-19 patients did develop systemic immune dysregulation of innate and adaptive immune responses, cytokine storm syndrome [23], abnormal leukocyte counts (e.g., neutrophilia and lymphopenia) [24], and clinical features resembling a systemic autoimmune disease [25–29]. Notably, severe COVID-19 is marked by higher autoantibody levels than HCs and those with mild COVID-19 [30,31]. In addition to autoantibodies targeting type I interferons (IFNs) [30], the exoproteome [31] and renin-angiotensin system (RAS)-related molecules, severe SARS-CoV-2 infection dysregulates the production of anti-GPCR autoantibodies [32]. Thus, similar to other viruses [33–36], SARS-CoV-2 infection can trigger the development of life-threatening autoimmune diseases [25–27,37]. Although different pathological events have been discussed (e.g., molecular mimicry [38–43] and hyperinflammatory reaction causing tissue damage [44]), the precise mechanism of SARS-CoV-2-induced dysregulation of autoantibody production remains to be further investigated.

Cabral-Marques et al. employed a systems immunology approach [45] to characterize various anti-GPCR autoantibodies found in COVID-19 patients at high titers and their association with clinical outcomes [32]. The results indicate the involvement of autoantibodies against multiple molecules with crucial functions in immune and vascular homeostasis [22,46–48] and the disruption of autoantibody correlation signatures in severe forms of COVID-19. Of note, at least some of these anti-GPCRs, i.e., those with the strongest association with COVID-19 severity (anti-AT1R and anti-CXCR3 autoantibodies), have agonist properties (e.g., on cell migration) and may associate with pulmonary fibrosis and cardiac death [25,27,49–52]. Thus, we postulate that these autoantibodies synergize with other natural ligands (CXCL9, CXCL10, CXCL11 [CXCR3 ligands], and angiotensin II [AT1R ligand]), contributing to the COVID-19 immunopathogenesis.

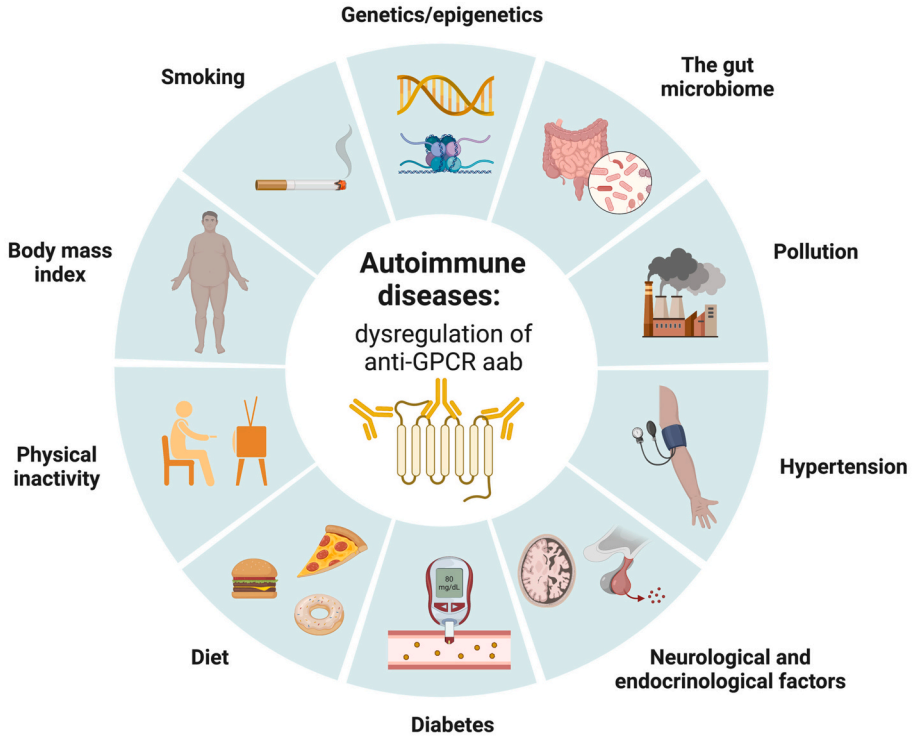

Fig. 1. Multifactorial causes of autoimmune diseases and the production of anti-GPCR abb. The figure shows several factors that influence the phenotype of autoimmune diseases and might influence the production of aab. Created with BioRender.com.

Furthermore, our collaborative working group has recently reported that functional autoantibodies against the thrombin receptor type-1 (PAR-1) seem to predispose to increased coagulation system activation, a characteristic complication of SARS-CoV-2 infection [53]. Our data indicate an association between severe COVID-19 and the generation of anti-PAR1 autoantibodies, which correlate with poor outcomes. While COVID-19 patients admitted to the intensive care unit (ICU) had high levels of circulating anti-PAR1 autoantibodies compared to HCs, this difference did not reach significant levels when comparing hospitalized non-ICU-treated COVID-19 patients with HCs. Elevated levels of anti-PAR1 autoantibodies within the ICU-treated cohort were associated with thromboembolic events and fatal outcomes. The circulating anti-PAR1 autoantibodies correlated with D-dimers, further indicating that anti-PAR1 autoantibodies are linked to coagulation processes in acute COVID-19. Thus, we hypothesize that anti-PAR1 autoantibodies, in combination with dysregulated coagulation proteases like activated protein C or matrix-metalloprotease-1, activate PAR1-dependent signals in endothelial cells and platelets, therefore contributing to immune-mediated micro thrombosis as suggested by the correlation of anti-PAR1 autoantibodies with D-dimers.

As recently shown by Simon et al., anti-PAR1 autoantibodies induce IL-6 secretion in microvascular endothelial cells (HMECs), which is associated with increased AKT, p70S6K, and ERK1/2 signaling, as well as increased c-FOS/AP-1 transcriptional activity [54]. However, in COVID-19 patients, no associations were found between anti-PAR1 ab levels and systemic IL-6 concentrations, suggesting the existence of other triggers of IL-6 production.

3.3. Post-acute COVID-19

Sotzny & Filgueiras et al. found dysregulated anti-GPCR autoantibodies targeting vaso- and immunoregulatory receptors in patients with post-acute COVID-19 syndrome (PCS), also known as long COVID [55], characterized by subacute long-term effects (e.g., fatigue, dyspnea, chest pain, cognitive disturbances, arthralgia) of COVID-19 known to affect multiple organ systems [56]. A subgroup of PCS patients suffering from long-lasting fatigue without clear evidence of organ dysfunction fulfills the Canadian consensus criteria (CCC) for myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). This is a severe, complex disease with patients suffering from fatigue, post-exertional malaise (PEM), cognitive impairment, pain, and autonomous dysfunction [57].

Contrasting with the autoantibody profile of severe acute COVID-19 patients, we found lower levels for various autoantibodies in PCS patients compared to seronegative HCs and asymptomatic SARS-CoV-2 infected individuals [55]. Group classification using random forest suggested autoantibodies targeting the β2 and α2a adrenergic receptors (ADRB2 and ADRA2A) among the strongest predictors of post-COVID-19 outcomes. Several autoantibodies correlated with symptom severity in PCS groups. For instance, the severity of fatigue and vasomotor symptoms associated with the levels of anti-ADRB2 autoantibodies in PCS/ME/CFS patients. We have discussed explanations for the contrast between high and low levels of autoantibodies in patients with COVID-19 and other diseases in a recent manuscript published elsewhere [58]. In summary, these results are in agreement with increasing evidence for an autoimmune etiology in ME/CFS [59] triggered by infections (e.g., Epstein-Barr virus [EBV] [60], human herpes virus [HHV]-6 [61], and the human parvovirus B19 [62]).

3.4. Myalgic encephalomyelitis/chronic fatigue syndrome

High levels of several autoantibodies, such as (ANAs) [63], anti-phospholipid, anti-ganglioside [64], and natural regulatory autoantibodies targeting GPCRs, have been reported in ME/CFS patients. Among the latter are elevated levels of anti-ADRB2 autoantibodies and autoantibodies against muscarinic acetylcholine receptors (mAChRs) [65,66]. As these receptors are essential vasodilators, their functional disturbance result in impaired circulation and oxygen supply [67]. Since ADRB2 activation by serum IgG is attenuated in ME/CFS patients with elevated levels of anti-β2AR autoantibodies [68], these autoantibodies possibly have antagonistic properties. Moreover, several studies showed that the autoantibodies mentioned above associate with ME/CFS severity, such as muscle weakness and neurocognitive impairment [69], altered structural brain networks [70], fatigue, and muscle pain [71]. These findings, and the observation that autoantibodies targeting therapies, including immunoadsorption and rituximab treatment, improve the disease severity of at least a subset of patients, reinforce the

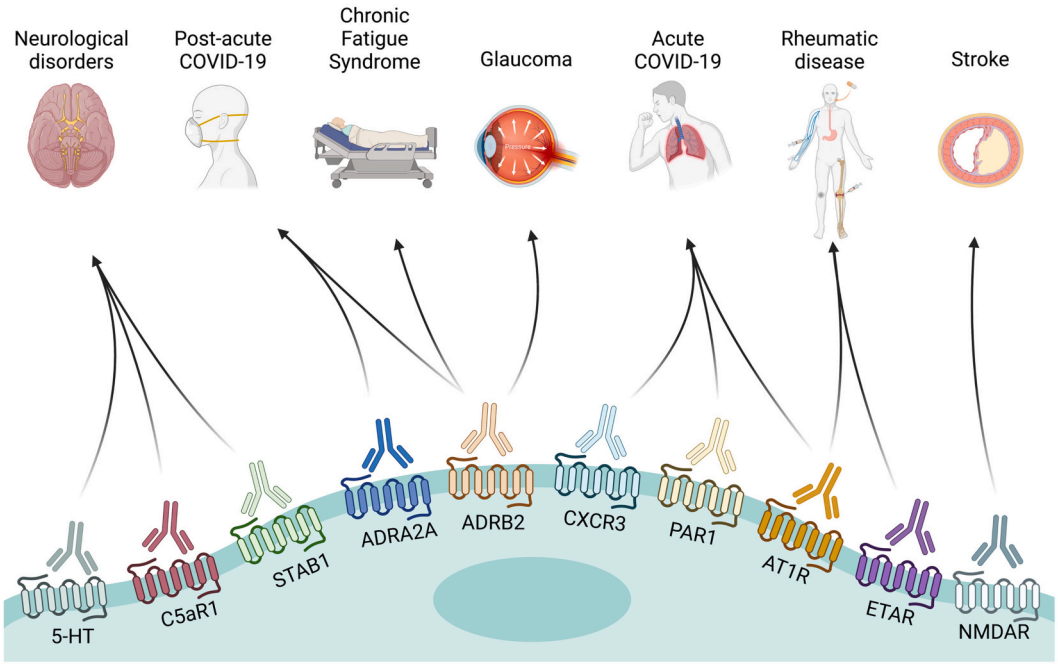

Fig. 2. The involvement of anti-GPCR abb in different pathological conditions. The diseases are shown on the top and anti-GPCR aab on the bottom of the figure. The arrows indicate their association. Created with BioRender.com.

importance of GPCR-autoantibodies in ME/CFS [65,72–74].

3.5. Neurological diseases

Increased evidence indicates that autoantibodies can also contribute to brain dysfunction, thus improving our understanding of nervous system autoimmunity and providing novel diagnostic and therapeutic opportunities for neurological diseases [75]. The importance of this subject was illustrated by the lecture delivered by Dr. Kamalanathan, who discussed the presence of autoimmune mechanisms, particularly humoral immune responses, in psychiatric disorders [77]. Proof of concept of an autoimmune contribution to schizophrenia (SCZ) and bipolar disorders (BPD) [76] was provided by demonstrating polyclonal IgG with DNAse-like catalytic activity. This phenomenon was confirmed by including neuro-psychiatric SLE (NP-SLE) patients as a comparator. The key observation was the presence of DNase-like activity mediated by polyclonal IgG antibodies in both the psychosis groups, especially in SCZ subjects, and in individuals with NP-SLE. A causal association could be established between IgG-induced DNAse activity and psychosis (symptomatic) clinical scores [77]. Overall, the results support an autoimmune process in a subset of SCZ patients and suggest a role of neuroinflammation in psychiatric disorders, significantly favoring the newer concept of "Autoimmune Psychosis" [78].

Remarkably, anti-GPCR autoantibodies have been characterized in patients with Alzheimer's disease (AD), a neurodegenerative disorder accounting for at least two-thirds of dementia cases [79]. Gill et al. reported high serum levels of autoantibodies targeting innate immunity (Stabilin-1: a scavenger receptor; C5aR1: C5a anaphylatoxin chemotactic receptor 1) and serotonin receptors (5-HT_{2A}R, 5-HT_{2C}R, and 5-HT_7R), which have been associated with impaired cognition and mood [80]. These results are compatible with the enhanced migration of immune cells into the brain [81], and the perturbed serotonin receptor function observed in AD patients [82]. Moreover, AD patients had significantly higher levels of anti-AT1R autoantibodies compared with HCs, which correlated with biomarkers of AD neuropathology, i.e., total and phosphorylated tau levels in the cerebrospinal fluid (CSF) [83]. In addition, AD patients may have agonist autoantibodies against the extracellular loop1 of the α1a adrenergic receptor (ADRA1A) associated with vascular dementia. The contribution of anti-ADRA1A autoantibodies to vascular damage has been well characterized in rats by demonstrating that these autoantibodies can stimulate the growth of vascular smooth muscle cells (VSMCs) [84].

3.6. Glaucoma

Physiological stimulation of the sympathetic and parasympathetic nervous systems in the context of infection or ischemia releases various molecules that antagonize the inflammatory processes [85–88]. Yet, overstimulation of autonomic receptors can dysregulate their homeostatic balance. It can be postulated that when this molecular imbalance (i.e., autonomic disbalance) exceeds a certain cellular level, clinical symptoms will occur. This hypothesis had been proposed as early as 1968 as the "imbalanced autonomic theory" for a specific receptor of the sympathetic nervous system (β-receptor) in asthma [89]. There is ample evidence that infectious diseases, necrosis, or ischemia can induce the development of autoantibodies [90,91]. In this context, the discovery of anti-GPCRs autoantibodies is of great interest, as GPCRs mediate diverse biologic functions in human biology. Various anti-GPCR autoantibodies exhibit substantial functional activities, leading to overstimulation of the receptor. This overstimulation, however, at least in some cases, differs from that generated by "normal" endogenous molecules or pharmaceuticals. For instance, the stimulation of the adrenergic receptors and their desensitization is of longer duration after exposure to anti-GPCR autoantibodies than exposure to physiological agents [92].

Glaucoma is a neurodegenerative disorder with progressive loss of retinal ganglion cells. Being one of the leading causes of irreversible blindness worldwide, glaucoma has an enormous impact on patients' quality of life and the economy. Autoimmunity is a known factor in the multifactorial pathogenesis of glaucoma [93–95]. Recent data revealed high levels of agonistic autoantibodies targeting ADRB2 in sera and aqueous humor of patients with glaucoma. ADRB2 receptors are present on cells of the trabecular meshwork [96] and the ciliary body [97], both involved in regulating the leading risk factor (intraocular pressure, IOP). The binding of anti-ADRB2 autoantibodies to the target receptor ß2-AR leads to overstimulation and loss of receptor desensitization, contrary to the effect of ß2-blockers, commonly used as anti-glaucomatous therapy. Thus, the presence of anti-ADRB2 autoantibodies results in chronic stimulation of the ADRB2 receptor [93]. A clinical proof-of-principal study demonstrated that IOP and the quantity of anti-glaucomatous eye drops used decreased after undergoing extra corporal immunoadsorption, eliminating anti-ADRB2 autoantibodies. A third target of anti-ADRB2 autoantibodies might be microcirculation. Anti-ADRB2 autoantibodies correlate with retinal vascular characteristics in patients with glaucoma, measured by non-invasive Heidelberg Retina Flow Meter [98] and OCT-angiography (OCT-A) [99]. This observation argues for a link between increased IOP and vascular dysregulation, both mediated by anti-ADRB2 autoantibodies. Interestingly, the neurodegenerative disorder Alzheimer's Disease (AD) may share a common molecular mechanism with glaucoma. A recent report suggested that this autoimmune dysregulation, mediated via an overstimulation of the ADRB2 signaling pathway, may play a role in both neurodegenerative disorders [100]. Based on these findings, it can be assumed that some neurodegenerative diseases have an adrenergic disbalance.

This pathogenetic hypothesis may show similarities to SARS-CoV-2 infection, which dysregulates the production of anti-GPCR-autoantibodies [101]. In addition, impaired microcirculation was observed in the retina of patients after severe SARS-CoV-2 infection, demonstrated by OCT-A [102], and blood rheology was altered in COVID-19 patients [103]. Based on this research data, a successful healing attempt was reported in a patient with glaucoma and PCS by neutralizing functional, active anti-GPCR autoantibodies using 007 BCE, a DNA aptamer drug with high affinity to GRCR-autoantibodies (Berlin Cures GmbH), consequently correcting the impaired microcirculation and improving the patient's symptoms [104]. It has been suggested that anti-GPCR-autoantibodies are functionally active in ischemic regions [105] and associated with various clinical features [106]. Ischemia-triggered autoimmune diseases have been linked to neurodegenerative (e.g., ocular) and post-viral disorders (e.g., Long/Post-COVID). Depending on the ischemic region, local (e.g., glaucoma) or systemic (Post-COVID) autonomic dysregulation appears to determine the clinical characteristics of each patient group [106].

3.7. Stroke

Stroke is one of the leading causes of death and the number one cause of disability in adulthood. In the Western world, about 9 in 10 stroke patients suffer from an ischemic stroke caused by thrombotic or embolic occlusion of a brain artery. Currently, evidenced-based therapies include intravenous thrombolysis and catheter-based thrombectomy, aiming at early recanalization of an occluded vessel [107].

The Prospective Cohort with Incident Stroke (PROSCIS)-B (ClinicalTrials.gov identifier NCT01363856), a prospective, hospital-based observational study of patients with first-ever stroke, was designed to investigate secondary risks. A total of >600 patients with ischemic stroke were enrolled in the study and followed up annually by telephone. Secondary events (i.e., recurrent stroke, myocardial infarction, death), functional outcomes, cognitive function, and depression were examined [108].

In the first step of the study, the potential role of serum anti-*N*-methyl-D-aspartate-receptor (NMDAR1) GluN1 (an ionotropic glutamate receptor, previously NR1) autoantibodies (NMDAR1-autoantibodies) was studied [109] for its role on long-term clinical outcome and

cognitive function [110,111]. Of note, several GPCRs (e.g., glutamate, acetylcholine, and dopamine receptors) regulate the function of NMDARs in several ways [112], including direct binding to the NMDAR, altering its trafficking, or modifying its activity. NMDAR1-autoantibodies play a role in new-onset cognitive impairment, encephalitis, and seizures [113]. A high prevalence of NMDAR1 IgA/IgM autoantibodies has been observed in different types of dementia [114]. However, anti-NMDAR1 autoantibodies of the IgA and IgM isotypes are also frequently detected in apparently healthy individuals and patients with ischemic stroke [115].

Interestingly, experimental evidence in mice indicates that an oral vaccine against NMDAR1 can provide neuroprotection against stroke, mediated by antibodies penetrating the blood-brain barrier and binding to NMDAR1 after the onset of brain ischemia [116]. NMDAR1-autoantibodies (i.e., IgM, IgA, and IgG) were measured in serum obtained from 583 ischemic stroke patients from PROSCIS-B within one week after the index event (39% female, mild to moderate stroke severity). >10% of ischemic stroke patients had (pre-existing) NDMAR1-autoantibodies at the time of stroke onset. NMDAR1-autoantibody seropositivity is statistically significantly associated with increased vascular risk. In contrast, NMDAR1-autoantibody seropositivity was not associated with functional outcome or cognitive function. However, higher titers were associated with poor functional and less favorable cognitive outcomes than negative patients [110,111].

Currently, the role of anti-GPCR autoantibodies in this stroke cohort is further investigated by Catar & Endres et al. Using serum samples obtained from PROSCIS-B patients during the first few days after the stroke had occurred, autoantibodies against AT1R, ETAR, complement receptors (C3aR and C5aR), PAR-1, PAR-2, VEGF1R, VEGF2R, VEGFA, VEGFB were measured to address the following research questions: 1) the levels of anti-GPCR-autoantibodies in stroke patients; 2) the relationship of anti-GPCR-autoantibodies and NMDAR1-autoantibodies; 3) the association of anti-GPCR-autoantibodies with different stroke subtypes (i.e., atherothrombotic, cardio-embolic, small vessel, etc.); 4) the association of anti-GPCR-autoantibodies with recurrent events and recurrent cardiovascular risk over three subsequent years; and lastly 5) the association of anti-GPCR-autoantibodies with functional outcome 12 months after the stroke (measured by both the modified Rankin scale as well as Barthel index).

4. Anti-GPCR autoantibodies against the autonomic nervous system: from breast silicone implants to “dysautonomia”

Seven clinical entities that include CFS, fibromyalgia, macrophagic myofasciitis, postural orthostatic tachycardia syndrome, complex regional pain syndrome, post-human papillomavirus vaccine syndrome/human papillomavirus vaccination associated neuro-immunopathic syndrome, and sick building syndrome have been noted to share several major clinical features. The manifestations entail cognitive impairment, memory loss, sleeping disturbances, severe and extreme fatigue, widespread pain, dry mouth and eyes, sweating disturbances, paresthesia, hearing disturbances, and other symptoms [117–120]. There is no common mechanism to explain all these clinical manifestations, and the affected individuals are often referred to multiple diagnostic procedures and frequently defined as “psychiatric patients.”

Recently, a group of individuals who underwent breast silicone implants was evaluated for symptoms similar to those described above [121]. Many of these individuals were found to have elevated levels of several anti-GPCR autoantibodies, which correlated with their clinical manifestations and disappeared following the removal of the silicone implants [119,122–124]. These findings indicate the involvement of autoimmune dysregulation in individuals undergoing breast silicone implants and in the aforementioned clinical entities (CFS, fibromyalgia, etc.), who frequently share manifestations of fatigue, dysautonomia, sensory disturbance, and cognitive impairment. Therefore, it is imperative to consider, in the future, the genetics, autoimmune comorbidities, immune cell subtype alterations, and detection of autoantibodies before performing breast silicone implantation.

These observations support the new concept of an autoimmune autonomic syndrome (dysautonomia) [117], with a common denominator of autoantibodies directed against GPCRs (e.g., adrenergic and muscarinic acetylcholine receptors) and the coexistence of small fiber neuropathy [118,125]. Therapeutic strategies that include targeting anti-GPCR autoantibodies (i.e., intravenous immunoglobulin therapy) may influence the extent of autonomic autoimmunity in patients with one of the seven syndromes outlined above, as previously reviewed [126]. By better understanding this new concept, it will be possible to identify the subgroups of patients who will benefit from targeted immunomodulatory therapeutic modalities [124].

5. The methodological approaches to quantify and understand the pathophysiological roles of anti-GPCR-autoantibodies

Figure 3 illustrates various approaches to investigate the pathophysiological roles of anti-GPCR-autoantibodies, as discussed below.

ELISA.

ELISA is a well-established and widely used method to characterize the levels of anti-GPCR autoantibodies, previously described in detail [45,127,128]. Autoantibodies are quantified directly from serum samples using, when available, commercial solid-phase sandwich ELISA Kits (CellTrend GmbH, Luckenwalde, Germany). This particular technique is a cell membrane-based ELISA method that detects autoantibodies against conformational epitopes within GPCRs [129] and has been validated by the Food and Drug Administration’s Guidance for Industry (Bioanalytical Method Validation). The autoantibody concentrations are defined as arbitrary units (U) created by extrapolating from a standard curve of five standards (ranging from 2.5 to 40 U/ml).

However, the ELISA approach has limitations [14]. For instance, it does not measure the avidity and affinity of autoantibodies to their target, potentially causing technical errors since these two properties can change the outcome of antibody-antigen interactions and binding, characteristics that are essential to determine the autoantibody pathophysiology. Furthermore, the ELISA approach is unable to characterize the functionality of autoantibodies. Hence, it is necessary to combine the ELISA technique with other methodologies, such as functional bioassays or luminometric methods [130,131], or with mouse models (described below) to determine autoantibody functionality, i.e., their agonistic or antagonistic properties.

5.1. *Multiplexed validation of anti-GPCR Antibodies*

Suspension bead array (SBA) is a valuable proteomics approach that permits the parallel capture and detection of multiple, unique, identifiable protein epitopes from a complex mixture of proteins in solution [132]. The SBA strategy is based on bar-coded magnetic microspheres with different colored dyes. Each bar-coded bead population can be coupled to a specific “capture” antibody (Ab), and the amount of analyte captured on the Ab-coated beads can be quantitated in a flow detector using a secondary “detection” Ab. The SBA approach was used recently to detect interactions between GPCRs and receptor-activity modifying proteins (RAMPs) expressed in mammalian cells in culture [132]. SBA’s multiplexed and miniaturized nature makes it particularly attractive for analyzing complex biological fluids such as serum, where up to several hundred analytes might be detected simultaneously from a single small sample [133]. In a pilot proof-of-concept study to assess the feasibility of developing an SBA assay tailored to detect autoantibodies against GPCRs in human serum, a library of approximately 220 GPCR clones with dual monoclonal Ab (mAb) epitope tags was created in a mammalian expression vector backbone. The clones each harbored a FLAG epitope sequence at the 5’-end and a 1D4 epitope sequence at the 3’-end. The expressed dual-epitope-tagged GPCRs can then be extracted from cell membranes and “captured” by the appropriate mAb coupled to

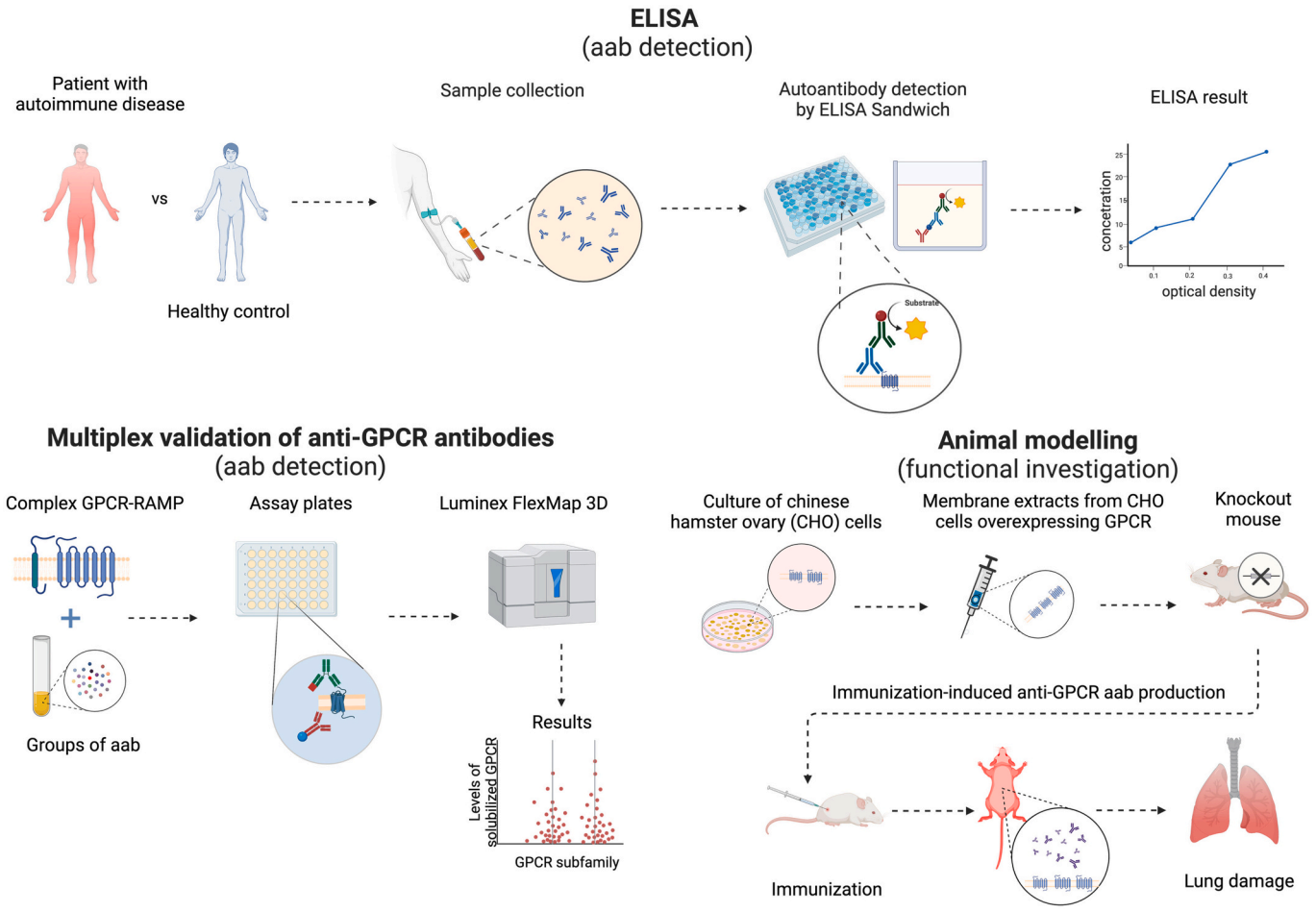

Fig. 3. Methodological approaches for understanding the pathophysiological roles of anti-GPCR aab. The image illustrates three approaches (in bold) used to investigate (for detection or functional evaluation) the involvement of anti-GPCRs in various diseases. The different steps of each approach are shown. Created with BioRender.com.

a bead. Alternatively, an SBA can be created using hundreds of anti-GPCR "capture" autoantibodies created as part of the Human Protein Atlas (HPA) and available commercially [134]. The HPA autoantibodies are raised against synthetic peptides corresponding to extracellular loops and tails of GPCRs, but their specificity has not been formally measured. The HPA autoantibodies must be validated to confirm the sensitivity and specificity of their binding to their intended GPCR target. To validate the HPA Ab, both mAbs (FLAG and 1D4) and the collection of HPA autoantibodies were used as capture-detection pairs in the SBA. The pilot study tested ~400 putative anti-GPCR autoantibodies from the HPA. A total of ~250 autoantibodies against 115 different GPCRs were found to be both sensitive and specific. The SBA methodology, along with the library of dual-epitope-tagged GPCRs and the validated library of anti-GPCR HPA autoantibodies, should be used to facilitate the development of a multiplexed detection assay for autoantibodies against GPCRs in human serum samples.

5.2. *Functional cardiomyocyte-bioassay*

Another method used in experimental and clinical studies to detect anti-GPCR autoantibodies' functionality is a cardiomyocyte bioassay, which was established several years ago [135,136]. The read-out of this approach is the function of cardiomyocytes, i.e., the beating rate. The difference between the basal beating rate of neonatal rat cardiomyocytes and the beating rate after incubation with the purified patients' serum immunoglobulins is monitored. The results of this approach can be expressed as an "increase in the number of beats/15sec". Identifying the specificity of the anti-GPCR autoantibodies to the receptor type is investigated by incubating the samples with specific blockers (e.g., ICI 118.551 for β2-AR). This method enables qualitative analysis of the presence and functionality of anti-GPCR-autoantibodies. However, it is crucial to control for the effects of specific blockers since it is possible that they themselves can inhibit the spontaneous and induced activity of some GPCRs.

5.3. *Animal modeling*

The 4th RAB Symposium also provided pertinent data demonstrating the paramount importance of animal models for addressing the multifactorial pathogenesis of autoimmune diseases, such as signaling pathways and immune response components, including autoantibodies [137,138]. The methodological strategies developed to induce experimental autoimmune disorders have been revised elsewhere [139–141]. Efforts have also been made to overcome the differences between the human and mouse immune systems [142,143].

Recently, a humanized mouse model for SSc has been established by transferring peripheral blood mononuclear cells (PBMCs) into immunodeficient mice [144]. After the transfer, mice receiving PBMC from SSc patients, but not those receiving PBMC from healthy subjects, develop systemic inflammation in multiple organs, including the lung, kidney, and liver. Notably, human IgG autoantibodies against AT1R are detected in murine sera and significantly elevated in mice who received PBMC from SSc patients compared to mice who received PBMC from healthy subjects. In contrast, levels of total human IgG are comparable in

both groups. These findings align with clinical observations that higher levels of autoantibodies against AT1R are characteristic of patients with SSc but not for healthy subjects [145]. Furthermore, in vitro depletion of T or B cells before the transfer of PBMC into mice demonstrates that both T and B cells are indispensable for producing autoantibodies against AT1R in the humanized mouse model, indicating that the production of anti-AT1R autoantibodies is a T cell-dependent response [146].

The immunization of immunocompetent mice with membrane-embedded human AT1R, which retains the conformational epitopes of the antigen in its native state, induces the production of functional autoantibodies against AT1R. These autoantibodies bind and activate the native receptor [147]. The hAT1R-immunized mice develop SSc-like symptoms, including perivascular and interstitial inflammation in the lung and perivascular inflammation and fibrosis in the skin. Moreover, applying an anti-AT1R monoclonal antibody generated based on this mouse model by hybridoma technology induces skin manifestations and interstitial lung disease [147]. The results of experiments in these mouse models support the concept that autoantibodies against AT1R contribute to the development of SSc [145]. Accordingly, since anti-AT1R autoantibodies are also present in healthy individuals at different levels, these abs could predispose to severe lung and skin inflammation in response to harmful stimuli, such as SARS-COV-2 infection [33].

The availability of animal models to investigate new pathophysiological roles of autoantibodies targeting other GPCRs promises to expand our current knowledge significantly. For instance, mice immunized with membrane extracts from Chinese hamster ovary (CHO) cells overexpressing human ETAR will enhance the production of anti-ETAR autoantibodies with chemotactic activity, which can induce the formation of neutrophil aggregates in vitro not seen if IgG from non-immunized mice is used. Another informative animal approach involves the transfer of serum with anti-GPCR autoantibodies collected from patients with primary Sjögren's syndrome (PSS) to animal models. In one such experiment, autoantibodies targeting muscarinic acetylcholine receptors were shown to interact with the rat parotid gland, activating these receptors and functioning as cholinergic agonists [148].

6. Mechanistic and functional studies of anti-GPCR autoantibodies

Mechanistic and functional studies performed with anti-GPCR autoantibodies demonstrated these autoantibodies' agonistic, synergistic, or antagonistic effects (Fig. 4). The results of these experiments are paramount for deciphering and understanding the intricate details involved in the development and manifestation of complex (immune) pathologies and diseases. Remarkably, these mechanistic and functional studies have been essential to establishing evidence-based proof for the pathophysiologic causality related to RABs, as previously demonstrated and reviewed [45,54,127,149–160].

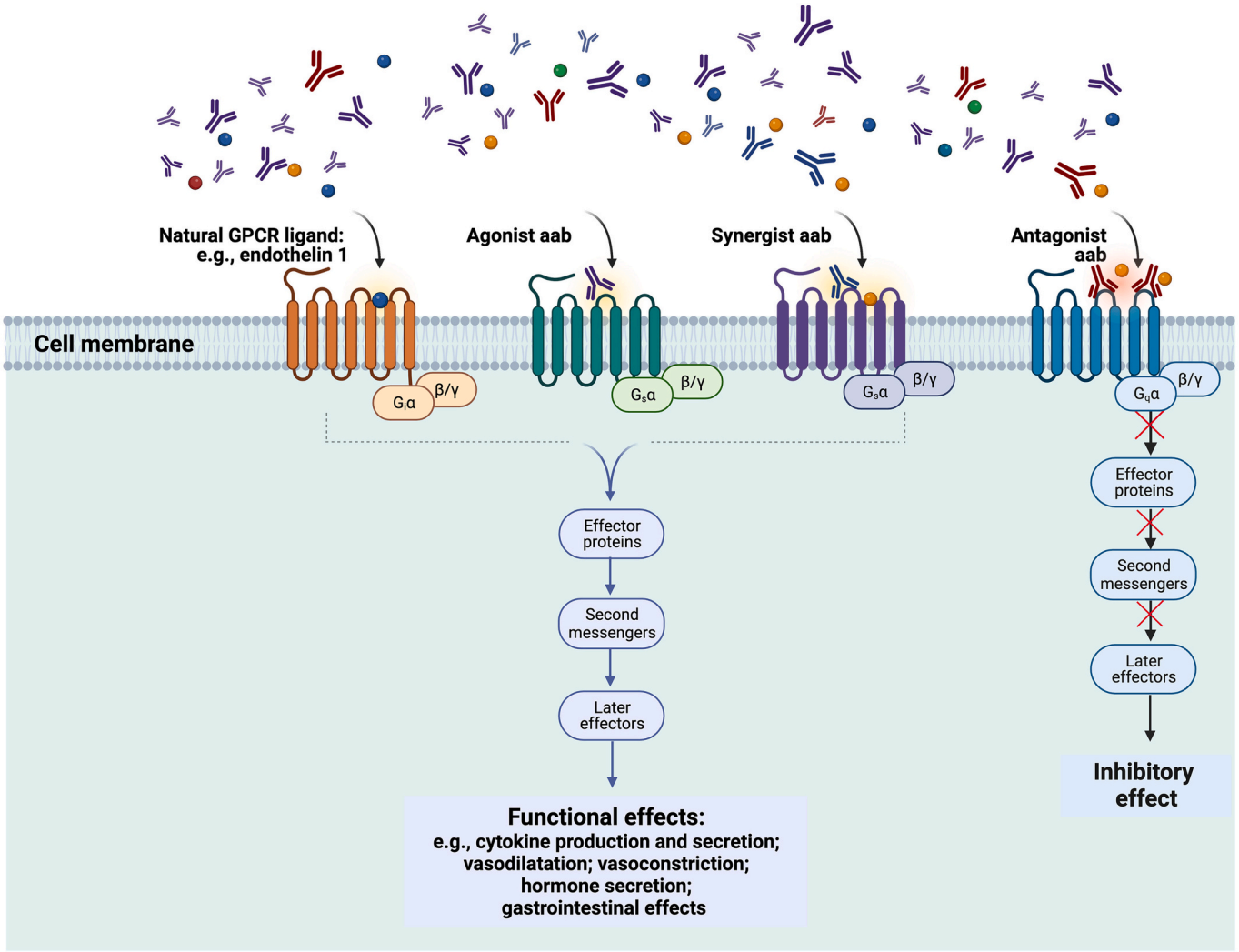

Fig. 4. Functional effects of anti-GPCR aab. These aab have agonistic, synergistic, or antagonistic effects upon receptor binding. Small circles represent other natural GPCR ligands. Created with BioRender.com.

6.1. Causal relationships

In particular, the pioneering experiments performed by Dragun et al. established the role of anti-AT1R-activating antibodies in renal allograft rejection [149] at a time when the critical role of alloantibodies directed against the human leukocyte antigens (HLA epitopes) in transplantation and rejection had already been comprehensively established. Using a cohort of 33 kidney transplant (KTx) recipients with refractory vascular rejections, of whom only 13 had donor-specific anti-HLA antibodies, whereas 20 had not, Dragun et al. were the first to provide solid proof for the involvement of non-HLA-directed antibodies in the development of kidney allograft rejection and vasculopathy. This study identified the direct role of agonistic RABs against the AT1R in the serum of transplanted patients with malignant hypertension without anti-HLA-antibodies but in none without agonistic RABs [149].

These RABs were identified to be of the immunoglobulin G (IgG) isotype, particularly IgG1 and IgG3 subclasses, and to bind to two different epitopes on the second extracellular loop of the AT1R. The expression of tissue factor (TF/CD142) was increased in renal tissue from KTx rejection patients who had developed these autoantibodies [149]. This mechanistic relationship was recently validated in detailed studies linking the role of vascular RABs targeting the AT1R and endothelin type A receptor (ETAR) to the triggering of coagulation [156]. Mechanistically, the in vitro stimulation of vascular cells with anti-AT1R-activating RABs induced phosphorylation of ERK 1/2 kinases and increased the DNA binding activity of the transcription factor activator protein 1 (AP-1) [149], while blocking studies with the AT1R antagonist losartan blocked the antagonistic AT1R-directed and RAB-mediated effects. Significantly, in a functional "proof-of-concept" approach, anti-AT1R-RAB-transfer induced vasculopathy and hypertension in a rat KTx-model [150]. These studies established solidly in vitro and in vivo proof, considering the molecular signaling pathways and respective functional outcomes in preclinical models and the concomitant clinical situation, that a non-HLA anti-AT1R-RAB-mediated signaling pathway contributes to refractory vascular rejection and that affected patients may benefit either from the removal of AT1R-directed antibodies or pharmacological blockade of the AT1R [150].

In support of these earlier studies on the role of AT1R- and ETAR-directed RABs, Guido Moll & Rusan Catar presented at the RAB Symposium in Lübeck new evidence for a clinical and functional role of protease-activated receptor 1 (PAR1)-directed RABs in KTx rejection (unpublished data). Similar to the role of coagulation in anti-AT1R-RAB-mediated initiation of vasculopathy, hypertension, and KTx rejection [150], PAR1-mediated signaling occurs via its natural ligand thrombin and may participate in clotting [161]. Here, both "pathogenic – disease-inducing" and "protective – pathology preventing" roles of PAR1-directed RABs might be involved, possibly contributing to the earlier described pathogenic role of AT1R in KTx rejection [149]. Indeed, recent systems biology RAB network analysis has shown a strong interdependency of GPCR-directed RABs in both immune pathology and homeostasis [14,20,32,45,54].

In conclusion, through close collaboration with the research groups of Cabral-Marques and Riemekasten, Moll & Catar et al. are now conducting an advanced, in-depth systems biology analysis of the underlying regulatory networks that include mechanistic side studies on the molecular, cellular, and functional levels. This approach will allow our research consortium to connect preclinical observations and clinical outcomes to decipher best the novel critical role of PAR1-directed RAB-signaling in solid organ transplantation. The results of these planned studies will contribute to a better understanding, timely diagnosis, and improved treatment options for rejection patients in the KTx setting and other related pathologies.

6.2. The binding of anti-GPCR autoantibodies to their receptors and the functional consequences

The mechanistic and functional analysis of anti-GPCR-directed RABs in the context of binding to GPCRs is significantly impacted by the complex structure of these receptors, with their 3D confirmation depending on the appropriate physiological embedding of the receptor subunits into the cell membrane. In addition, the presence of accessory molecules can further influence the conformation of these receptors and, thus, the accessibility of epitopes to RAB-binding [162]. Indeed, a crucial step in RAB research has been the development of an accurate detection system for RAB titers, which has been introduced by biotechnology companies such as CellTrend, who now offer suitable whole-cell-lysate detection systems with overexpression of specific receptors for a comprehensive panel of GPCRs, now exceeding assays for >20 different receptors, thus allowing systems biology analysis [32,45,163]. Functional and mechanistic assessment requires the presence of intact GPCRs in living cells. To control the degree of complexity of the system and allow for stepwise-validated conclusions, Catar et al. have developed a three-step model to study RABs in 1) Yeast cells (transfected with individual or combined GPCRs); 2) HEK cells (for studies of receptor internalization); and 3) Adult Human Cell Lines (HMECs, human microvascular endothelial cells) that most closely resemble their corresponding in vivo counterparts in cell signaling and functionality.

Notably, it is essential to distinguish autoantibodies that bind to GPCR with a "functional effect", by triggering or blocking intracellular signaling pathways, resulting in agonistic or antagonistic effects, respectively [14]. A challenge in this context is to distinguish orthosteric autoantibodies that are "bound" and "active" in promoting a response by themselves (thus substituting for the natural ligand, e.g., thrombin) or allosteric autoantibodies that are "bound and inactive by themselves," but can augment the response to a natural GPCR ligand (e.g., potentiating angiotensin or thrombin), for instance by changing the GPCR conformation or ligand binding.

7. Research challenges and opportunities

Despite the development of an increasing interest in anti-GPCR autoantibody biology, several challenges remain to be overcome, and they represent unique opportunities to expand this research field (Fig. 5). Studies that are designed to clarify how the binding of anti-GPCR autoantibodies to the extracellular regions/loops of GPCRs biochemically modulates/blocks the binding of their natural ligands, for instance, through steric inhibition of access or through altering the 3D conformation and flipping properties of the GPCR subunits, will considerably improve the understanding of anti-GPCR biology. Moreover, because autoimmune diseases are highly complex and multifaceted in their appearance, they typically affect selective or multiple organ systems, sometimes the whole body (systemic autoimmune diseases, e.g., SSc), or only affect compartmentalized body regions (organ-specific autoimmune disorders, e.g., type 1 diabetes). Therefore, one challenge to overcome is to characterize how anti-GPCR autoantibodies contribute to systemic vs. organ-specific autoimmune diseases. In this context, anti-GPCR autoantibodies are generally measured on blood samples due to its physiological importance as "high-way conduits" but also because it is one of the most accessible, and consequently, most studied sample material used in immunological studies that can be obtained from patients or healthy subjects in well-planned regular intervals, and the results often correlate with disease outcome. However, to investigate further the presence and action of anti-GPCR autoantibodies, it will be necessary to study other affected tissues, for instance, from patients with autoimmune diseases affecting the musculoskeletal system, joint cavities, or skin.

In parallel, considering the relevance of GPCRs for human physiology and pathophysiology, another essential issue that needs to be

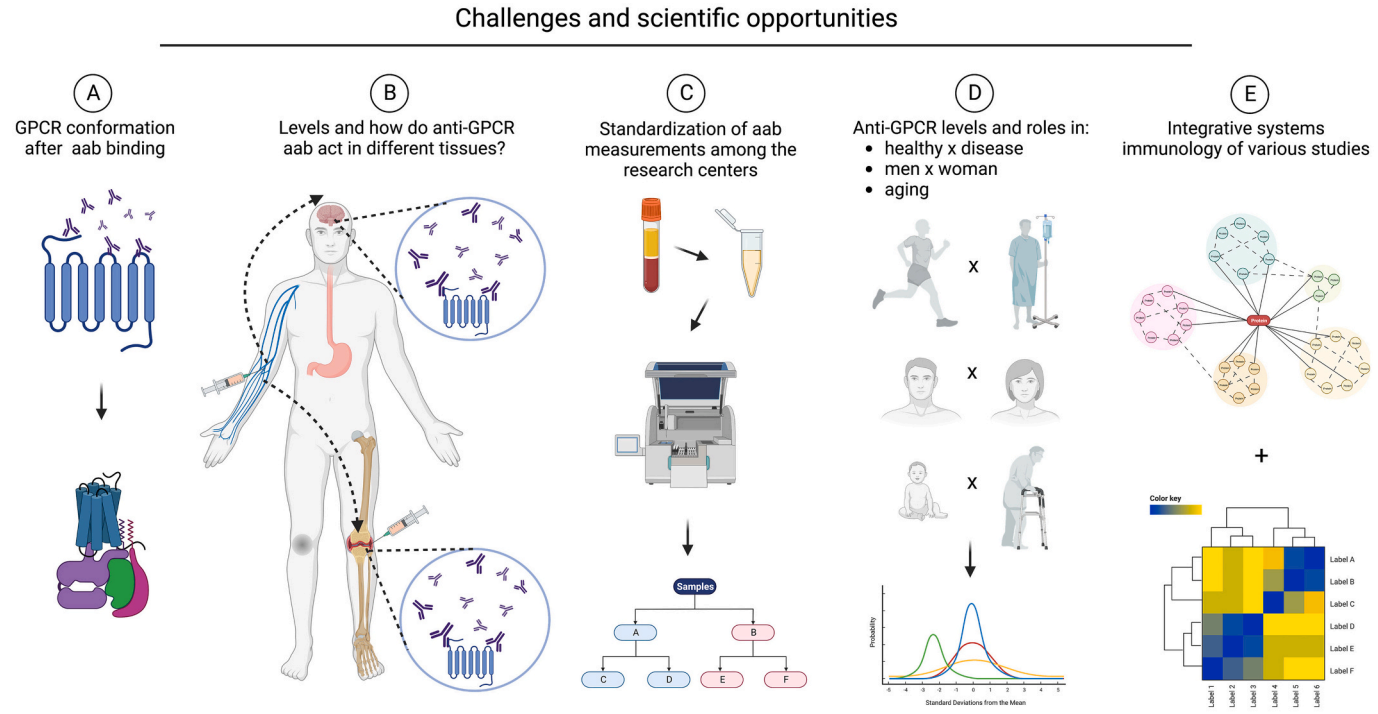

Fig. 5. Research challenges and opportunities that will expand the field of anti-GPCR aab investigation. From A to E, are issues that remain to be addressed. Created with BioRender.com.

addressed is the standardization of the serological tests among the different research groups investigating anti-GPCR autoantibodies. Since anti-GPCR autoantibody levels, depending on their specificity, have several confounders, such as age, sex, smoking status, co-morbidities, or BMI, identifying the autoantibody levels in HCs is challenging and must be taken into consideration. To reach this goal, it will be crucial, for instance, to perform systems integrative approaches that we plan to develop soon and to determine the expected levels of anti-GPCR autoantibodies in healthy individuals and disease conditions.

7.1. Are anti-GPCR autoantibodies natural components of the immune system?

We are still in the process of characterizing the biology of potential regulatory anti-GPCR autoantibodies. Accumulating evidence suggests that some autoantibodies are natural components of the immune system. Despite Paul Ehrlich's warning of the horror autotoxicus, and Macfarlane Burnet's postulate justifying the elimination of forbidden clones, autoantibodies are universally present in all healthy individuals and are considered to play beneficial homeostatic roles [6]. It is generally accepted that functional autoantibodies, including those targeting GPCRs, are also present in the sera of healthy individuals [14], although in lower quantity than in the sera of patients with autoimmune diseases [38,39,164–167]. Notably, GPCR-specific autoantibody signatures are associated with physiological and pathological immune homeostasis [45]. Since GPCR expression is found in immune cells and is highly variable among different tissues, the level of anti-GPCR autoantibodies could determine the localization and the strength of immune cell migration into various tissues. Thus, another challenge we must overcome to expand our comprehension of anti-GPCR biology is to investigate the anti-GPCR autoantibodies' biological evolution and conservation to distinguish when they act as modulators of homeostasis in healthy individuals and when they induce dysregulation, making them biomarkers of autoimmune and non-autoimmune pathologies [14,20,32,45,145,168]. Deciphering the individual function of each autoantibody will contribute to our understanding of relevant pathways and variations in disease phenotype and the different degrees of disease predisposition and severity.

8. Conclusions

In conclusion, the 4th Symposium in Lübeck provided an excellent opportunity to share and discuss the evolving investigation of anti-GPCR autoantibodies, serving as an international meeting that successfully expanded our understanding of autoimmunity. After several highly informative talks and discussions (some presented in this review), the highlights of the Symposium were summarized by the leaders of the Scientific Committee (Gabriela Riemekasten, Yehuda Shoenfeld, and Carmen Scheibenbogen). Since numerous therapies targeting GPCRs have been developed, the international scientific collaboration of scientists dedicated to investigating the biology of anti-GPCR autoantibodies may open new horizons for the development of novel therapeutic interventions, not only for autoimmune diseases but also for the reduction of morbidity and mortality caused by cancer, infectious diseases, metabolic disorders, or inflammatory conditions, in which the role of autoantibodies targeting GPCRs remains to be investigated.

Data availability

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

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