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Functional autoantibody diseases: Basics and treatment related to cardiomyopathies

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1. ABSTRACT

In the 1970s, autoantibodies directed against G-protein-coupled receptors (GPCR, GPCR-AAB) were discovered. After receptor binding, GPCR-AAB trigger uncontrolled receptor mediated signal cascades, thus producing pathologies. Diseases associated with such functionally active autoantibody type (functional autoantibodies) can be called "functional autoantibody diseases". Here we focus exclusively on GPCR-AAB directed against the GPCR's extracellular loops. The GPCR's role in the pathogenesis and progression is accepted in idiopathic dilated cardiomyopathy and is increasingly considered in diseases such as Chagas' cardiomyopathy, peripartum cardiomyopathy, hypertension, diabetes mellitus and scleroderma and even dementia, complex regional pain syndrome and postural orthostatic tachycardia syndrome. We briefly summarize the mechanistic background of GPCR-AAB induced pathologies, mainly focused on autoantibodies targeting the β1-adrenergic and muscarinic 2 receptors, due to their importance for cardiomyopathies. Furthermore, treatment strategies for "functional autoantibody diseases", such as for GPCR-AAB removal (therapeutic plasma exchange, immunoadsorption) and *in vivo* GPCR-AAB attack (intravenous IgG treatment, B-cell depletion, GPCR-AAB *in vivo* binding and neutralization) are critically reflected with respect to their patient benefits focused on but not exclusive to patients with dilated cardiomyopathy.

2. INTRODUCTION

The immune system recognizes potentially harmful foreign material invading the body and organizes the body's defense against it in order to minimize the material's pathogenic potency and prevent disastrous consequences for the organism. The body's own materials such as macro-molecules, cells and tissues are generally excluded from immune attacks. However, this self-tolerance of the body can be broken; the immune system can generate antibodies (autoantibodies) and activate cell-mediated immune processes, both directed against the body's own material (self-antigens).

For the loss of self-tolerance, which is called "autoimmunity", the innate and adaptive immune system are highly interconnected at several levels (1). Subliminal autoimmunity is always present in all individuals, but is frequently more pronounced in older subjects. Autoimmune diseases are the result of over-boarding autoimmunity. The individuals' genetic and epigenetic background (2) as well as environmental conditions (3) are important prerequisites for breaking self-tolerance and consequently autoimmune diseases. As derived from animal experiments (4) and demonstrated for individual autoimmune diseases such as Sjögren Syndrome (5), dysregulation may arise in youth and manifest as an autoimmune disease later in life.

The American Autoimmune Related Disease Association (6) listed 80 to 100 different autoimmune diseases. Furthermore, at least 40 additional diseases are suspected to have an autoimmune basis. In accordance with this, the Autoimmune Registry, Inc., ARI (7), a non-profit corporation founded in 2016, published a list including more than 150 diseases based on medical sources at the National Library of Medicine (PubMed) that are most likely caused by autoimmunity. This means that 6.5.-14% of US-Americans (between 23 and 50 million people) suffer from an autoimmune disease. The typical hallmark of these autoimmune diseases are "classic autoantibodies", which were very frequently found in the affected patients. Classic autoantibodies predominantly induce regular immune responses, ultimately leading to destruction of affected tissues.

However, in the second half of the 1970s, an additional antibody type was discovered in patients with Grave's disease (Morbus Basedow) (8) and in those with cardiomyopathy (9, 10). Inflammatory or destruction injury is not invariably the consequence of the autoantibodies' attacks on cells, tissues or organs, which is clearly in contrast with classic autoantibodies. This new type of autoantibody (GPCR-AAB) specifically binds to receptors on the cell surface, to the best of our knowledge, to G-protein coupled receptors (GPCR). The extracellular domains of the receptor protein are targets of autoimmune recognition. The GPCR-AAB in patients with Grave's disease, specifically the highly-affine and stimulating autoantibodies directed against the TSH receptor, are directed against N-terminal located epitopes (11). The GPCR-AAB indicated in Tab. 1 are directed against the extracellular loops of the GPCR. Therefore, this new type of autoantibody can be called "functional autoantibodies". In the following discussion, "GPCR-AAB" stands exclusively for this type of autoantibodies. GPCR-AAB compete with physiologically-specific receptor ligands. After binding to GPCR, the autoantibodies can reduce the activity of physiological ligands and exert stimulatory and inhibitory effects on the receptors. In this way, the autoantibodies lead to modulation of the receptor-mediated signal cascade with an impact on physiological functions. After physiological and pharmacological ligand binding, over-boarding receptor modulation is controlled by prevention mechanisms such as receptor down-regulation and the desensitization of signal transduction. Such mechanisms, most importantly, are missing when GPCR-AAB bind to the related receptors. Consequently, GPCR-AAB induce disturbed metabolic balance and pathological conditions, which are crucial points in GPCR-AAB associated autoimmunity.

The story of functional autoantibodies in patients with cardiomyopathy primarily focused on patients with Chagas' cardiomyopathy was initiated by the Argentinian group of Sterin-Borda, who discovered an antibody in patients' serum that acts as an agonist of the β1-adrenergic receptor (β1-AAB) (9, 10).

These agonistic GPCR-AAB recognize epitopes localized on the first or second extracellular loop of the β1-adrenergic receptors (9, 10, 12), as do the majority of the other agonistic GPCR-AAB discovered to date and indicated in Table 1. Inhibitory GPCR-AAB such as autoantibodies directed against the β2-adrenergic receptor, found in patients with allergic asthma, block receptor activation by relevant agonists. These autoantibodies target the third extracellular receptor loop (13).

As is already known for classic autoantibodies, GPCR-AAB are present in a subset of healthy individuals at low levels. In the case of the excessive generation of GPCR-AAB, the related pathophysiology introduces a new class of autoimmune diseases which we have named "functional autoantibody diseases". Table 1 presents diseases with their corresponding GPCR-AAB that can be classified as "functional autoantibody diseases".

Many of these diseases (e.g. scleroderma, systemic lupus) were, due to their association with different classic autoantibodies, already categorized as autoimmune diseases according to (6, 7). The finding of GPCR-AAB in patients suffering from such diseases supplemented and manifested the diseases' autoimmune background. However, more research is necessary to clarify whether GPCR-AAB possess any causal or at least supporting role in the whole autoimmunity concert in these diseases. This is also true for diseases (like e.g. diabetes mellitus type 2, Alzheimer's disease, prostate hyperplasia) where GPCR-AAB are possibly supportive in the disease manifestation. Once it is better understood which role the GPCR-AAB are playing, this, in our view, would possibly implicate different treatment options. In contrast, there are other diseases indicated in Table 1 (e.g. dilated cardiomyopathy, regional pain syndrome, different form of hypertension, preeclampsia) for which, based on animal and human studies, evidence exists for the role of GPCR-AAB as predominant pathogenic players. This knowledge, in our view, should be exploited for the development of novel treatment strategies clearly focused on GPCR-AAB.

Since the beginning of the story of GPCR-AAB as causative or at least supportive players in disease pathology, two different treatment lines have become obvious: first, the elimination of GPCR-AAB from the patients' circulation, and second, *in vivo* treatment for GPCR-AAB attack. In both strategies, there are technologies designed which either globally attack the autoimmunity or are specifically directed against any group of autoimmune players or even specific players. For the elimination of GPCR-AAB from the patient's circulation, technologies were developed such as unselective plasmapheresis (therapeutic plasma exchange; TPE) or immunoadsorption of all immunoglobulins, any IgG class, a specific IgG subclass and even a specific GPCR-AAB. Therapeutic approaches to directly suppress the generation of pathogenic GPCR-AAB and/or their activity in the patients' blood are intravenous IgG treatment (IVIG) and B-cell depletion therapies. In the future, these could be supplemented by strategies for the *in vivo* binding and neutralization of GPCR-AAB which are presently under development.

Due to the relevance of cardiovascular diseases, mainly that of cardiomyopathies, in the history of GPCR-AAB research and due to the evidence for any causal or at least supportive role of GPCR-AAB in pathological processes in the cardiovascular system, GPCR-AAB-directed treatment started in patients with cardiomyopathies. Cardiomyopathies, together with diseases that are tightly associated with cardiovascular alterations, is also currently the predominant indication and will remain the focus for now. However, there are further diseases with GPCR-AAB positivity listed in Table 1 such as thromboangiitis obliterans (41, 52), dementia and Alzheimer's disease (43-46) and benign prostate hyperplasia (53), as well as disorders presenting with signs typical for postural orthostatic tachycardia syndrome (POTS) (34-36), complex regional rain syndrome (CRPS) (48-50), and fatigue syndrome (51). However, also these diseases are currently gaining more attention because patient benefit is expected from GPCR-AAB-directed treatment. With respect to the neurologic disorders and their serum GPCR-AAB positivity, no studies, to the best of our knowledge, have been published which presented data for GPCR-AAB presence parallel in the liquor but data from such studies would be of high interest to clarify whether GPCR-AAB cross the blood-brain barrier or are generated by the CNS immune system.

With respect to the number of diseases presently summarized under autoimmune diseases (6, 7), it would be not surprising to us if, step by step, other functional autoantibody diseases extend that list.

3. BASICS

3.1. G-protein-coupled receptors

"… G protein coupled receptors (GPCR)… represent by far the largest, most versatile and most ubiquitous of the several families of plasma membrane receptors. They comprise almost a thousand genes

Table1. Diseases with the properties of functional autoantibody diseases

Disease	GPCR-AAB directed against ( ) - receptor	Activity	BC 007 sensitivity	References
Idiopathic dilated cardiomyopathy	β1-adrenergic	agonistic	+	(12, 14-19)
	muscarinic M2	agonistic	+	(17, 19)
Peripartum cardiomyopathy	β1-adrenergic	agonistic	+	(20, 21)
	muscarinic M2	agonistic	+	(20, 21)
Chagas' cardiomyopathy	β1-adrenergic	agonistic	+	(22, 23)
	muscarinic M2	agonistic	+	(22, 23)
	β2-adrenergic	agonistic	+	(22)
Myocarditis	β1-adrenergic	agonistic	+	(24)
Electric cardiac abnormalities	β1-adrenergic	agonistic	+	Rev. (25-27)
	muscarinic M2	agonistic	n.t.	Rev. (25-27)
	β2-adrenergic	agonistic	n.t.	Rev. (26)
	serotoninergic 5HT4	n.d.	n.t.	(28, 29)
Refractory hypertension	α1-adrenergic	agonistic	+	(30)
Idiopathic pulmonary hypertension	α1-adrenergic	agonistic	+	(31)
	endothelin 1 ETA	agonistic	+	(31)
Malignant hypertension	angiotensin II AT1	agonistic	+	(32)
Preeclampsia	angiotensin II AT1	agonistic	+	Rev. (33)
	endothelin 1 ETA	agonistic	+	u.p.
Orthostatic hypotension	β2-adrenergic	agonistic	+	Rev. (33)
	muscarinic M3	n.d.	n.t.	Rev. (33)
Postural orthostatic tachycardia syndrome (POTS)	β1-adrenergic	agonistic	+	(34, 35)
	β2-adrenergic	agonistic	+	(34, 35)
	α1-adrenergic	agonistic	+	(35, 36)
	muscarinic M2	agonistic	+	u.p.
	angiotensin II AT1	agonistic	+	(36)
Diabetes mellitus type II	α1-adrenergic	agonistic	+	(37)
Vascular renal rejection	angiotensin II AT1	agonistic	+	(38, 39)
Scleroderma	angiotensin II AT1	agonistic	+	Rev. (40)
	endothelin 1 ETA	agonistic	+	Rev. (40)
Thromboangiitis obliterans	α1-adrenergic	agonistic	n.t.	(41)
	endothelin 1 ETA	agonistic	n.t.	(41)
	angiotensin II AT1	agonistic	n.t.	(41)
Systemic lupus erythematosus	serotoninergic 5HT4	antagonistic	+	(28)
Allergic asthma	β2-Adrenergic	inhibitory	n.t.	(42)
Open angle glaucoma	β2-Adrenergic	agonistic	+	rev. (33)
Vascular dementia / Alzheimer's dementia	α1-adrenergic	agonistic	+	(43-45)
	β2-adrenergic	agonistic	+	(43-45)
	endothelin 1 ETA	agonistic	+	(45)
	angiotensin II AT1	n.d.	n.t.	(46)
Benign prostate hyperplasia	endothelin 1 ETA	agonistic	+	(47)
Complex regional pain syndrome (CRPS)	muscarinic M2	agonistic	+	(48-50)
	β2-adrenergic	agonistic	+	(48-50)

Sjögren's syndrome	muscarinic M3	agonistic	n.t.	rev. (33)
Fatigue syndrome	β2-adrenergic	agonistic	+	(51)
	muscarinic M2	agonistic	+	(51)
	muscarinic M3	n.d.	n.t.	(51)
	muscarinic M4	n.d.	n.t.	(51)
Post cancer chemotherapy	α1-adrenergic	agonistic	+	(rev.) (33)
	angiotensin 1–7 Mas	agonistic	+	(rev.) (33)
Periodontitis	β1-adrenergic	agonistic	+	(rev.) (33)

Based on representative references, their corresponding autoantibodies directed against G-protein coupled receptors that are potentially targets for treatment such as the application of the aptamer BC 007 (see 4.2.3.2.2.) for the *in vivo* autoantibody inhibition (n.d. - not determined, n.t. – not tested; u.p. – unpublished; rev. – review)

which regulate virtually all known physiological processes in humans including the sensory modalities of vision, taste and smell. Moreover, these receptors are the targets for drugs accounting for more than half of all prescription drug sales in the world." This was stated by Robert J. Lefkowitz (54), to which Brian Kobilka (55) added "… (GPCR) respond to a broad spectrum of chemical entities ranging from photons, protons and calcium ions, and small organic molecules (including odorants and neurotransmitters), to peptides and glycoproteins (including functional autoantibodies). … The classical role of a GPCR is to detect the presence of an extracellular agonist, transmit the information across the plasma membrane, and activate a cytoplasmic heterotrimeric G protein, leading to modulation of downstream effector proteins"; both of these statements were made by the Nobel Prize laureates, awarded in 2012 *"for studies of G-protein-coupled receptors",* in their Prize lectures.

GPCR are integral membrane proteins. Their amino acid chain forms seven transmembrane regions resulting in the extracellular N-terminal, intracellular C-terminal domains, and three extracellular and three intracellular loops. Glycosidic moieties and disulfide bridges contribute to GRCR stability and functionality and are consequently involved in the regulation of receptor response to agonists and antagonists. The classic physiological and pharmacological ligands target a hydrophobic pocket of the GPCR for binding. However, GPCR "… cannot be described as simple bimodal 'on–off' switches, but should rather be viewed as highly dynamic systems that exist in a multitude of functionally distinct conformations … (whereby). ligands can regulate the receptor activity through conformational selection of distinct states…"(56). Related to stabilize the state of activation, as summarized in (57), a shift from the monomeric to the dimeric state of the GPCR with formation of either homo- or hetero-oligomeric receptor complexes have been discussed for many of the GPCR. Compared with monomers, the dimers show different agonist affinity and efficacy resulting at last in different biological answers as discussed in (58).

It is well accepted, that extracellular ligand binding induces a cycle of G-protein activation and inactivation localized on the intracellular receptor side, which modulates the activity of enzymes and ion channels downstream, regulating the formation and concentration of cytosolic second messengers. However as recently summarized in (58), novel modes of GPCR activation including different G protein subtypes and regulatory proteins, such as arrestins have been proposed to supplement the classic G-protein associated signaling which allow to understand better the very complex functional and structural changes seen after ligand binding to GPCR.

It was suggested that dimerization of GPCR switches the signal transduction from G-protein to arrestin. Due to the importance of GPCR in the regulation of signal transduction from the extracellular environment to the internal metabolic machinery, disturbances in the regulation of these highly-complex signaling pathways can cause a shift in the metabolic balance and may induce pathological conditions.

3.2. Autoantibodies against G-protein coupled receptors

As already mentioned above, the extracellular loops of the receptor protein are targets of autoimmune recognition and after binding to receptors, GPCR-AAB can exert stimulatory and inhibitory effects on the receptors as indicated in Table 1. The stimulatory or agonistic GPCR-AAB activate the receptors as do physiological and pharmacological agonists. This leads to activation of the receptor-mediated signal cascade. Inhibitory GPCR-AAB, e.g. β2-AAB, block the receptors and prevent their activation through the relevant agonists. These GPCR-AAB act via the third extracellular receptor loop. We have first indication that GPCR-AAB can also synergistically work together with physiological or pharmacological ligands; e.g. if spontaneously beating rat cardiomyocytes were pre-incubated with autoantibodies directed against the β2-adrenergic receptor and thereafter treated with the agonist clenbuterol (unpublished data).

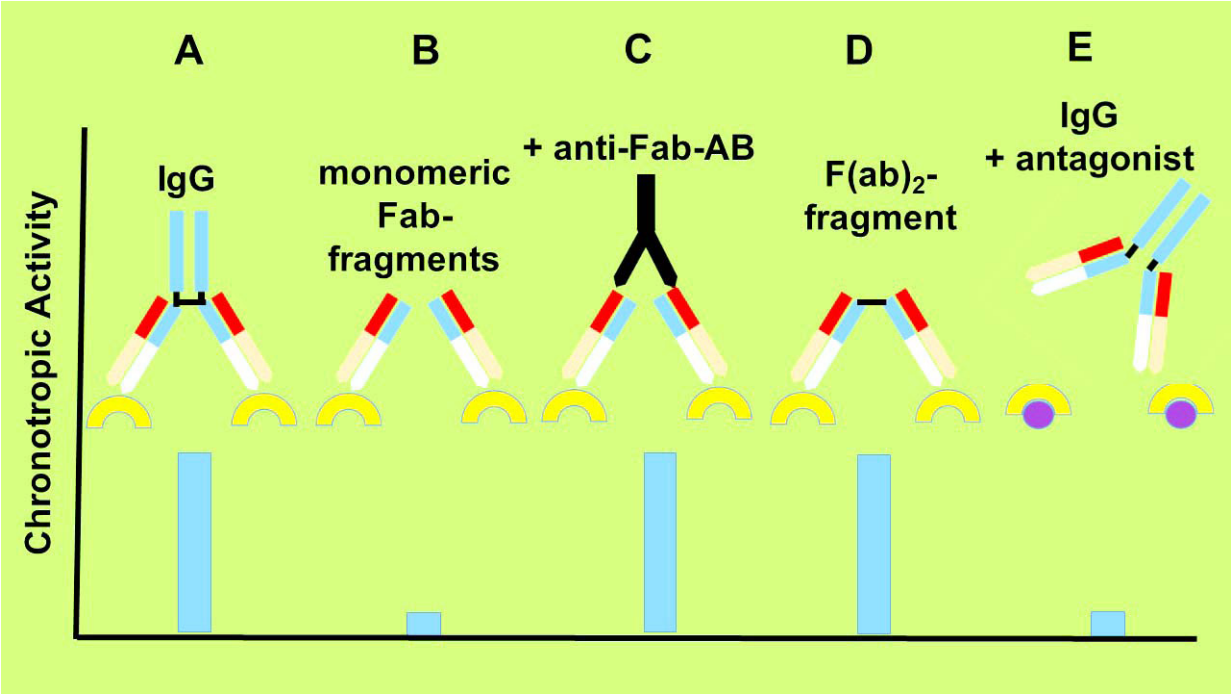

Figure 1. Cross-linking of G-protein coupled receptors by the corresponding autoantibody as prerequisite of signaling, illustrated using results of (59, 60, 63) The chronotropic activity of autoantibodies directed against the β1-adrenergic receptor was demonstrated using the bioassay of spontaneously beating cultured neonatal rat cardiomyocytes. A: receptor cross-linking by the autoantibody à activity, B: Fab monomers bind to receptor (no receptor cross-linking) à no activity, C: receptor cross-linking by Fab monomers cross-linked with anti-Fab-antibodies à activity, D: bivalent Fab construct à activity, E: chronotropic activity can be blocked by receptor antagonists (control experiment).

3.2.1. Signaling and pathophysiological consequences of autoantibodies against G-protein-coupled receptors

3.2.1.1. Subcellular and cellular level

It has been hypothesized that GPCA-AAB, due to their bivalent IgG structure, are ideally suited to cross-link receptors and therefore probably are capable to induce and stabilize the active receptor state like physiological and pharmacological ligands by inducing receptor dimerization. This was demonstrated for β1-AAB, for autoantibodies directed against the β2-adrenergic receptor (β2-AAB), and muscarinic 2 receptor (M2-AAB) (59-63); however, in our view, possibly being a general phenomenon of GPCR-AAB.

In support of this hypothesis, as illustrated in Figure 1 using results from (59, 60, 63), it was shown that monovalent Fab fragments of the GPCR-AAB, despite being the obvious receptor binding site, are unable to induce any agonistic-like effect such as cAMP accumulation and chronotropic response in spontaneously beating neonatal rat cardiomyocytes. When the bound monovalent Fab fragments were cross-linked with an anti-Fab antibody, cardiomyocytes responded with an agonistic effect which was comparable with the effect of the intact GPCR-AAB and a bivalent Fab construct.

As an alternative mechanism of GPCR-AAB receptor interaction (64) - which is not in agreement with the GPCR activating potency of bivalent Fab constructs as well as with the activity of monomeric Fab cross-linked by anti-Fab antibodies as demonstrated in Figure 1 - GPCR-AAB could bind via their highly-variable region to the receptor epitope, but their Fc fragments subsequently crosslink to the cardiac Fc (gamma) - receptor IIa. However, this concept was derived from only a single set of experiments. Further supporting experiments by the investigator or confirmation by others have not been performed, to the best of our knowledge.

GPCR-AAB induced receptor cross-linking is thought to be one of the key events which are responsible for the lack in regulatory mechanisms such as receptor desensitization and internalization with the consequence of over-boarding and long-lasting receptor stimulation which can result in disturbed metabolic balance and pathologic conditions. This is clearly in contrast to the receptor state after the binding of physiological or pharmacological ligands, where receptor internalization and desensitization counteract over-boarding and long-lasting receptor activation and signal transduction, thus protecting individuals from disturbed metabolic balance and pathologic conditions. The lack of tachyphylaxia has been observed for several GPCR-AAB and therefore very likely plays a

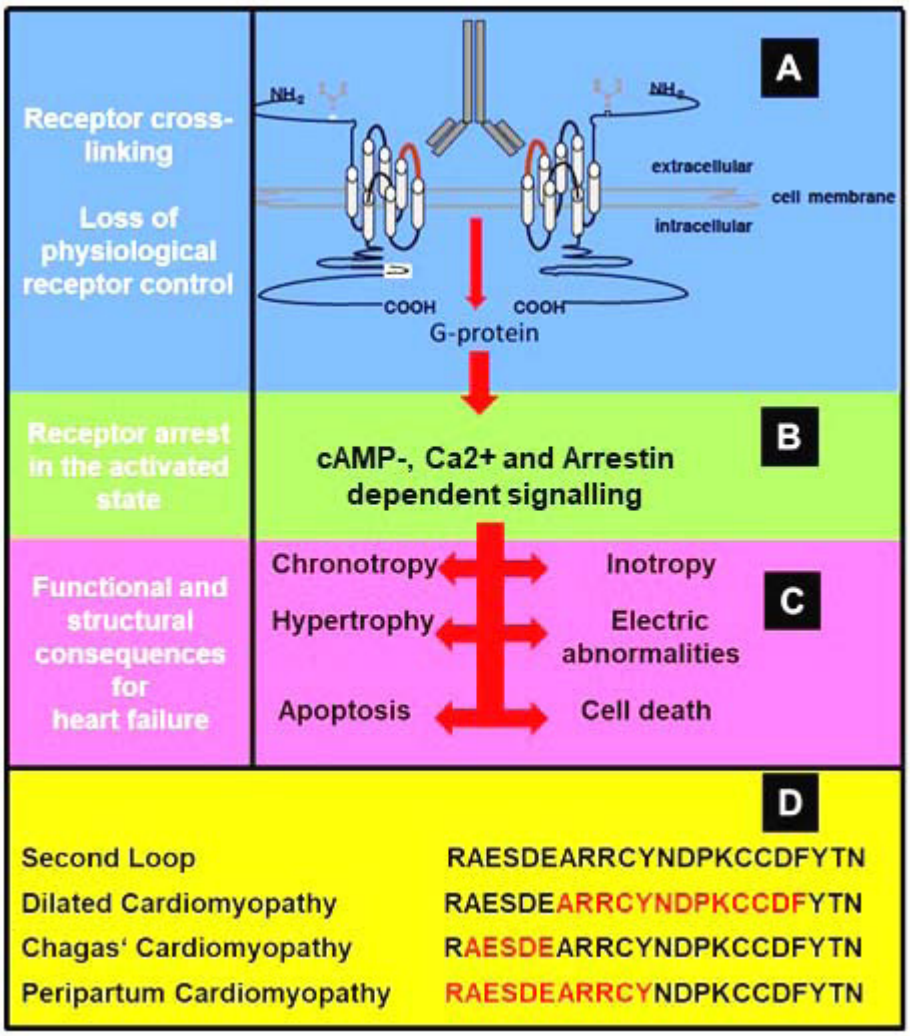

Figure 2. Schematic view of functional autoantibody diseases induced by autoantibodies directed against G-protein-coupled receptors, exemplarily demonstrated for cardiomyopathy and autoantibodies directed against the β1-adrenergic receptor. A: GPCR-AAB cross-link two receptors for activation which block receptor mechanisms for activity control in parallel. B: Permanent activation of "physiologic" second messenger mechanisms. C: Activation of cellular mechanisms for functional and structural cell and tissue alterations. D: Disease-related epitope on the second extracellular loop of the β1-adrenergic receptor.

key role in the pathogenesis of GPCR-AAB-associated diseases (65-68). In a recent study focused to β1-AAB, this effect was demonstrated for IgG preparations of the majority of patients with DCM, but for IgG of a minority this effect failed (69). Interestingly, an even opposite effect was described for β1-AAB in the first publication of this subject (70). In a more detailed experimental setting, the opposite effect of β1-AAB on cardiomyocytes was only seen following strongly extended incubation time (71).

Figure 2, exemplarily for cardiomyopathies and β1-AAB, illustrates schematically the pathway from GPCR-AAB binding to pathology. The schematic structure of GPCR and the communication with a GPCR-AAB directed to the second extracellular receptor loop, to receptor cross-linking and the resulting loss of receptor control are demonstrated in (A). GPCR-AAB which target the first loop of the GPCR work in the same way. The resulting arrest of the receptor in the activated state associated with the over-boarding and long-lasting stimulation of intracellular signaling mechanisms are indicated in (B). In consequence, this leads to disturbed metabolic balance, resulting in pathologies (C). In (D), it is demonstrated that, with regards to the β1-adrenergic receptor and its communication with the related autoantibody directed against the second extracellular receptor loop, the autoantibodies target slightly different epitopes on the second extracellular receptor loop depending on the underlying disease (idiopathic dilated cardiomyopathy, Chagas' cardiomyopathy, peripartum cardiomyopathy).

Specific metabolic conditions such as those induced by hypoxia, ischemia and/or inflammation

could be a prerequisite for or at least supportive of full GPCR-AAB activity. In line with this hypothesis, autoantibodies directed against the angiotensin II receptor type 1 (AT1-AAB) induce their vasoconstrictive effect in ischemic but not in non-ischemic arteries (72) and β2-AAB realize their effects only in cultured cardiomyocytes that were partially under-supplied with oxygen; this was not seen in cells with an optimal oxygen supply. However, the latter cells became β2-AAB-sensitive after the addition of lactate (42). In summary, it appears that GPCR autoantibodies only have pathogenic effects on damaged tissue.

Downstream of the GPCR-AAB receptor binding, different effects are documented. Those that are associated with cAMP dependent cascades and the MAPK/ERK pathway are most prominent. After β1-AAB receptor binding, the activation of adenylate cyclase and consequently elevated concentration of cyclic AMP have been observed (14, 73). However, not all of the patients' β1-AAB preparations showed this effect (14). Activation of protein kinase A (74) was evidenced. Elongation of the action potential duration and an increase of the L-type Ca++ (ICa) current are also seen (62, 75, 76), as well as changes in the mitochondrial structure and membrane potential (77), apoptosis induction and cell death (78-81). For cardiac mast cells, maturation and degranulation were observed (82). Via activation of the MAPK/ERK pathway, β1-AAB could support mechanisms important for cardiac remodeling (83, 84). When β1-AAB targeted their related receptor on T cells, changes in T-cell proliferation and secretion occurred via activation of the β1-AR/cAMP/PKA and p38-MAPK pathways (85). In the rat atrium, β1-AAB led to increased prostaglandin E2 production and induced the release of soluble CD40L, mediating a pro-inflammatory response (86).

For M2-AAB, a negative chronotropic effect on cardiomyocytes has been evidenced, associated with the blocking of cardiac parasympathetic innervation (87). This effect was attributed to the M2-AAB-induced inhibition of the L-type Ca^{2+} current. Cyclic GMP increase was also demonstrated (25). Furthermore, an increase of the outward potassium current in the presence of M2-AAB was observed. All of these factors together could be responsible for the electric abnormalities in the heart found in the presence of M2-AAB (25, 88, 89). M2-AAB interfere with the regulation of COX-2 and iNOS mRNA to produce pro-inflammatory conditions (90).

With regards to receptor stimulation by AT1- and ETA-AAB, and specifically focused on systemic sclerosis, downstream events have been summarized in (40) which affected the interleukins, oxygen species and growth factor balance, chemotaxis, cell migration, proliferation, angiogenesis thrombosis and fibrosis, among others.

3.2.1.2. Animal models

The role of GPCR-AAB as pathogenic drivers was substantiated in animal models with the main focus on the development of signs for cardiomyopathies.

After the immunization of rabbits with peptides corresponding to the second extracellular loop of the β1-adrenergic receptor and muscarinic 2 receptor, the generation of β1- and M2-AAB has been evidenced, which was followed by structural and functional heart alterations which are typical for heart failure: left ventricular hypertrophy with mild inflammatory cell infiltration, mild or moderate fibrosis and electron microscopically evidenced focal myofibrillar lysis, loss of myofilament, mitochondrial swelling and condensation, sarcoplasmic vacuolation, and deposition of dense granules in the sarcoplasm and myofibrils (91, 92). Using a comparable study design for rat immunization, a peptide derived from the β1-AAB epitope of the second extracellular loop, induced anatomical, hemodynamic and echocardiographic alterations that are typical of heart failure. Most importantly, if previously healthy animals were treated with β1-AAB and M2-AAB, produced by immunization, the animals developed heart failure (93, 94). Signs of heart failure were also developed if preparations and/or lymphocytes from cardiomyopathic rabbits or DCM patients were transferred into mice (95). Rats immunized for β1-AAB generation presented with cardiac arrhythmias (96).

For Chagas' cardiomyopathy, as recently summarized in (97), some evidence exists for breaking self-tolerance: Antigens presented by the *T. cruzi* parasite, such as ribosomal P and B13 proteins, and animal and human heart antigens are cross-reactive; these include the β1- and β2-adrenergic receptors as well the muscarinic 2 receptor. In accordance with this finding, the related autoantibodies were frequently found in Chagas' heart failure patients (22). Already demonstrated in the 1970s and 1980s, the injection of *T. cruzi* subcellular preparations induced inflammation in rabbit and mouse hearts (98, 99). In addition to second extracellular loop targeting β1- and M2-AAB in Chagas' heart failure patients, autoantibodies against the third intracellular loop of the muscarinic 2 receptor were identified by Western blotting (100) but, to the best of our knowledge, any confirmation of this finding is missing.

Another strategy for proving the pathogenic role of GPCR-AAB, mainly that of the β1-AAB, relies on animal studies designed to prevent the AAB effects. In case of immunized rats for β1-AAB generation, the development of heart failure symptoms was completely prevented when animals were treated with a peptide competing with the β1-AAB for receptor binding (101). "Therapeutic peptides" have also been used in a

mouse model of Chagas' disease (102). The treatment of Chagasic mice, which display acetylcholine receptor-related dysfunction, with peptides derived from the muscarinic 2 receptor prevented the typical dysfunctions observed in Chagas' mice, such as a decrease in heart contractility, impaired response to carbachol and a significant reduction of acetylcholine receptor-binding sites.

However, in the future, to manifest the pathogenic function of GPCR-AAB in general but more importantly to specify their role in each of the diseases where this potential pathogen could play any role, it will be necessary to use already-existing specific disease-related animal models or to establish new models representing distinct diseases with the presence of GPCR-AAB. The model of spontaneously hypertensive rats (SHR), which present with positivity for β1- and M2-AAB in older age (103) and Doberman dogs suffering from DCM and being positive for β1-AAB could also be used to establish GPCR-AAB-related treatment strategies. As another example, to clarify the role of AT1- and autoantibodies directed against the endothelin A receptor (ETA-AAB) in preeclampsia and to develop GPCR-AAB-related treatment concepts, the reduced uterine perfusion pressure (RUPP) rat model is available for studying cardiovascular-renal dysfunction (104, 105). A GPCR-AAB-related mouse model of systemic sclerosis is currently under development.

3.2.1.3. Clinical findings related to healthy subjects

The majority of the GPCR-AAB which are listed in Table 1 were also found with low prevalence in healthy subjects, just like classic autoantibodies. In the largest study currently available, a prevalence of approximately 10% for the β1-AAB and M2-AAB was found in 408 healthy individuals, with GPCR-AAB titers increasing with age. A remarkably high co-existence of both of these AAB of nearly 65% was found (106). Clustering of GPCR-AAB seems to be a typical phenomenon in both healthy subjects and even more in patients. Reviewing the currently available literature, we calculated a prevalence of β1-AAB and M2-AAB in healthy subjects of up to 20% each, which seems to be slightly higher than e.g. that for classic autoantibodies such as AAB against cardiac myosin and troponin (107). For other GPCR-AAB such as β2-, α1-, and ETA-AAB, the prevalence in healthy subjects is <15% in general (108-110). In conclusion, it can be assumed that a significant proportion, probably higher than 30%, of healthy individuals could be carriers of at least one GPCR-AAB. However, the data used for this assessment are based predominantly on measurements, with well-known problems when used for the GPCR-AAB measurement (111-114). Using the bioassay of cultured spontaneously-beating neonatal rat cardiomyocytes, which was unfortunately applied only in a small cohort of healthy individuals, the prevalence of β1-, β2- and M2-AAB was each clearly lower than 5%.

It is unclear whether finding a specific GPCR-AAB or a specific cluster of GPCR-AAB in the healthy subject can predict the development of any of the related diseases. However, for patients with systemic sclerosis, a higher GPCR-AAB level predicted disease severity and patient mortality (108) We showed that among patients with Chagas' disease who are asymptomatic (asymptomatic Chagas' disease), 30% are carriers of β1-, β2-, and M2-AAB. Because approximately 30% of the Chagas' patients develop life-threatening complications (preferentially cardiomyopathy), sometimes after decades, as shown by epidemiological data, and since nearly all of these patients were positive for GPCR-AAB, the symptomless but GPCR-AAB positive Chagas' patients could be those who progress to the development of Chagas' cardiomyopathy and/or gastrointestinal mega syndromes (22). Consequently, finding GPCR-AAB in asymptomatic Chagas' patients may indicate their risk for the life-threatening complications of Chagas' disease. For supporting this hypothesis, we found in a 31 month follow-up study that from 21 primarily asymptomatic but GPCR-AAB positive Chagas' patients, two developed symptomatic Chagas' disease whereas none of the asymptomatic but AAB-negative patients reported clinical symptoms or had other characteristics indicative of disease progression (115). Furthermore, the β1-AAB activity in Chagas' patients increased from GPCR-AAB-positive asymptomatic patients via those with mild cardiomyopathy to the patients with severe cardiomyopathy (22). This finding could support the predictive value of GPCR-AAB in Chagas' disease in general and specifically with regards to severity.

3.2.1.4. Clinical findings related to diseased subjects

3.2.1.4.1. General remarks

For the diseases listed in Table 1, the GPCR-AAB prevalence can be as high as 100% with a high co-existence of several GPCR-AAB, indicating disease-specific clustering of the GPCR-AAB. The cluster e.g. of cardiomyopathy patients predominantly contained β1- and M2-AAB, while that of patients with hypertension often contained α1-AAB, AT1-AAB and ETA-AAB. In patients with scleroderma, a predominant co-existence of AT1-AAB and ETA-AAB was found. Clusters also exist for many other diseases, such as diabetes mellitus and Alzheimer's disease.

In the case of co-morbidities like DCM associated with diabetes mellitus or with hypertension,

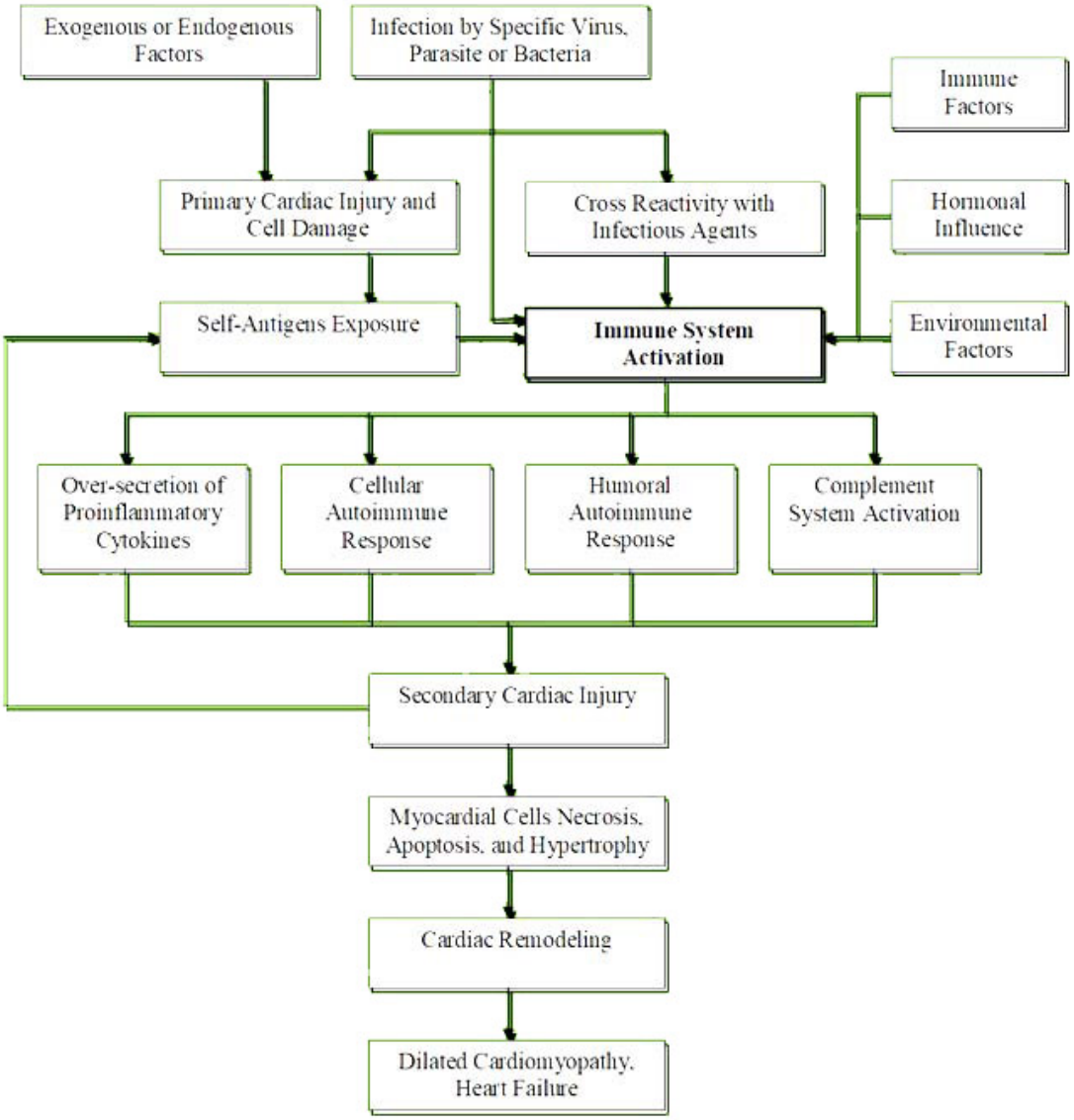

Figure 3. The pathogenesis of dilated cardiomyopathy due to immune-mediated response; reproduced with the permission from (116).

the primary GPCR-AAB clusters can be combined with the GPCR-AAB of the associated diseases. It is therefore often difficult to identify a relationship between a disease-specific phenotype and a specific GPCR-AAB.

Since the first indications that GPCR-AAB play a role in human diseases, the pathogenic role of GPCR-AAB in cardiomyopathies and consequently in heart failure were the focus of many research activities. Consequently, cardiomyopathies and heart failure, especially idiopathic dilated cardiomyopathy and to a lesser extent Chagas' cardiomyopathy and peripartum cardiomyopathy, will be at the center of the following chapters. Additionally, typical co-morbidities such as electric abnormalities, hypertension and diabetes mellitus will be considered.

3.2.1.4.2. Functional autoantibodies in cardiomyopathy and heart failure

The immunologic background including genetic, environmental and specific metabolic conditions which can induce autoimmunity that might lead to DCM development, is described (116) and illustrated in Figure 3. However, it seems to be evident that several of the events depicted in Figure 3 could also contribute to the pathogenesis of Chagas' cardiomyopathy (Chagas' heart disease) and possibly even to that of peripartum cardiomyopathy.

For cardiomyopathies and associated diseases, Figure 4 summarizes (without claiming completeness) the GPCR-AAB that were found in related patients.

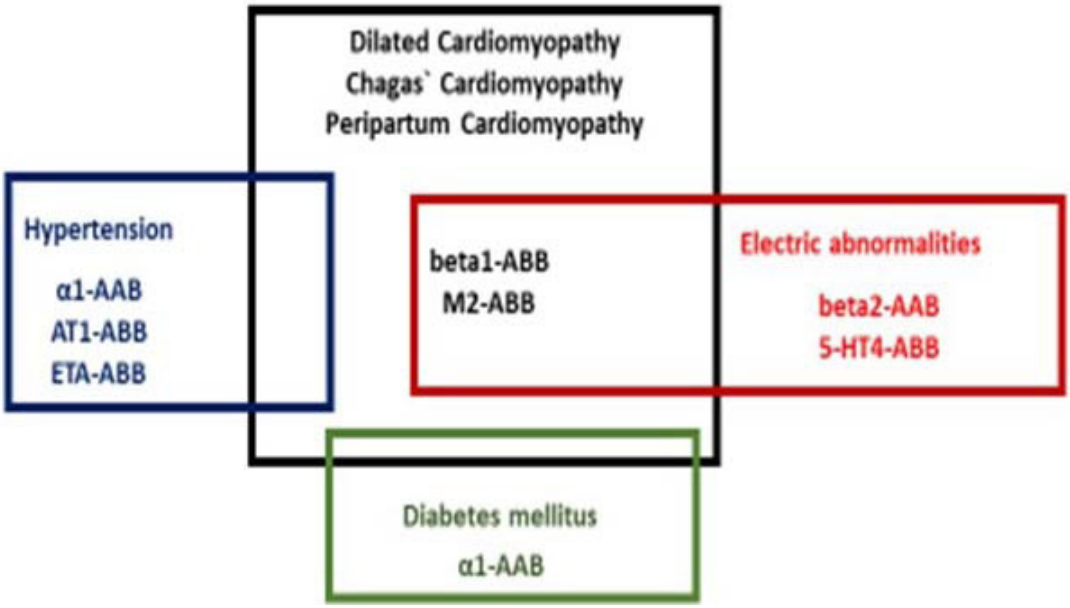

Figure 4. Functional autoantibodies (autoantibodies directed against G-protein coupled receptors) in cardiomyopathies and related co-morbidities

3.2.1.4.2.1. Functional autoantibodies in patients with idiopathic dilated cardiomyopathy

Idiopathic dilated cardiomyopathy (DCM) (117) "… is a progressive, usually irreversible, disease causing global systolic (contractile) dysfunction with heart failure. Often, there are ventricular and supraventricular arrhythmias, conduction system abnormalities, and thromboembolism; sudden death may occur, typically in later stages of disease. From a pathologic standpoint, the term dilated cardiomyopathy is generally used to designate an idiopathic process, in the absence of long-standing hypertension, toxin exposure, or chronic alcoholism (secondary dilated cardiomyopathy). Most patients are middle aged or older at the time of onset; younger patients with dilated cardiomyopathy often have a family history and a genetic predisposition (familial dilated cardiomyopathy)". According to a recent re-calculation, a prevalence of 1:250 (118) for DCM was published for the US. DCM belongs to the most common causes of heart failure. With regard to the 10 year survival, a rate of less than 50% was documented for patients suffering from DCM. For patients who are refractory to medical therapy, heart-assist device implantation and/or heart transplantation will become necessary.

Based on bioassay as well as ELISA measurements, a β1-AAB prevalence in DCM patients ranging between 26% and 95% with high-coexistence with M2-AAB has been published (15, 16, 19). However, several ELISAs used for data acquisition were only able to detect the β1-AAB directed against the second extracellular loop (119, 120). Together with obvious problems in the design and performance of ELISA for measuring β1-AAB (111-114), this could be one of the reasons for the spread in prevalence data. Therefore, the ELISA data should be critically questioned before being used for strategic decisions such as specific patient selection for GPCR-AAB-related treatment options. Derived from bioassay measurement, a β1-AAB prevalence in DCM patient of 70–80% was calculated (14, 17). This prevalence seems to fit clearly better because nearly 60% of the DCM patients unselected with regards to their autoantibody presence but treated with immunoadsorption for autoantibody removal profited from this treatment (121). Interestingly, it was shown that nearly all DCM patients who need mechanical assist devices were positive for β1-AAB (122).

In addition to β1-AAB directed against the second extracellular receptor loop, there are such β1-AAB in DCM patients that target the first extracellular receptor loop (12, 17). In European countries with a total β1-AAB positivity of 70–80%, both β1-AAB directed against the first and second receptor loop were found to have, with small regional differences, a nearly equal frequency. For a small DCM cohort in the US, a β1-AAB frequency of 62% vs. 38% for the first and second receptor loops has been calculated (18). Although it has been mentioned that the second loop targeting β1-AAB are cardiopathogenic (14), we have no doubt about the pathogenic potency of first loop β1-AAB because their functional activity was clearly demonstrated *in vitro* by the chronotropic effect (12, 17). Furthermore, patients with DCM unselected for either first or second loop β1-AAB profited in the same extent from autoantibody removal (123).

As summarized in (124, 125), the presence of β1-AAB in DCM patients correlated with the patients' negative prognosis, the all-cause mortality, the risk of electric abnormalities and sudden death, although the absence of some of these correlations was also documented (125). A high co-existence of the β1-AAB with M2-AAB has already been mentioned (19), but it was shown that the affinity of M2-AAB to the related receptor compared to β1-AAB was 100-fold lower (17, 126). What the different affinities of β1- and M2-AAB mean for the pathogenesis of DCM is still under discussion (127). M2-AAB were preferentially seen as associated to electric abnormalities, as summarized in (25, 26).

Based on the present knowledge, M2-AAB with a prevalence of 15–50% in DCM patients exclusively target the second extracellular receptor loop

3.2.1.4.2.2. Functional autoantibodies in patients with Chagas' heart disease

Chagas' disease, caused by *Trypanosoma cruzi* infection, was discovered nearly 110 years ago (1909) by the Brazilian physician Carlos Chagas. Chronic Chagas' disease is still ranked as the most serious parasitic disease in Latin America. Infected patients remain lifelong parasite carriers. Following *T. cruzi* infection, *T. cruzi* transmission by blood transfusion or congenital transfer (56), nearly one third of chronic *T. cruzi* carriers develop life-threatening complications: the majority develop Chagas' heart disease (90%). Gastrointestinal disorders (megaesophagus, megacolon) and neuronal afflictions mainly affecting the parasympathetic nerve system were found in the others. In 2016, the World Health Organization estimated that 6-8 million people are infected with *T cruzi*, mainly in endemic areas of 21 Latin American countries, but not exclusively, and that about 12,000 deaths each year can be attributed to Chagas' disease, preferentially to Chagas' heart disease (128). Due to the increase in migration and tourism, Chagas' disease is becoming a world health care problem (129).

Chagas' heart disease, presenting with sudden death, heart failure, malign cardiac arrhythmia, and thromboembolism, and morphologically with an enlarged heart, is currently the major cause of morbidity and mortality in Latin America, thus enormously burdening economic resources and dramatically affecting the patients' social and employment situations.

Both for β1-AAB and M2-AAB, a high frequency was demonstrated in Chagas' heart patients (23), but any correlation between levels of autoantibodies and clinical parameters such as contractile dysfunction was not shown in this study. Using the bioassay of cultured spontaneously beating neonatal rat cardiomyocytes, we found that 100 of 102 patients (98%) with Chagas' heart disease carry β1-AAB and coexisting M2-AAB (22). As already mentioned, among the 96 analyzed asymptomatic Chagas' patients, nearly one-third of asymptomatic patients already carried β1- and M2-AAB. Furthermore, the autoantibody activity was significantly higher in patients with manifested Chagas' heart disease compared with the autoantibody-positive asymptomatic patients. β2-AAB were additionally found in both asymptomatic patients and in those with heart disease. In contrast to the β1-AAB in DCM (affinity in nM range), the affinity of the Chagasic β1-AAB is in the μM range (126, 130).

3.2.1.4.2.3. Functional autoantibodies in patients with peripartum cardiomyopathy

"Peripartum cardiomyopathy is an idiopathic cardiomyopathy that presents with heart failure secondary to left ventricular systolic dysfunction toward the end of pregnancy or in the months after delivery, in the absence of any other cause of heart failure. …. Although the left ventricle may not be dilated, the ejection fraction is nearly always reduced below 45%" (131). The prevalence is reported to be the highest in Haiti, with 1 case per 350-400 live births, followed by 1 in 1000 in South Africa, 1 in nearly 2300 in the US (with higher incidence in African American women) and 1 in 6000 in Japan. While 30-50% of the diseased women recover to health, the others face the same survival prognosis as general patients suffering from common DCM (132). According to the typical feature of peripartum cardiomyopathy, with the high incidence of myocarditis (133, 134) and related to the already presented data for the well-known relationship between myocarditis, cardiomyopathy and autoimmunity, the finding of functional autoantibodies in patients with peripartum cardiomyopathy is not really surprising.

With respect to β1-AAB, positivity was found in nearly 100% of the women (20). In a recent study, which enrolled 37 women diagnosed with peripartum cardiomyopathy and 36 matched controls, nearly 60 % vs. 19 % were positive for β1-AAB and 46% vs. 17% for M2-AAB. The GPCR activity was correlated with increased left ventricular dimension and worse cardiac contraction function and, moreover, increased the risk of the onset of peripartum cardiomyopathy (21).

3.2.1.4.2.4. Functional autoantibodies and myocarditis, cardiac electric abnormalities, hypertension and diabetes mellitus

Patients with myocarditis were found to be positive for β1-AAB (24). Although a high prevalence was assumed, significant data are missing. Up to 50% of the patients suffering from cardiac electric

abnormalities can carry GPCR-AAB, as summarized in (25-27), preferentially M2-, β1- and β2-AAB. In patients with DCM, M2-AAB predicted atrial fibrillation. 5-HT4-AAB was seen in relation to complete heart block (28, 29).

For patients with the most severe forms of hypertension, the presence of α-AAB either directed against the first or second extracellular loop were documented (30-32, 135, 136), e.g. with a prevalence of up to 60%. AT1-AAB directed against the second extracellular receptor loop are further GPCR-AAB, which were found in hypertensive patients (137-139), e.g. with a prevalence of up to 35% in patients with malignant hypertension (138). ETA-AAB were present in the sera of patients with pulmonary hypertension (31). In patients with pulmonary hypertension, the ETA-AAB recognize an epitope on the second extracellular receptor loop.

In patient with type II diabetes mellitus, α1-AAB were found (37), although their prevalence in diabetic patients is not accurately known.

4. TREATMENT STRATEGIES TARGETING GPCR-AAB. THE PRESENT STATE AND THE VIEW IN THE FUTURE

For basic researchers, and especially for immunologists, it is becoming clear that functional autoantibodies and functional autoantibody disease form a platform to explain the pathophysiology or at least some of the pathophysiological events of the diseases listed in Table 1. This community is therefore more open minded to the idea of a GPCR-AAB-directed treatment than clinicians, of which many are still not fully convinced of functional autoantibody disease. Consequently, clinicians are often reluctant to apply functional autoantibody-targeted treatment strategies. The reason for this is somewhat unclear. However, to overcome the clinicians' hesitation to accept the concept of functional autoantibody disease for diagnostics, monitoring and even more for treatment in affected patients, two main problems should be addressed: first of all, in-house assays must be converted to and replaced by, respectively, assays for functional autoantibody measurement which are validated and standardized related to the EMA and FDA (140, 141) requirements. Only this will guarantee correct and reproducible patient selection and monitoring after treatment. There are currently two strategies for GPCR-AAB measurement available. The first strategy, as summarized in (33), is based on the measurement of a second messenger signal in living cells that is induced after the binding of GPCR-AAB to their related receptors. The advantage of such bioassays is their ability to only measure functionally active GPCR-AAB. Among the bioassays, there is one based on cultured spontaneously beating neonatal rat cardiomyocytes that can be used for the measurement of different GPCR-AAB in parallel (33, 45) Furthermore, as described in detail in (45), this bioassay can be used as a research tool to: 1) explore the GPCR-AAB-specific carriers (IgG sub-classes), 2) indicate the targets of GPCR-AAB on the receptors (loop finding, epitope mapping), and 3) screen substances for a) binding/neutralizing of GPCR-AAB and b) receptor protection against GPCR-AAB attack. For the read out, the global cell function (change in beat rate) caused by the chronotropic activity of GPCR-AAB is monitored. Additionally, the bioassay of cultured spontaneously beating neonatal rat cardiomyocytes can be applied for the monitoring of other cell function parameters influenced by GPCR-AAB, such as contractility and conductivity, as well as for the measurement of specific signaling molecules in the cells such as Ca^{2+} or cAMP formed in the presence of GPCR-AAB downstream in the signaling cascade. To specifically measure one of the GPCR-AAB, the neonatal rat cardiomyocytes can be replaced by cells designed for expressing the related human receptor. Among the bioassays that can be constructed following this concept, one was realized for human β1-AAB, where the β1-AAB induced an increase in intracellular cAMP in HEK293 cells (stably expressing the human β1-adrenergic receptor) which was measured by fluorescence resonance energy transfer using a highly sensitive cAMP sensor (14).

However, the main disadvantages of the bioassays are problems of standardization, which are necessary to make the bioassays practical for individual patient care under field conditions.

The second measurement strategy for GPCR-AAB is based on the direct detection of GPCR-AAB after binding to GPCR epitope mimics using ELISA (33), or chip technologies (142). More recently, GPCR-AAB measurement by FACS (143) was introduced. For many of the disease-associated GPCR-AAB, ELISA has been applied in human studies. However, the crux of the technologies based on GPCR-AAB binding is their inability to provide information about the functionality of the measured GPCR-AAB. Consequently, using direct assays in patient studies, their comparison with GPCR-AAB data measured with a bioassay would be necessary in order to guarantee data validity (111, 112). Unfortunately, such a comparison is missing in general. Furthermore, as already mentioned, data for the sensitivity and specificity of the direct assays are often missing, such as validation and standardization related to the requirements of the EMA and FDA (140, 141). For each of the GPCR-AAB, a specific ELISA must be developed, but this disadvantage of the ELISA could be overcome using chip technology (142).

The second reason why clinicians still hesitate to apply GPCR-AAB-directed treatment strategies in clinical practice is related to cost, logistical effort and

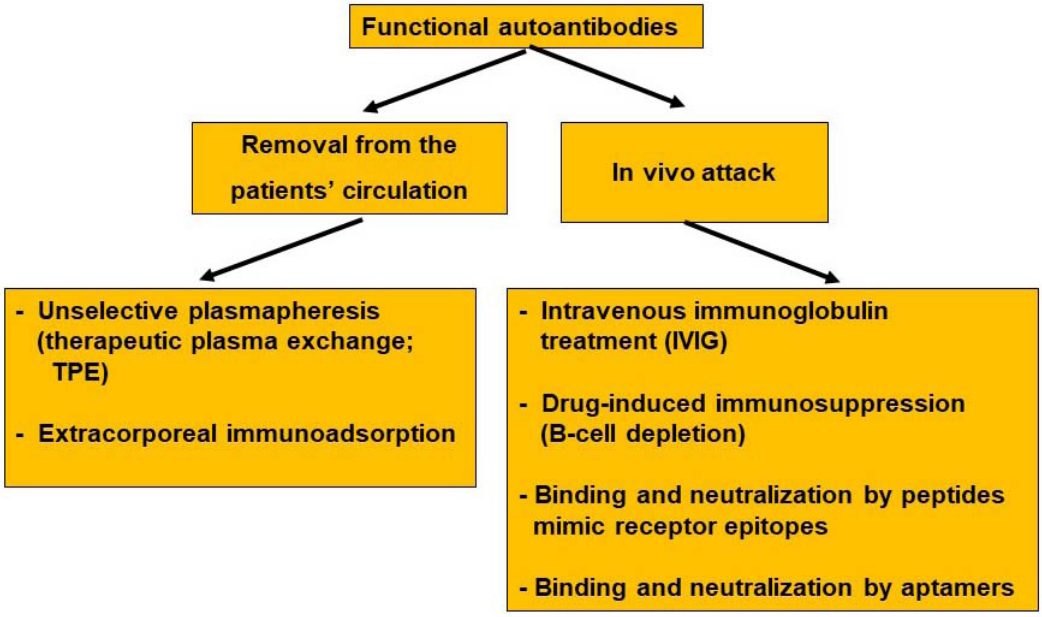

Figure 5. Treatment strategies for functional autoantibody disease

patient burden of the treatment concepts, such as for GPCR-AAB removal studied in the past.

However, due to the patient benefit of GPCR-AAB directed treatment that has been proofed in several studies already, it is time for refining the existing treatment strategies and for developing new treatment concepts in order to overcome the clinicians' restraints towards the GPCR-AAB targeted therapy. In principle, all the functional autoantibody diseases indicated in Table 1 could have a benefit from GPCR-AAB directed treatment strategies.

However, cardiomyopathy in general and specifically dilated cardiomyopathy was the pioneer for establishing and forwarding the concept of "functional autoantibody disease", as well as for studying the patient benefit of treatments directed to GPCR-AAB in general and specifically to β1-AAB. Consequently, existing and emerging GPCR-AAB-targeting treatment strategies will be discussed preferentially but not exclusively with focus on cardiomyopathies.

Among these treatment strategies indicated in Figure 5, there are two different approaches: first, the elimination of GPCR-AAB from the patients' circulation, and second, the patients' *in vivo* treatment for GPCR-AAB attack.

4.1. Technologies for GPCR-AAB removal

Unselective plasmapheresis (therapeutic plasma exchange; TPE) and immunoadsorption (IA) for the adsorption of immunoglobulins, specific IgG subclasses and even specific GPCR-AAB (immunoadsorption; IA) are available for the elimination of GPCR-AAB from the patients' circulation.

4.1.1. Therapeutic plasma exchange (TPE)

In TPE, an extra-corporeal therapy, the patient's blood is cleared of pathological substances (144), among which are GPCR-AAB, if present. As shown schematically on the left side of Figure 6, the patients' circulation is connected to the TPE device, which separates cells and plasma. The blood cells are re-transfused to the circulation, whereas the plasma containing the harmful components such as GPCR-AAB is discarded. To compensate for patient plasma loss, donor plasma or albumin/saline solutions are substituted.

For several autoimmune disorders, TPE is one of the preferred therapeutic options. TPE is often supplemented with immune-suppressive therapy. Guillain Barré syndrome, lupus erythematosus and thrombotic thrombocytopenic purpura are, among a variety of other autoimmune diseases, examples of diseases treated with TPE (144).

Concerning heart disease, TPE was discussed with regard to cardiac allograft rejection (145), but without any relationship to any GPCR-AAB removal. Congenital heart block associated with neonatal lupus was considered as another indication for TPE to remove anti-SSA/Ro52 autoantibodies, among others (146). Anti-SSA/Ro52 are directed against the cardiac 5HT4 serotoninergic receptor 5-hydroxytryptamine receptor 4, which belongs to the GPCR, and are discussed for their contribution

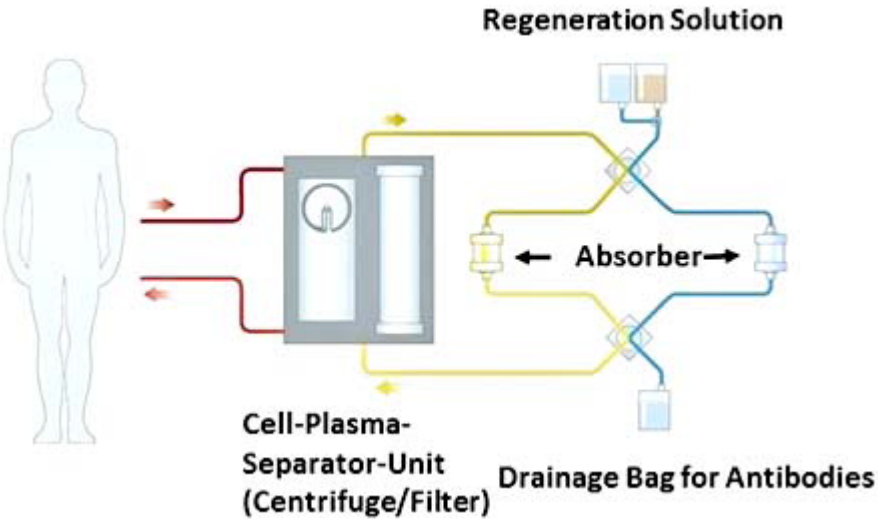

Figure 6. Principle of extra-corporal removal of autoantibodies directed against G-protein-coupled receptors; reproduced with permission of Fresenius Medical Care AG, Germany, from http://www.fmc.nl/media/1129/ia_brochure_algemeen_01_14_gb_w.pdf

to the development of congenital heart block found in neonates suffering from lupus (28, 29). TPE to prevent cardiac neonatal lupus could therefore be considered as a door opener for the more intensive use of GPCR-AAB removal therapy.

For other cardiac patients with GPCR-AAB positivity, there are, until now, only case reports and small observation studies that have demonstrated any TPE benefit. Moreover, due to the un-selectivity of TPE with regards to the removed harmful blood component, it is difficult to substantiate any patient benefit after TPE solely with the removal of GPCR-AAB and even more with the removal of a specific GPCR-AAB such as e.g. β1-AAB. However, it was documented in a case report that TPE supplemented with immunosuppressive therapy decreased the β1-AAB serum titer in a 28-year old man with heart failure which was associated with the improvement in NYHA functional class, exercise test and cardiac reserve. This patient, before being listed for heart transplantation, could be removed from the transplant list. One year after the primary treatment, the recurrence of cardiac deterioration was diagnosed, but the authors did not inform about the recurrence of β1-AAB; knowing this would be helpful to verify the role that β1-AAB play in the pathogenesis. The second TPE led to patient benefit again (147). Another case report presented a five-year old boy suffering from DCM who was positive for β1-AAB and profited from TPE. Associated with the β1-AAB loss after treatment, a clear benefit indicated by decreased BNP was seen after three months. Unfortunately, the benefit was not sustainable thereafter (148).

In 6 children with refractory DCM who were positive for β1-AAB and M2-AAB, repeated TPE improved cardiac function; heart failure symptoms were attenuated. Three of the 6 patients who were exemplarily analyzed for GPCR-AAB loss were free of GPCR-AAB after the second TPE run (149).

A further study enrolling patients with non-ischemic heart failure demonstrated improvement in LVEF and quality of life 6 months after TPE applied due to anti-cardiac antibodies in the patients as suggested by the authors. However, the exact specification of the autoantibodies was not presented but when comparing myocardial biopsies sampled at baseline vs. 6 months after treatment, the patient benefit was accompanied by reduced IgG immunostaining in the myocardium (150).

For patients with Chagas' disease, and specifically those suffering from Chagas' heart disease, there are - to the best of our knowledge- presently no information available about the treatment with TPE.

Regarding peripartum cardiomyopathy and TPE treatment, there are until now only a few case reports of treatment with TPE. For just one woman with peripartum cardiomyopathy who was treated with TPE, normalized (24 month follow-up) and improved (22 month follow-up), respectively, LVEF was published (151). In a 24-year-old female suffering from severe peripartum cardiomyopathy with biventricular mechanical circulatory support, TPE was associated with rapid hemodynamic recovery (152). Unfortunately, information about the women's positivity regarding cardiac-pathogenic autoantibodies, especially β1-AAB, and the influence of TPEs on the autoantibody titer was not presented. Despite the poor evidence for TPE benefit in women with peripartum cardiomyopathy, this treatment option has been suggested to hold promise for the future (153).

Outside of the heart diseases and specifically cardiomyopathies, TPE treatment aimed at the removal of GPCR-AAB has demonstrated patient benefit in several diseases. Kidney transplant recipients with refractory vascular rejection who were positive for autoantibodies directed against AT1-AAB, which seem to be the dominant pathogenic GPCR-AAB in these patients, profited from TPE with prolonged allograft survival in comparison to conventionally treat patients (38, 39). Based on case studies summarized in (48, 49), of the 6 GPCR-AAB-positive patients suffering from complex regional pain syndrome (CRPS), 3 reported meaningful improvements of mood and fatigue following TPE. For 2 other CRPS patients who did not profit from conventional treatment, TPE resulted in the loss of autoantibodies directed against the β2-adrenergic receptor (β2-AAB), which was accompanied by a strong improvement in pain and autonomic symptoms. Benefit has also been demonstrated for a young lady with CRPS and positivity for β2-AAB and M2-AAB. However, this young lady was treated with rituximab in addition to TPE (50). In later publications, the authors reported that the first CPRS symptoms appeared two weeks after the patient's first human papillomavirus (HPV) vaccination (154, 155). Without speculating about any causality between HPV vaccination and GPCR-ABB generation, we found, however, β2-AAB and M2-AAB in two additional young women who presented with symptoms similar to those of CPRS and fatigue which started – as detailed by the patients - soon after HPV vaccination (unpublished data). In conclusion, although TPE was indicated as a first- and second-line therapy in many diseases associated with an autoimmune background (144), the currently available data regarding the benefit of TPE in patients with positivity for GPCR-AAB has mainly come from case studies, which means that there is no strong indication for the application of TPE in GPCR-AAB-positive patients to date.

4.1.2. Extra-corporeal immunoadsorption (IA)

There is a quite different situation – in our view –for IA treatment of patients with cardiomyopathy, mainly for those suffering from DCM and being positive for GPCR-AAB. Despite still missing results from randomized double-blind studies such as the "Multicenter, Randomized, Double-blind" study (36) started in 2007, the currently available study data for IA in DCM patients, which are summarized below in Table 3, point to a level of evidence B and class I recommendation (rather than class II) for IA treatment of GPCR-AAB-positive DCM patients presenting with NYHA class II-IV. These data pave the way to study IA as a treatment for further diseases related to GPCR-AAB-positivity.

In IA (right site of Figure 6), the TPE machinery is connected with a second device containing an immunoadsorption column for the removal of cardio-toxic autoantibodies such as GPCR-AAB. After separation of the patient's blood in cells and plasma, the plasma is not discarded as in TPE, but is transferred to the immunoadsorption unit where the plasma is then passed through a column carrying special ligands to bind the plasma carrier of the GPCR-AAB or specifically the GPCR-AAB. In the majority of apheresis machine, two IA columns work in parallel. After saturation of the first column, the plasma is transported into the second column, while the first is regenerated.

The outflow of the columns, which is then free of GPCR-AAB, rejoins the bloods cells and is infused back into the patient (156). Depending on the ligands of the IA column that were used to bind GPCR-AAB, either the total immune globulin fraction, a specific class of immune globulins or more specifically any of the IgG sub-fractions are removed to clear the patient plasma of GPCR-AAB. Table 2 lists the currently available columns that were used to study the removal of cardio-toxic autoantibodies.

The majority of studies are aimed at the removal of GPCR-AAB, and specifically β1-AAB. There was also an IA column in the past which was designed specifically for exclusively binding β1-AAB. This column is no longer commercially available. The first generation of IA machines contains two columns that work in parallel; while the one column is working for adsorption, the other is being regenerated. Subsequently, the trend appeared to move towards single use columns.

In the majority of IA studies for the removal of whole IgG, but with the aim of removing GPCR-AAB from the blood, but not in all (157-160), IgG was replaced after IA. As found in a systematic study that analyzed the benefit or risk of IgG application following immunoadsorption in patients with autoimmune diseases, no evidence was found to show that the non-replacement of immunoglobulins after IA would produce more frequent infections. There were even more adverse effects in the IgG replacement group, so the IgG replacement was discontinued. During the subsequent IA without IgG replacement, no side-effects were seen (161). This means in our view in general for IA, and also for IA designed for the removal of GPCR-AAB, the following should be kept in mind in cases of immunoglobulin replacement: foreign immunoglobulins contain several antigens that may induce pro-inflammatory reactions in treated patients, which may attenuate the positive effect of IA.

Table 3 summarizes, without claiming to be exhaustive, clinical studies on IA treatment in patients with heart failure. Unfortunately, the GPCR-AAB status of patients was not analyzed in all of the studies. Table 3 also includes IA studies directed against other

Table 3. Clinical studies (CC, case control study; CS, case study; RA, retrospective analysis) using IA treatment (A: Ig-Therasorb®, B: Coraffin®, C: Immunosorba®, D: Immunsorba TR®; E: IgAdsopak®, F: Globaffin®) for patients with heart failure and other diseases

Trial (Ref.)	Study Design	Intervention (Immunoadsorber)	IA treated Patients / Controls	Baseline Characteristics	Follow-up	Results
Wallukat *et al.* 1996 (162)	CS	1 course of IA wit 4/5 sessions + IgG substitution (A)	8/0	NYHA II-IV, β1-AAB (+)	2.5. month	Pre-IA vs. post-IA: day 5 n 7/8 patients (β1-AAB (100 vs. 8% LU); NYHA class reduction); 2.5. month (β1-AAB return to β1-AAB (+), pre-IA NYHA class
Dorffel *et al.* 1997 (163)	CS	1 course of IA with 5 sessions (A)	9/0	NYHA III/IV, LVEF <25%, β1-AAB (+)	5 days	Pre-IA vs. post-IA: β1-AAB (6.4.±1.3. vs 1.0.±0.5., p<0.0.01 LU); CO (3.7.±0.8. vs. 5.5.±1.8. L/min, p<0.0.1); MAP (76.0. ± 9.9. vs. 65.0. ± 11.2. mm Hg, p<0.0.5)
Müller *et al.* 2000 (159)	CC	1 course of IA with 5 sessions (A)	17/17 conventionally treated	NYHA II-IV, LVEF <30%, β1-AAB (+)	12 months	Patients vs. controls post-IA: β1-AAB (<1.0. vs. 5.0.±1.3. LU, p<0.0.01); LVEF 37.9.±7.9. vs. 25.2.+5.9.%, p<0.0.001); NYHA class improvement (p<0.0.01)
Felix *et al.* 2000 (164)	RCT	1 course of IA with 3 sessions + IgG substitution (A)	9/9 conventionally treated)	NYHA III/IV, LVEF <30%, β1-AAB (+)	3 months	Pre-IA vs. post-IA: CI in the treatment group (2.3.±0.1. L/min/m^2 vs.3.0.± 0.3. L/min/m^2; p<0 01). β1-AAB (>4 LU vs. <2 LU) No change in the controls
Schimke *et al.* 2001 (165)	CC	1 course of IA with 5 sessions (A)	17/17 conventionally treated	NYHA II-IV, LVEF <30%, β1-AAB (+)	12 months	Patients vs. controls post-IA: β1-AAB (<1.0. vs. 5.0.±1.3. LU, p<0.0.01); LVEF (37.9.±7.9. vs. 25.2.+5.9.%, p<0.0.001); NYHA class improvement (p<0.0.01), reduced serum markers for oxidative stress (TBARS (p<0.0.5), LPO (p<0.0.5) and anti-oxLDL-AB (p<0.0.5))
Wallukat *et al.* 2002 (160)	CS	1 course of specific β1-AAB IA wit 5 sessions (B)	8/0	LVEF <35%, β1-AAB (+)	12 months	Pre-IA vs. post-IA: LVEF (28.5.±6.1. vs.36.6.±10.7., p<0.0.5); β1-AAB (5.0.±0.5. vs. <1.2.±0.6. LU), serum oxidative stress markers (TBARS (8.4.±4.1. vs. 3.7.±1.6. µmol/l, p<0.0.5)
Felix *et al.* 2002 (166)	CC	1 course of IA with 3 sessions + IgG substitution (A)	11/9 (healthy)	NYHA III/IV, LVEF <30%, cardio-depressant AAB (+)	3 days	Pre-IA vs. post-IA CI (2.2. ± 0.1. vs.2.7. ± 0.2. L/min/m^2; p<0.01). Serum cardio-depressive AAB were found in the column eluate after IA
Staudt *et al.* 2002 (167)	CC	IA in 4 courses at 1 month intervals until month 3	9 patients IA with protein A column 9 patients IA with anti IgG column	NYHA III/IV, LVEF <30%, AAB (+)	3 months	AAB were only removed by anti IgG column after one course. Protein A group showed no hemodynamic improvement after 3 month, anti IgG IA showed persistent hemodynamic improvement
Mobini *et al.* 2003 (168)	CS	1 course of IA with 3 sessions + IgG substitution followed by 2 courses once per month for 3 months (A)	22/0	NYHA III-IV, LVEF <30%, β1-AAB (+) and (-)	3 months	Pre-IA vs. post-IA: LVEF (21.5.±6.4. vs. 26.8.±7.3.% (p<0.0.5), CI: 2.2.±0.3. vs. 2.7.±0.7. l/min/m^2, p<0.0.01), No difference in CI and LVEF between β1-AAB (+) and (-)
Knebel *et al.* 2004 (169)	RA	1 course of IA with 5 sessions + IgG substitution (AD)	17/17 conventionally treated	NYHA II,III, LVEF < 35%, AAB (+) not declared	3 years (median 2.3. years)	Patients vs. controls post-IA: reduced days of hospitalization (p<0.0.1)

Hessel *et al.* 2004 (170)	CC	1 course of IA with 5 sessions (A)	17/17 conventionally treated	NYHA II-IV, LVEF <30%, β1-AAB (+)	5 years	Post-IA patient vs. controls: The 5-year mortality (10/17 vs. 3/17), survival rates (82 vs. 41%), Medical cost for annual treatment based on survival time and medical cost Germany 2000 (€24,900 vs. €28,900) with resulted in incremental costs per life year gained for IA of about €35,000 seen as cost-effective due to the cited limit of US$50,000 per quality-adjusted life-year gained
Dörffel *et al.* 2004 (171)	CS	1 course of IA with 5 sessions (A)	9 patients on standard heart failure therapy, beta blocker therapy started 1 day before IA	NYHA III/IV, LVEF <25%, β1-AAB (+)	3 years	One patient underwent heart transplantation, two patients died by cardiac death after a reincrease of β1 adrenergic AAB, 7 patients improved in EF over 3 years.
Staudt *et al.* 2005 (172)	CC	4 courses of IA with 2/3 sessions with improved IgG3 binding vs. normal IA columns (C)	9/9	NYHA III/IV, LVEF 21.6.±2 vs. 24.3.+2%, AAB not declared	3 months	Pre-IA vs. post-IA: IgG3 reduction (-65±4 vs. -36.4.%, p<0.0.01), LVEF (34.7.±4 vs. 24.4.±2%, p<0.0.5)
Schimke *et al.* 2005 (173)	CS	1 course of specific β1-AAB IA with 5 sessions (B)	8/0	LVEF <35%, β1-AAB (+)	12 months	Pre-IA vs. post-IA: Oxidative stress: TBARS (LVEF (28.5.±6.1. vs.36.6.±10.7., p<0.0.5); β1-AAB (5.0.±0.5. vs. <1.2.±0.6. LU)), serum oxidative stress markers (TBARS (8.4.±4.1. vs. 3.7.±1.6. µmol/l, p<0.0.5))
Staudt *et al.* 2006 (174)	RCT	1 course of IA with 5 sessions + IgG substitution vs. 4 courses for 5 days + IgG over 3 months (C)	11/11	NYHA III/IV, LVEF <35%, AAB (+) not declared	6 months	Pre- vs. post-IA: NYHA improvement (10/11 vs. 11/11), LVEF improvement (26.5.±2.2. vs. 34.8.±2.9.%, p<0.0.1 and 28.1.±2.9. vs. (37.0.±1.6.%), p<0.0.1; no difference between groups
Staudt *et al.* 2006 (175)	CC	4 courses of IA with 5 sessions + IgG substitution over 3 months (C)	15/15 conventionally treated	NYHA III, IV, LVEF <35%, AAB (+) not declared	3 months	Pre-IA vs. post-IA: NYHA improvement in patients, p<0.0.1 vs. unchanged NYHA in controls; LVEF patients (29.7.±1.0. vs. 38.6.%), controls (28.1.±1.0. vs.26.4.±1.0.%), p<0.0.01; NT-ProBNP patients (>1400 vs. <800 pmol/l), controls (>1400 vs. >1500 pmol/l), p<0.0.01
Cooper *et al.* 2007 (158)	CS	1 course of IA with 5 sessions (C)	4/0	NYHA II/III, LVEF 34.6.±12. 3%, AAB (+) not declared	6 months	Pre-IA vs. post-IA: LVEF (26.3.+9.4. vs. 28.7.+11.4.%, p<0.0.5), exercise capacity (82.0.+30.8. vs. 92.1.+34.3. Watt, p<0.0.1), NT-proBNP (1230 vs. 829 ng/l, p<0.0.01)
Doesch *et al.* 2009 (176)	CS	1 courses of IA with 5 sessions (C)	27/0	NYHA II-IV, LVEF 24.1.+7.8.%, 6/27 TnI-AAB	6 months	Pre-IA vs. post-IA: NYHA class improvement in 33%, LVEF improvement >5% in 33% which were all diabetics and TnI (-), exercise capacity improvement >15 Watt in 48%, in 5/6 TnI-AAB were cleared by IA but TnI-AAB returned
Doesch *et al* 2010 (177)	CS	1 course of IA with 5 sessions (C)	51/0	NYHA II-IV, LVEF <50%	6 months	Pre- vs. pos-t-IA: LVEF (34.6.+12.3. vs. 44.1.+15.3.%, n.s.)

Herda *et al.* 2010 (178)	CC	1 course of IA with 5 sessions + IgG substitution (C)	30/30 conventionally treated	NYHA II-IV, LVEF < 45%, AAB (+) for cTnI-AAB and/or KChIP2-AAB in a subset of 14patients and controls	3 months	Pre-IA vs. post-IA: LVEF patients (33.0.±1.2. vs. 40.1.±1.5., p<0.0.01) controls 30.1.±1.2. vs. 32.0.±1.5., n.s.), exercise capacity improvement patients (114.2.±7.4. vs. 141.9.±7.9. Watt, p<0.0.5), improvement of spiroergometry parameters, p<0.0.5-0.0.1), no improvement in the controls; only improved peak VO_2, p<0.0.1 in patients with AAB (+)
Baba *et al.* 2010 (179)	CS	1 course of IA with 3/5 sessions, in 3 patients due to return of β1- and/or M2-AAB 2nd course after 3 months (D)	18/0	NYHA III/IV, LVEF <30%, β1-AAB (+), M2-AAB (+), cardio-depressant AAB (+)	3 moths	Pre-IA vs. post-IA: Six-min walk test improved (p<0.0.1), LVEF improvement (p<0.0.1 only in patients with complete removal of AAB
Trimpert *et al.* 2010 (180)	CS	1 course of IA with 5 sessions + IgG substitution (C)	17/0 (11 cardio-depressant AAB (+), 6 (-))	NYHA II-IV, LVEF <45%,	12 months	Pre-IA vs. post-IA: LVEF AAB (+) (33.8.±1.7. vs. 51.8.±1.7.%, p<0.0.01, AAB (-) (no change); LVIDd AAB (+) (66.6.±1.2. vs.61.2.±2.2. mm, p<0.0.5); no return of the AAB (+)
Bulut *et al.* (2010) (181) Bulut *et al.* (2011) (182)	CS	IA not specified (C)	10/0 13/0	NYHA II-IV, LVEF <35% AAB not declared	6 month	Pre-IA vs. post-IA: LVEF (25.5.±4.9. vs. 37.3.±10.1.%, p<0.0.5; regulatory T cells increased, activated T cells decreased Pre-IA vs. post-IA: LVEF (25.5.±4.9. vs. 37.3.±10.1.%, endothelial derived microparticels decreased
Nagatomo *et al.* 2011 (183)	CS	1 course of IA with2/5 sessions within 1 or 2 weeks (D)	16/0	NYHA III/IV, LVEF 18+2%, β1-AAB (+), M-AAB (+)	3 months	Pre-IA vs. post-IA: LVEF (18+2 vs. 21.+2%, p<0.0.5), BNP (752+156 vs. 432+96 ng/l), six minute walk distance (31+39 vs. 369+30, p<0.0.1)
Dandel *et al.* 2012 (123)	RA	1 course with 5 sessions of unspecific or specific β1-AAB IA (A,B,F)	216 (195 β1-AAB (+), 140 IA, 116 unspecific IA, 24 specific β1-AAB IA, 55 non-IA), (21 β1-AAB (-), 21 unspecific IA)	NYHA II-IV, LVEF <30%, β1-AAB (+)	5 - 14.5. years	Post-IA 5 years HTX/VAD free survival probabilities: β1-AAB (+) vs. (-) (69.4.+4.4. vs. 47.4.+11.5.%), β1 AAB (+) with vs. without IA (69.4.+4.4. vs. 25.5.+11.4.%), Unspecific IA vs. specific IA for β1-AAB (+)(88.0.+8.5. (column1) vs. 78.8.+8.4. (column3) vs.91.3.+5.9. column2), IA responders vs. non-responders (89.3.+3.6. vs. 24.7.5.+7.5.%), post-IA 5 years HTX/VAD free survival probabilities tended to continue up to 10 years after IA
Bulut *et al.* 2013 (184)	CC	1 course of IA with 5 session + IgG substitution (C)	18/5 DCM conventionally treated/12 ischemic cardiomyopathy conventionally treated	NYHA II-IV, LVEF <35%, AAB (+) not declared	6 months	Pre-IA vs post-IA: LVEF (27.1.+5.3. vs. 36.8.+8.2.%, p<0.0.5, n=12 responder; 28.4.+6.0. vs. 28.2.+5.8.%, n.s., n=6 non-responder; no change in conventional treated DCM and ischemic cardiomyopathy patients), regulatory T cells (2.3.2+0.2.2 vs. 4.0.6+0.6.8%, p<0.0.5, n=12 responder; 4.8.6+0.2.8 vs. 4.5.6+0.8.1%, n.s., n=6 non-responder)

Pokrovsky *et al.* 2013 (185)	CC	1 course of IA with 5 session (E)	9/7	NYHA II-IV, LVEF <35%, β1-AAB (+)	6 months	Pre-IA vs. post-IA: LVEF (patients tended to improvement), BNP improved (507+279 ng/l vs. 272+185 ng/l, p<0.0.5, no change in the controls),
Reinthaler *et al.* 2015 (121)	RA	2 courses of IA with 5 sessions, 1st course followed by 2nd course after 41.7.+27.4. month (A,C)	15/0	1st course: NYHA improvement ≥ 1 class, LVEF improvement <5% but thereafter subsequent deterioration, AAB not declared	6 months	Pre-IA vs. post-IA: 1st session NYHA (2.8.7±0.6.4 vs. 2.3.3±0.7.2, p<0.0.5), LVEF (33.0.±.4 vs. 43±7.9.%, p<0.0.01); 2nd session NYHA 2.8.7±0.6.4 vs. ± p<0.0.5), LVEF (29.7.±4.6. vs. 34.9.±8.3., p<0.0.5)
Dandel *et al.* 2015 (186)	RA	1 course of IA with 4 sessions (F)	31/31 (DCM/ DCM+Diabetes mellitus)	NYHA III/IV, LVEF <30%, β1-AAB (+) 79%	5 years	Post-IA 3- and 5-years HTX-free survival probabilities for all patient (79.6.+5.6. and 63.5.+7.9.%) without significant differences between the groups as it was also for post-IA 3-year freedom from β 1-AAB reappearance
Yoshikawa *et al.* 2016 (187)	RCT	1 course of IA with 5 sessions within 2 weeks followed by 1 course of IA (with 5 sessions within 2 weeks) (D)	22 with 1st and 2nd IA courses/22 with only 2nd IA course	NYHA III/IV, LVEF <30%, β1-, M2-, NA/K-ATPase-, TnI- and/or myosin-AAB (+)	12 months	Pre-IA vs. post-IA: NYHA improvement (p<0.0.01), (LVEF: 23.8.+1.3. vs. 25.9.+1.3.%) at 4 months after IA, no additional effect of the 2nd course of IA
Ohlow *et al.* 2016 (188)	CS	1 course of IA with 5 sessions within 5 consecutive days + IgG substitution (D)	93/0	NYHA II-IV, LVEF<45%, CAD excluded, AAB (+) not declared	12 months	43 patients responded towards IA, 46 patients did not. LVEF improvement in responders 11% (p<0.0.001)
Baumann *et al.* 2011 (189)	CS	1 course of IA with 5 sessions within 5 consecutive days (A)	10/0	Buerger's Disease, AAB (+) not declared	6 month	Pain intensity decreased rapidly in immunoadsorbed patient. After one month patients were persistently without pain. Walking distance increased subsequently. Ischemic ulcerations healed in all patients during the follow up period. Decreased tissue oxygenation normalized 1 month after immunoadsorption.
Dandel *et al.*, 2013 (190)	CS	1 course of IA with 4 sessions within 4 consecutive days + IgG substitution on the last day (F)	5/0	Pulmonary hypertension, α1-AAB (+), ETA-AAB (+)	24 month	With the first 3 weeks systolic PAP decreased, stroke volume increased partial oxygen uptake and 6 MWT performance increase in all 5 patients. After reoccurrence of AAB in 3 of 5 patients clinical symptoms worsened after 3, 17 and 24 months. The other two patients showed stable clinical improvement.
Klein-Weigel *et al.* 2014; (41)	CS	1 course of IA with 5 sessions within 5 consecutive days (F)	11/0	Buergers Disease 9 GPCR-AAB (+), 2 GPCR (-)	5 days	AAB were absent in the (+) patients after treatment
Hempel *et al.*, 2016 (191)	CS	1 course of IA with 4 session within 4 consecutive days + IgG substitution on the last day. In 4 patients IA had to be stopped after 2 or 3 sessions. (F)	8/0	Dementia α1-AAB (+)	12-18 month	Patients treated over 4 days (n=4) showed no reoccurrence of AAB during the follow up-period. Whereas AAB reoccurred in 75% of patients with incomplete IA. Neutralization of AAB correlated with stabilized cognitive development and clearly improved living skills.

Klein-Weigel *et al.* 2016; (52)	CS	1 course of IA with 5 sessions within 5 consecutive days (F)	22	Buergers Disease 14 GPCR-AAB (+), 8 GPCR (-)	3 (0-36) months for 15 patients	Healing of skin lesions, pain scale values decreased from 7.0. (5-9) to 2.0. (0-5).
Nagel *et al.*, 2017 (192)	CS	1 course of IA with 5 sessions within 5 consecutive days (A)	10 / 0	Pulmonary hypertension, α1-AAB (+), AT1-AAB (+), ETA-AAB (+)	6 month	After 3 months the pulmonary vascular resistance improved significantly. Overall, patients with high AAB levels improved the most from IA treatment.
Scheibenbogen *et al.*, 2018 (193)	CS	1course of IA with 5 sessions within 7 days (F)	10 / 0	Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME), β2 –AAB (+)	12 month	3 patients: long lasting moderate to marked improvement for 6–12 months, 2 patients: short improvement only, 2 patients improved for several months following initial worsening.

Table 2. Commercially available systems for immunoadsorption suitable for GPCR-AAB blood clearing. Reproduced with permission from (53).

Producer	Trade Name	Specification	Binding Ligand	Mechanism	Use
Miltenyi Biotec GmbH, Bergisch Gladbach Germany	TheraSorb™ – Ig pro Adsorber TheraSorb™ – Ig flex Adsorber	IgG 1-4 IgM IgA	Anti-human polyclonal Ig from sheep/Sepharose	Binding of κ and λ light chain and heavy chain of human Ig	M S
Fresenius Medical Care, Bad Homburg, Germany	Immunosorba® Globaffin® Ligasorb® Coraffin® (presently not available)	IgG1, 2, 4 (IgG3), IgM, IgA IgG 1, 2, 4 (IgG3), IgM, IgA IgG 1, 2, 4 (IgG 3), IgM, IgA Autoantibodies directed against the first and second loop of the β1-adrenoceptor	Recombinant Protein A from *Staphylococcus aureus*/Agarose Synthetic Peptide-GAM®/Sepharose Recombinant Protein A/ Agarose Synthetic peptides representing the first (PDCM349;14 mer) and second (PDCM075; 18 mer) extracellular loop of the β1-adrenoceptor/ Sepharose	Constant (Fc) region of Ig Constant (Fc) region of Ig Constant (Fc) region of Ig Antigen binding site of autoantibodies directed against the first and second loop of the β1-adrenoceptor	M M S M
Asahi-Kasei Medical Co., Japan	Immusorba TR-350® Immusorba PH-350®	IgG, Fibrinogen, CRP,	Tryptophan/Polyvinyl Alcohol Phenylalanine/Polyvinyl Alcohol	Ionic and hydrophobic interaction to Ig	S S
POCARD Ltd., Russia	IgAdsopak®	IgG 1-4 IgM IgA IgE	Anti-human polyclonal Ig from sheep/Sepharose	Binding of κ and λ light chain and heavy chain of human Ig	M
Kaneka Corp. Japan	Selesorb®	IgG 1-4 IgM IgA	Dextran sulfate/ Cellulose	Ionic interaction to Ig	S

diseases which are associated with GPCR-AAB. As indicated in Table 3, the story of IA for the treatment of GPCR-AAB-positive patients started in 1996, when IA was first applied in β1-AAB-positive patients with DCM (162). In this initial report, 8 patients with DCM (NYHA classes II-IV) were treated with Ig-Therasorb® to remove the whole IgG and thereby the β1-AAB. Thereafter, β1-AAB levels were significantly reduced within the range of healthy subjects and remained low during the 2.5 month follow-up in seven of the eight patients. In parallel, these seven patients presented with improved NYHA classification. The β1-AAB titer returned to the pathological level in only one of the patients, which was associated with recurring NYHA class deterioration, making heart transplantation necessary. Using an adequate protocol for the Ig-Therasorb® treatment of DCM patients, improvement of the cardiovascular function demonstrated by invasively measured hemodynamic parameters was already seen one day after the IA, whereas LVEF

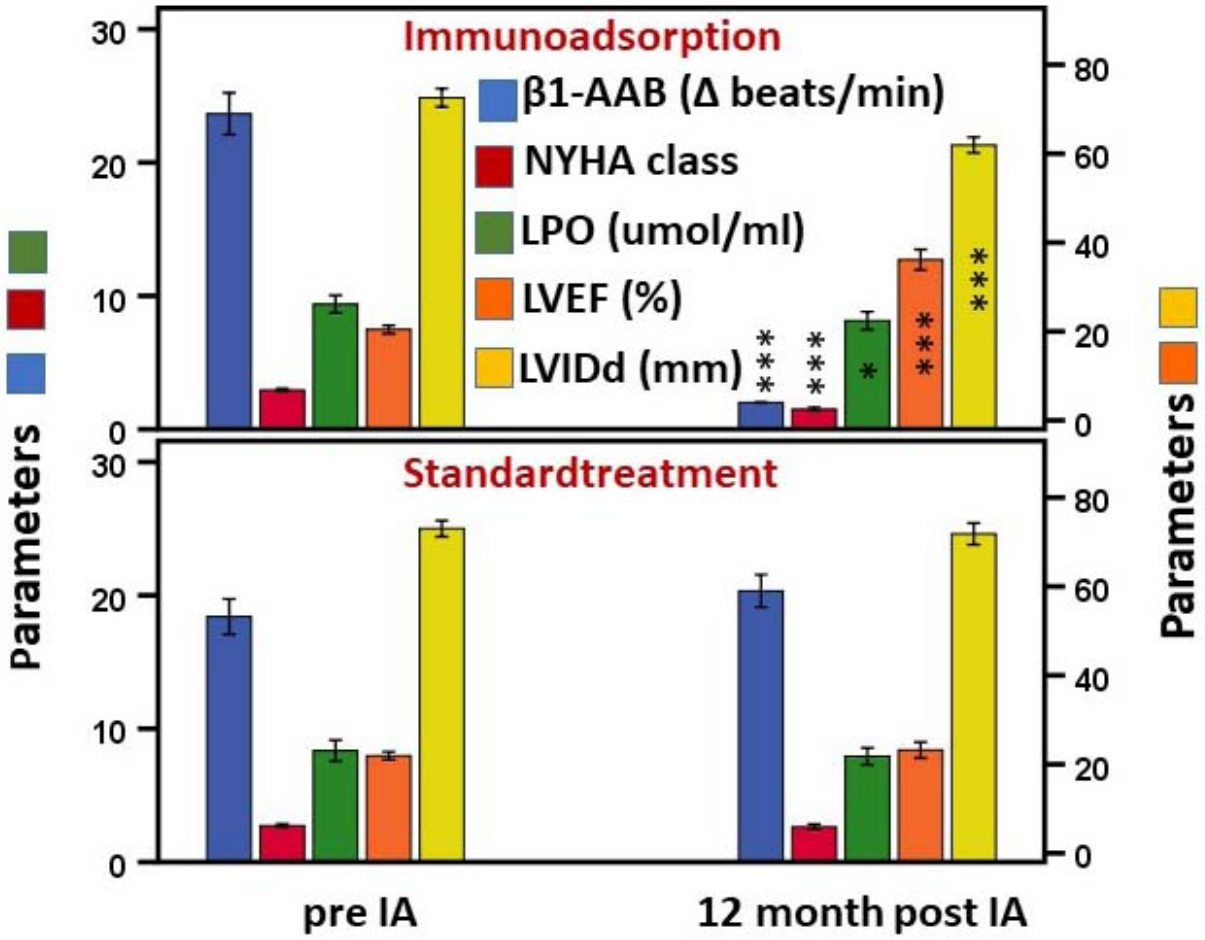

Figure 7. Benefit of immunoadsorption on patients with DCM 12 month after treatment. Reduction of autoantibodies directed against the β1-adrenergic receptor (β1-AAB) resulted in significantly (* p<0.0.5), *** p< 0.0.01 increased left ventricular ejection fraction (LVEF) decreased left ventricular diastolic diameter (LVIDd) and oxidative stress (LPO); The Figure merges data from the same patient cohorts published in (159, 165). However, outcome analysis provides the strongest evidence for the benefit of any treatment. With respect to β1-AAB positive patients with DCM, Dandel *et al.* published the most convincing evidence for IA as a successful treatment strategy in 2012 (123). For this retrospective analysis, patients with end-stage DCM and listed for HTx at the "Deutsches Herzzentrum Berlin, Germany" were enrolled. The study design, which included initially 216 patients, is presented in (123).

improvement still failed at this time (163). The fact that significant LVEF changes were missing immediately after the IA run has also been confirmed in IA using Immunosorba®, but there were even studies where LVEF increase together with improved cardiac index and stroke volume index immediately after IA (164, 166-168).

With respect to the IA benefit, 1) heart function, mainly demonstrated by echocardiography and in the majority indicated by increased LVEF, six minute walk distance and the patients' long-time outcome clearly improved, independent of which IA column was applied. This was documented, depending on the study design, after three, 6, or 12 months, or after three years of follow-up, respectively (121, 157-160, 164-169, 171-185, 187, 188). The patient benefit of IA was also visible by the reduction of biochemical and immunologic markers such as natriuretic peptides, oxidative stress markers, regulatory immune cells, markers for endothelial function, total and endothelial microparticles (MPs) in the blood (165, 173, 179, 181, 182, 184, 185), gene expression measured in myocardial biopsies, and fibrosis markers (194)

Figure 7 merges the data of two studies but of the same cohorts of patients (159, 165) for demonstration of the parallel improvement of structural, functional and metabolic markers after IA in patients with DCM.

However, outcome analysis provides the strongest evidence for the benefit of any treatment. With respect to β1-AAB positive patients with DCM, Dandel *et al.* published the most convincing evidence for IA as a successful treatment strategy in 2012 (123). For this retrospective analysis, patients with end-stage DCM and listed for HTx at the "Deutsches Herzzentrum Berlin, Germany" were enrolled. The

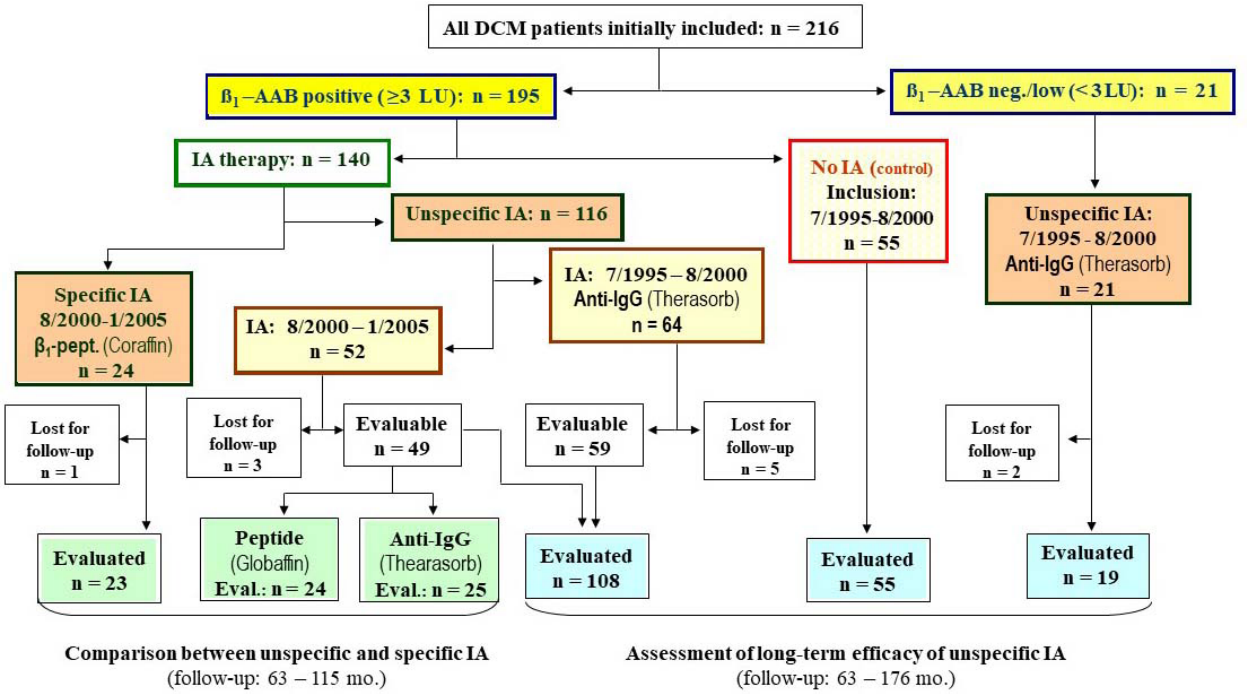

Figure 8. Study design for evaluation of immunoadsorption technologies with respect to the patients' long-term outcome; reproduced with permission from (123)

study design, which included initially 216 patients, is presented in Figure 8

In this study, a significantly prolonged survival free of heart transplantation or ventricular-assist device support (HTx/VAD-free survival) was demonstrated for the IA-treated patients.

All patients – where 140 were positive and 21 were negative for β1-AAB (for inclusion and exclusion criteria see (123) – who have been treated with IA between 1995 and 2005 were evaluated for their outcome (follow-up 5.3.–14.7. years). In comparison, β1-AAB-positive DCM patients (n=55) referred to HTx but who did not receive IA were also enrolled. To assess the efficiency of unspecific IA of the complete IgG vs. specific β1-AAB removal, the 116 β1-AAB-positive DCM patients treated with unspecific IA and the 24 patients with IA for specific β1-AAB removal were analyzed.

Related to the patients' five year follow-up concerning the HTx/VAD-free survival, DCM patients who were positive for β1-AAB benefited significantly more from IA than patients who were β1-AAB-negative, as illustrated by the Kaplan-Meier Estimator (shown in Figure 9A). This is in accordance with the results of a multi-center study performed in Japan (187), where a subgroup analysis revealed an improvement of echocardiographic LVEF after IA in patients with higher baseline autoantibody scores but not in those with lower scores. In Figure 9B, it is shown that the survival rate of patients who were positive for β1-AAB but did not receive IA was significantly reduced compared to the IA-treated patients. In this figure, we additionally indicate that the benefit of IA prolonged probably for more than 10 years.

The authors of the study also calculated a significantly improved survival rate 5-year post-IA for β1-AAB-positive patients who showed LVEF improvement of ≥20% of the pre-IA value within one year after IA (recognized as IA responders) vs. patients with an LVEF that did not increase to this extent (non-responders). No difference in survival rates were evidenced between β1-AAB-positive DCM patients who were treated with either unspecific IA for whole IgG removal or specific IA for selective β1-AAB removal. The authors interpreted the comparable efficiency of un-specific and specific IA as being a strong evidence for the specific driving role of β1-AAB in the pathogenesis of DCM. In view of the presented data, we agree. However, one should keep in mind with respect to DCM patients in general: there is a significant number who suffered from co-morbidities such as electric abnormalities, diabetes mellitus and hypertension and therefore present additionally to β1-AAB with further GPCR-AAB such as M2-, α1-, AT1 and ETA-AAB. (see Table 1) that part some in the pathogenesis of DCM could presently still be underestimated.

This would explain why an even stronger IA benefit has been observed in DCM patients who

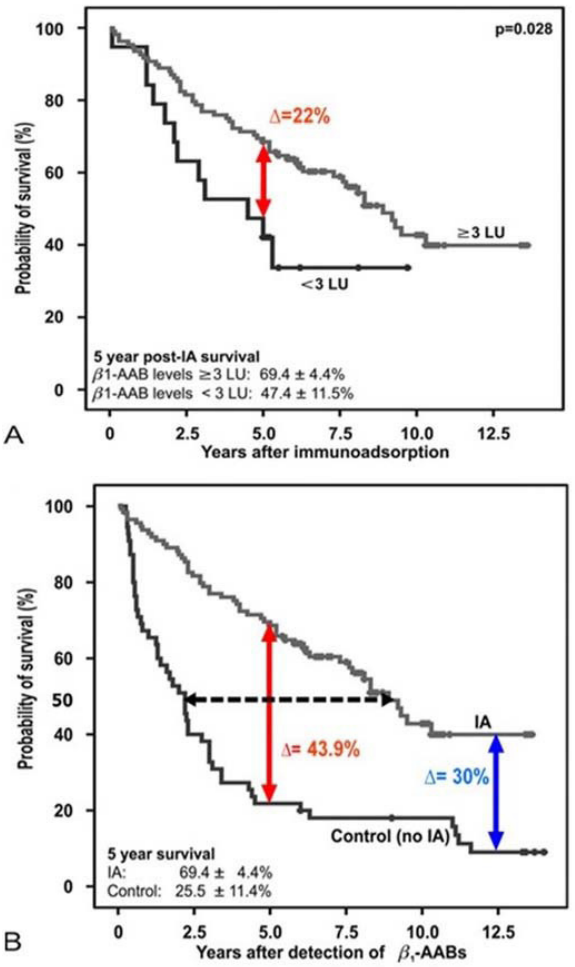

Figure 9. Kaplan–Meier estimates of heart transplantation/ventricular assist device (HTx/VAD)-free survival for dilated cardiomyopathy (DCM) patients after immunoadsorption. A: Comparison between β1-AAB-positive patients (≥3 laboratory units (LU) and β1-AAB negative patients (<3 laboratory units (LU); B: Comparison between the DCM patients positive for β1-AAB treated with IA and non-treated with IA (control). Reproduced with permission from (53).

additionally suffer from diabetes mellitus (186). Patients with diabetes mellitus frequently carry α1-AAB (37) which were reported to be pathogenic in these patients. Consequently, and in relation to the applied unspecific IA, the additional removal of α1-AAB the elimination of β1-AAB could be responsible for this observation.

Derived from an experimental *in vitro* study using the Coraffin® column for the specific β1-AAB clearance of serum from patients with Chagas' cardiomyopathy, IA was already suggested in 2007 as a treatment option for patients with Chagas' disease, but mainly for those with Chagas' heart disease (195, 196). However due to the GPCR-AAB pattern in Chagas' heart patients (see Table 1), unspecific IA for the concerted GPCR-AAB removal of β1-, β2, and M2-AAB should be superior compared with only β1-AAB removal. However, to the best of our knowledge, IA treatment has not been studied to date in patients with Chagas' heart disease; however, IA in 2013 has again been listed in a recommendation for the treatment of Chagas' heard disease (197). IA has also been suggested for patients with peripartum cardiomyopathy, although indicated as a still "unconventional treatment" (198, 199) but, as already mentioned for TPE, to hold promise for the future. However, there is no information published about applying IA in women with peripartum cardiomyopathy.

Furthermore, IA has been studied in patients with pulmonary arterial hypertension (PAH) (190), where all five of the patients who were studied responded to IA with the removal of α1-AAB and ETA-AAB, both of which are believed to drive vascular alterations generally in hypertension and specifically in PAH (200). The strong GPCR-AAB reduction was combined with a reduction of the pulmonary arterial pressure, improvement of RV function, and exercise capacity. In two of the patients whose autoantibodies did not return, stable clinical improvement was achieved for more than 24 months. In the other three patients, the autoantibodies recurred, which was associated with severe worsening of the clinical situation. After renewed IA, one patient recovered to a stable clinical condition at the date of publication. The other two patients died due to ventricular arrhythmia and pulmonary embolism. The benefit of IA for patients with PAH has been recently confirmed (192).

IA for GPCR-AAB removal is increasingly considered for the treatment of diseases such as thromboangiitis obliterans, fatigue syndrome and dementia. In 10 patients with advanced thromboangiitis obliterans treated with un-specific IA for IgG removal, decreased pain intensity was already shown at the second post-IA day and one month later. All patients were without pain over the follow-up period of 6 months, healing of ischemic ulcerations was observed during follow-up and improved walking distance was documented for the patients. Unfortunately, the patients were not analyzed before and after IA for the presence of any GPCR-AAB (189). However, a subsequent study demonstrated α1-AAB and ETA-AAB in patients with thromboangiitis obliterans that disappeared after IA (41). During the mean follow-up period of 3 months available for 15 patients, skin lesions healed in all but one patient. Pain scale values decreased from 7.0. (5-9) to 2.0. (0-5) (52).

Recently, 10 patients with chronic fatigue syndrome indicated as positive for β2-AAB (results of further GPCR-AAB were not reported) were treated with IA. β2-AAB were reduced in 9/10 patients, 7 patients improved, 3 of them for 12 months (193). Furthermore, IA treatment was attempted in 8 patients with Alzheimer's or vascular dementia selected based

on α1-AAB positivity. After IA, negativity for α1-AAB immediately after IA and stabilization of cognitive and mental condition during a 12-18 month follow-up were documented (191). A second IA study that aimed to remove a1-AAB in Alzheimer's patients is presently underway (48). We believe that α1-AAB positivity is only an indicator of GPCR-AAB autoimmunity in patients with Alzheimer's or vascular dementia who often carry, based on our recent findings (45)), further GPCR-AAB such as β2-AAB and ETA-AAB; the latter were mainly found in patients with vascular dementia who most frequently presented with all three GPCR-AAB. Consequently, we see the IA benefit in such patients rather in association – due to the un-specific IA used for the treatment – with the removal of all the GPCR-AAB.

Based on a cost-effectiveness analysis related to 5-year survival rates of patients with moderate and severe heart failure and β1-AAB positivity treated either with IA or conventionally, IA is initially cost-intensive, but considering the significant survival improvement of the IA treated patients, reasonable costs per life-year gained were calculated (170). Nevertheless, cost factors, logistical problems and the patients' burden associated with IA must not be underestimated, which in our view is one of the reasons for the still restricted use of this treatment option. This could explain why IA for GPCR-AAB removal has not entered the therapy of millions of patients with Chagas' heart disease.

4.2. *In vivo* treatment for GPCR-AAB attack

Therapeutic approaches for fighting against GPCR-AAB by drugs directly in the patients' blood could overcome the problems limiting the extensive use of IA. To demonstrate this concept, two approaches were investigated. The first approach aimed to prevent or minimize the generation of GPCR-AAB; intravenous IgG treatment (IVIG) and B cell depletion therapies have already been studied. The second approach implicates drugs derived from peptides or aptamers that are able to bind and neutralize GPCR-AAB in the patients' blood.

4.2.1. Intravenous immunoglobulin treatment (IVIG)

In IVIG, patients were treated with pooled plasma prepared from several thousand healthy donors. Such plasma contains numerous antibodies and, after treatment, these antibodies can react with different antigens in the recipient (201). Complement activation, the suppression of idiotypic antibodies, the saturation of Fc receptors on macrophages, and the suppression of various inflammatory mediators belong to the multiple activities of the pooled plasma preparation believed to be responsible for patient immunomodulation (202). Among the diseases with an autoimmune background, idiopathic thrombocytopenic purpura (203) was the first disease treated with IVIG. Today, IVIG is successfully used in a wide range of autoimmune and inflammatory conditions, mainly in Kawasaki disease, Guillain-Barré syndrome and other autoimmune neuropathies, myasthenia gravis, dermatomyositis, and several rare diseases.

Although, down-regulation or inhibition of cardio-pathogenic autoantibodies could be expected following IVIG (204), "pros and cons" were seen, as summarized in (205), for patients with heart failure and among those whose disease based on DCM.

Compared to placebo treatment, a significant increase in LVEF associated with a decreased level of natriuretic peptide but elevated plasma levels of the anti-inflammatory mediators has been documented for patients with heart failure; however, IVIG did not differ in patients whose heart failure was related to either ischemic or dilated cardiomyopathy (206).

With respect to the assumption of any profound pathogenic role of GPCR-AAB and specifically of β1-AAB in DCM patients, it is surprising that patients suffering from ischemic cardiomyopathy and those with DCM responded equally to IVIG. Furthermore, a controlled trial failed to evidence an IVIG benefit in DCM patients (207), although cardio-pathogenic AAB have been reported as to be sensitive to IVIG treatment (204). Even more confusing for IVIG treatment is that β1-AAB increased after IVIG in DCM patients, although a benefit to cardiac function was evidenced. It is speculative, whether that could be in agreement with above-mentioned pro-inflammatory side-effects of IgG replacement after IA.

For women with peripartum cardiomyopathy, based on case reports and small studies, IVIG benefit has been observed by improved left ventricular function and prognosis (134, 208, 209); however, none of these reports referred to a reduction of cardio-pathogenic autoantibodies that could contribute to the IVIG treatment benefit.

To the best of our knowledge, no study data have been available until now for IVIG treatment in patients with Chagas' heart disease. There is only a mice study of acute experimental Chagas' disease where IVIG restored electrical abnormalities and prolonged survival. In this publication, the authors mention their own unpublished data, where they also saw IVIG benefit in a study with chronic Chagasic mice (210).

A confusing situation with contradictory results of IVIG also exists for patients with myocarditis, as reviewed in (211). Consequently, well-reasoned recommendations for the use of this treatment strategy in myocarditis have not been given (212, 213).

Considering the above referenced data, we see no strong evidence for recommendation of IVIG in cardiomyopathy patients.

4.2.2. B-cell depletion

Rituximab was the first and still most prominent member of a group of agents that can be used for the selective depletion of CD20-positive B-cells (214). Rituximab was studied in a wide range of diseases with an autoimmune background, as extensively discussed in (215). However, among the diseases associated with GPCR-AAB, Graves' disease was the only one where patients were frequently treated with Rituximab; the study results were inconsistent regarding patient benefit (216, 217). Recently, for the treatment of chronic regional pain syndrome (CRPS), Rituximab was applied after TPE (50). When considering B-cell depletion with Rituximab or comparable drugs for the treatment of heart failure patients with the aim of preventing GPCR-AAB generation, the cardio-toxic potential of these drugs must be kept in mind (218). Although pre-existing cardiovascular disease is not considered as an absolute contraindication to rituximab use, more pronounced adverse cardiac effects specifically in patients with prior history of cardiovascular diseases, cannot be absolutely excluded (219-221).

4.2.3. *In vivo* neutralization of GPCR-AAB

4.2.3.1. Peptide-based neutralization of GPCR

Peptides that mimic epitopes of GPCR have the potential for *in vivo* competition with the cellular receptors for the related GPCR-AAB; thus, they could reduce or abolish the pathogenic potency of GPCR-AAB. For this concept, for each of the GPCR-AAB, a specific peptide has to be created such as COR-1, a cyclic peptide homologue of the second extracellular loop of the β1-receptor that was designed to bind β1-AAB directed against the second loop of the β1-adrenergic receptor in patients with DCM (222).We have no information about whether COR-1 targets also β1-AAB of patients with Chagas' disease or peripartum cardiomyopathy. In a key experiment, rats were immunized with a peptide representative for the second extracellular domain of the human β1-adrenergic receptor. This resulted in β1-AAB generation and, over time, in typical signs of heart failure. The treatment of these rats with COR-1 neutralized the β1-AAB and reduced the rats' heart failure symptoms (222). Beside the neutralization of β1-AAB by COR-1, it was recently demonstrated that COR-1 treatment depleted memory B-cells involved in the production of antibodies (223). For forwarding the COR-1 concept to the therapy of patients with DCM, COR-1 has been transferred to humans for clinical phase 1 trials (50). Based on the trial data, it was stated that "…COR-1 was shown to be safe after i.v. administration *in vivo*; no relevant side effects occurred." (224). In the following "Pilot Study of COR-1 in Heart Failure", a phase 2 clinical trial performed between 2011 and 2014, patients with heart failure due to DCM and positivity for β1-AAB were repeatedly treated with increasing dosages of COR-1 vs. placebo. For study design and results including the adverse events, see (51). Unfortunately, a renewed clinical phase 1 trial performed in parallel (35, 225) presented with serious adverse events such as "*the drug* … was possibly immunogenic, and two participants reported thromboembolic serious adverse events…", which halted any further clinical development.

Additionally it must be kept in mind that by design, COR-1 exclusively targets β1-AAB against the second extracellular loop of the β1-adrenergic receptor. Other pathogenic GPCR-AAB present in DCM patients such as β1-AAB targeting the first receptor loop and M2-AAB as well as such GPCR-AAB associated with co-morbidities like hypertension and diabetes mellitus (e.g. α1-, AT1-, ETA-AAB) are not affected, which is illustrated in Figure 10. Nearly 50% of the DCM patients carry β1-AAB targeting the first receptor loop. Their biologic activity is equivalent to that of the β1-AAB against the second loop, as demonstrated in the bioassay of spontaneously beating neonatal rat cardiomyocytes (12, 17, 18). However, in cohorts of DCM patients who were declared for their β1-AAB positivity but were unselected for either the first or second loop targeting β1-AAB and treated with IA, 60–80% of the patients responded with benefit (121, 123, 159, 160, 162). This means that, beside patients with β1-AAB directed against the second loop of the β1-adrenergic receptor, those with first loop-targeting β1-AAB also profit from removal. We therefore consider those treatment strategies that would be able to fight against the whole family of GPCR-AAB to be superior. This strategy has already been demonstrated by the unspecific IA for whole IgG removal. In view of *in vivo* fighting against the whole GPCR-AAB family, the treatment with the aptamer BC 007, described in the following, principally offers the possibility of such a fight. Figure 10 illustrates what happens when DCM patients who were positive for β1-AAB but present an additional GPCR-AAB, here M2-AAB, were treated with either COR-1 or BC 007. COR-1 fights successfully against β1-AAB but the M2-AAB and therefore its pathogenic potency remains untouched. BC 007 switches off both GPCR-AAB which, in our view, should be of clearly greater benefit for patients.

4.2.3.2. Aptamer-based neutralization of GPCR

4.2.3.2.1. Basics of aptamers and their characteristics favoring therapeutic purposes

The pioneering steps in the discovery of aptamers were: 1) the finding that the HIV-originating

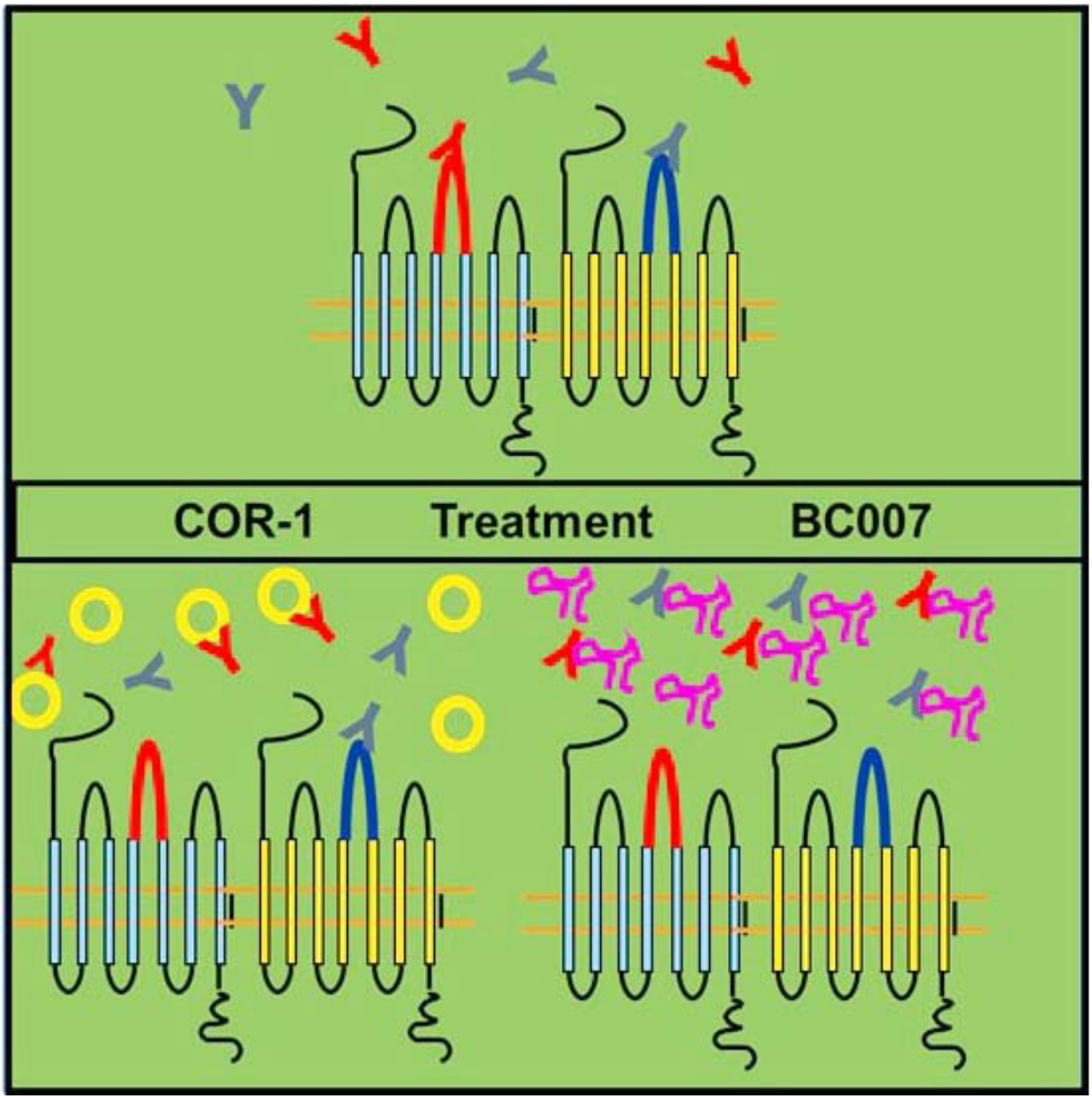

Figure 10. Strategies of GPCR-AAB binding and neutralization. Typically, several GPCR-AAB are present in patients with functional autoantibody diseases, such as β1-AAB (**Y**) and M2-AAB (**Y**) in DCM. The binding of both GPCR-AAB to their receptors in the absence (above) and presence (below) of drugs for GPCR-AAB inhibition is demonstrated: peptide COR-1 (yellow circle, left site) binds to the autoantibodies' high variable region (CDRs) and can therefore only the β1-AAB bind but none of the other GPCR-AAB (e.g. M2-AAB). BC 007 (violet symbol, right site) binds to autoantibodies outside of the CDRs, but to a conserved region present in most different GPCR-AAB and can therefore bind all these GPCR-AAB additionally to β1-AAB, outperforming the peptide concept.

TAR aptamer (an RNA aptamer) was capable of acting as a decoy for TAR trans-activation responsive element of the human immunodeficiency virus type 1 (HIV-1), which is an essential protein for HIV replication, meaning that it is able to stop HIV replication when over-expressed in a human T-lymphoid cell (226); and 2) the selection of an RNA aptamer directed against the bacteriophage T4 DNA polymerase (227), which was successful against small organic dye molecules (228). Some of the basic information about aptamers is summarized in Table 4. For an overview of the current methods and developments in the aptamer selection sector, such as the "tailored SELEX" mentioned above, but also for developments such as "mirror-image SELEX", "covalent SELEX" and many others, see (229, 230).

Aptamers are regularly compared to antibodies, since the purpose of their application is often the same. Both are specific binders for their respective targets and are used for diagnostic and therapeutic purposes. Whereas antibodies carry all

Table 4. Basics of aptamers and their characteristics favoring therapeutic purposes

APTAMERS (Latin: *aptus* - fit, Greek: *meros* - region**)**
• Single- or double-stranded RNA or DNA oligonucleotides containing 10–80 bases (MW: 3,250–26,000 D) that bind target molecules according to their 3D structure
• targets are single ion, complex molecules, or complex biological structures such as viruses, bacteria or whole cells
• Selected against the target of interest from a large pool of oligonucleotides, the aptamer library, by sophisticated chromatographic and washing procedures followed by PCR amplification (*Selex process*)
• Once selected, can be chemically and therefore cost-effectively produced in high quantity and quality
• Heat stability favors production, processing, transportation and storage and enables easy sterilization
• Possess - if well-selected – high affinity and specificity to their targets, both comparable with those of antibodies, therefore were also called “chemical antibodies”
• When bound to functional or active molecules can be used to modulate the molecules’ function
• Possess low toxicity and immunogenicity
• Easy to modify for *in vivo* protection and stability and for modulation of pharmacokinetic and pharmacodynamic properties
• For therapeutic use, a complementary oligonucleotide can be easily designed and provided as antidote

of the advantages and disadvantages associated with their protein nature, aptamers, as oligonucleotides, show as summarized in Table 4 all of the characteristics of either short RNA or DNA sequences; this means, among others, low toxicity and immunogenicity (231-233), most importantly if aptamers are applied to humans. Being synthetically produced, aptamers can easily be modified for *in vivo* protection and stability, as well as for steering their pharmacokinetic and pharmacodynamic properties when used as a drug. The aptamers’ heat stability is another advantage that can be exploited for production, processing, transportation and storage and also facilitates their easy sterilization. Important for the therapeutic use of any aptamer, a complementary oligonucleotide with the same favorable properties, as above indicated in general for aptamers, can be easily designed and provided as an antidote.

4.2.3.2.2. Aptamers as GPCR-AAB binder and neutralizer

We recently selected and characterized, respectively, two aptamers. The first is a 21 mer single strand DNA oligonucleotide (5’-ACA GTA ACC GCG TGA GGT CGA-3’) that binds and neutralizes specifically β1-AAB directed to the second extracellular receptor loop of the β1-adrenergic receptor of DCM patients (234). Due to the aptamer’s potency to also neutralize second loop β1-AAB of patients with Chagas’ heart disease and those β1-AAB present in patients with peripartum cardiomyopathy, its indication area would cover besides DCM also both other cardiomyopathies (78, 235); but unfortunately excluding those DCM patients with first loop β1-AAB. We have successfully tested the *in vivo* neutralizing potency of this aptamer for β1-AAB in spontaneously hypertensive rats which are positive for these autoantibodies. After the aptamer treatment (five times within 4 weeks), β1-AAB titer clearly decreased below the lower limit of detection. The reduced β-AAB titer was stable for months (103). As demonstrated (236), this aptamer could in principal also be used as binder for β1-AAB in the apheresis technology.

The second aptamer, a 15 mer single strand DNA oligonucleotide (5’-GGT TGG TGT GGT TGG3’), named BC 007 (237), binds and neutralizes concentration-dependently all of the GPCR-AAB, as indicated in Table 1.

The concentration dependent decrease by BC 007 of the GPCR-AAB induced chronotropic activity on cardiomyocytes is exemplarily demonstrated for β1-AAB in Figure 11.

The “broadband” potency of BC 007 for GPCR-AAB neutralization (237, 238) enables principally its use for the treatment of all of the diseases indicated in Table 1 and most importantly even if patients are suffering from diseases characterized by presence of several GPCR-AAB (e.g. DCM) or suffering from co-morbidities affected by additional GPCR-AAB (e.g. Diabetes mellitus with α1-AAB positivity). Figure 12 shows an *in vitro* experiment demonstrating the “broadband” neutralizing potency of BC 007 for most several GPCR-AAB such as indicated in Table 1 (239).

Despite our idea of using BC 007 for the treatment of functional autoantibody disease in general, our main focus is currently to develop BC 007 as a drug for the GPCR-AAB neutralization in patients with DCM. As one of the necessary steps, we had to demonstrate the neutralizing potency of BC 007 *in vivo*. For this purpose, as demonstrated Figure 13, spontaneously hypertensive rats (SHR) characterized as being positive for β1-AAB were treated with BC 007 (239); BC 007 was administered five times at weekly

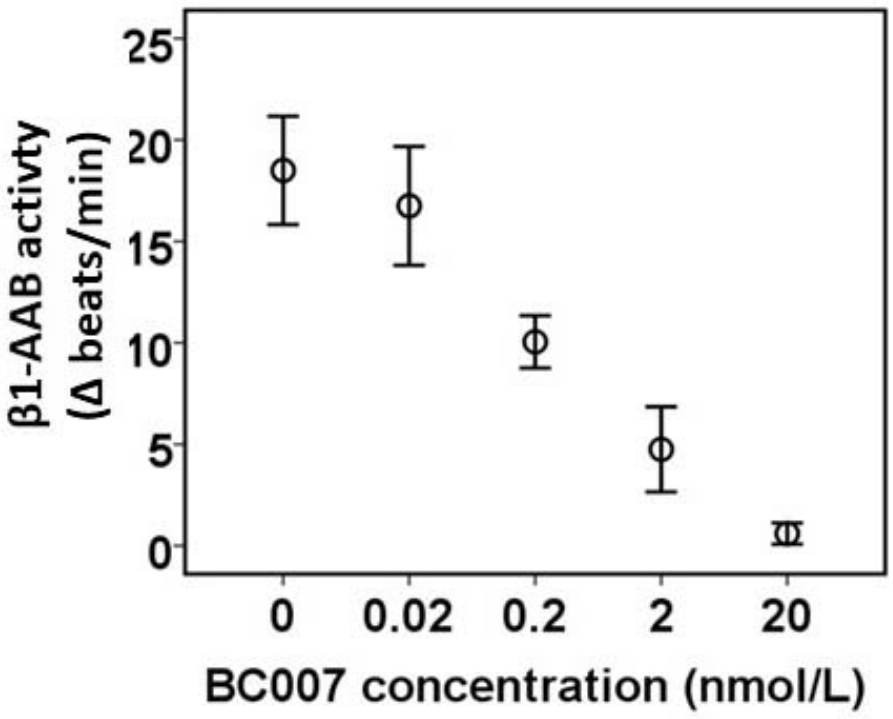

Figure 11. *In vitro* study demonstrating the concentration-dependent neutralization by BC 007 of β1-AAB in the IgG of serum sampled prepared from patients with DCM (n=7). Reproduced with permission from (239).

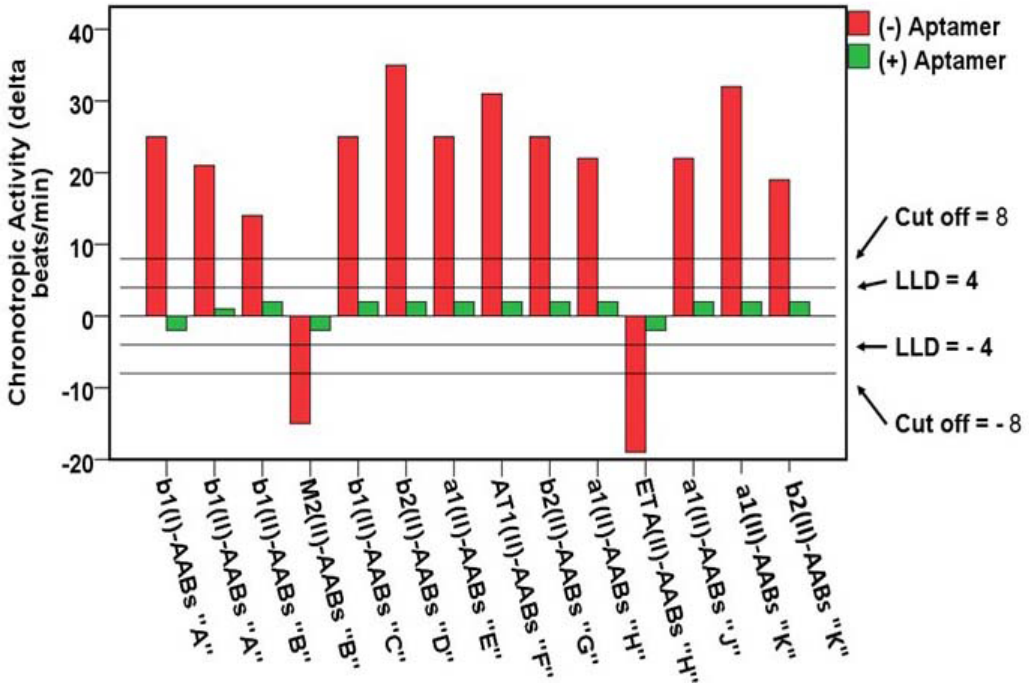

Figure 12. *In vitro* neutralization by the aptamer BC 007 of autoantibodies directed against G-protein coupled receptors isolated from the serum of patients with different diseases. To demonstrate the activity of autoantibodies, patient IgG was prepared and, using the bioassay of cultured neonatal rat cardiomyocytes, the autoantibodies' chronotropic activity was analyzed in the absence and presence of the aptamer BC 007. "A" – Dilated cardiomyopathy; "B" – Chagas' cardiomyopathy; "C" – Peripartum cardiomyopathy; "D" – Glaucoma; "E" – Hypertension; "F" – Malign hypertension; "G" – Chagas' megacolon; "H" – Pulmonary hypertension; "J" – Diabetes mellitus; and "K" – Alzheimer's disease. Autoantibodies directed against the first extracellular loop of the: beta1-adredergic receptor – b1(I)-AABs, beta2-adrenergic receptor – b2(I)-AABs; directed against the second extracellular loop of the: beta1-adrenergic receptor – b1(II)-AABs, beta2-adrenergic receptor – b2(II)-AABs, muscarinic2 receptor – M2(II)-AABs, alpha1-adrenergic receptor – a1(II)-AABs, angiotensin II receptor type I – AT1(II)-AABs, endothelin A receptor – ETA(II) – AABs. Reproduced with permission from (239).

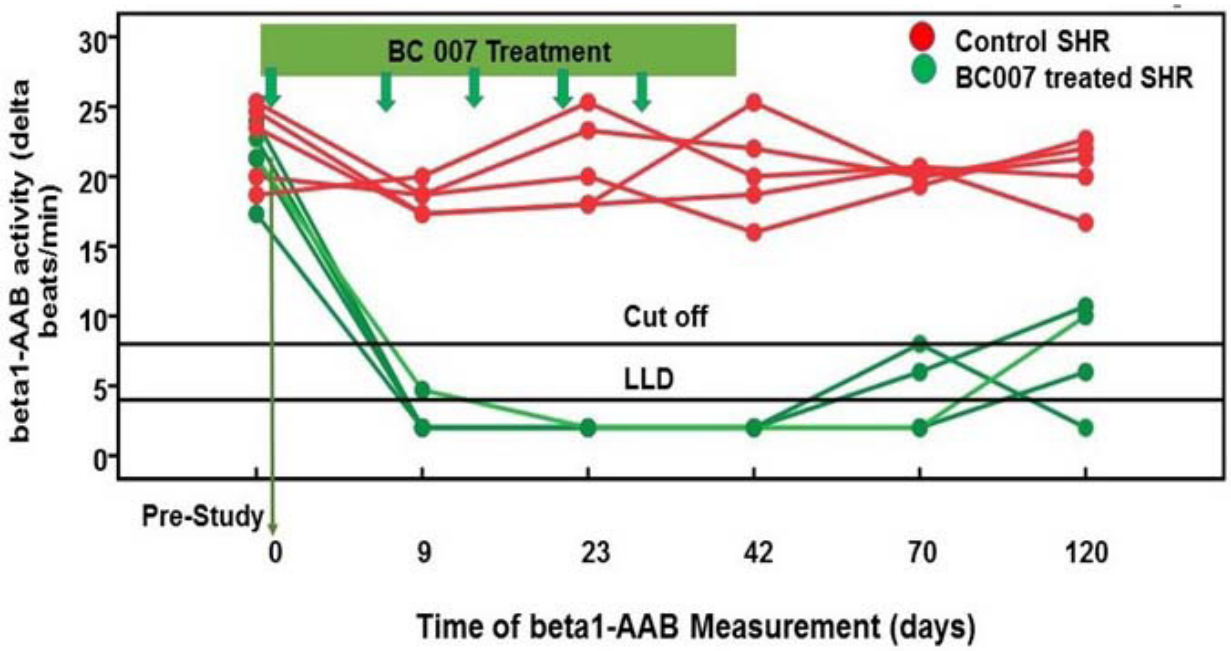

Figure 13. Elimination of autoantibodies directed against the beta1-adrenergic receptor (beta1-AAB) from the blood of spontaneously hypertensive rats (SHR). BC 007 was administered five times at weekly intervals (4 mg/kg BW IV) and was followed-up for beta1-AAB activity for 3 months after the last administration. Control rats received 0.9.% NaCl solution. Rats treated with BC 007 showed a continuous decrease of AAB activity over time (LLD = lower limit of determination; Cut-off = threshold for pathological beta1-AAB activity. Reproduced with permission from (239).

intervals (4 mg/kg BW i.v.). The serum titer of β1-AAB was checked starting one day after the second treatment until 3 months after the last administration. Control rats received 0.9.% NaCl solution. Rats treated with BC 007 responded with a strong reduction in the β1-AAB titer, which was already seen at the first measurement on the first day after the second BC 007 application. In the follow-up, β1-AAB did not substantially return until study end. Visual examination of the heart, liver, and kidney, or the measurement of plasma CK, ALT, and creatinine, did not reveal any signs of aptamer toxicity. Doberman dogs which suffer frequently from DCM and are positive for β1-AAB are another animal model that is presently under study to demonstrate the *in vivo* GPCR-AAB neutralization potency of BC 007.

Based on the presented data for the BC 007 dependent neutralization of GPCR-AAB, our hypothesis is that BC 007 treatment in patients will reduce their GPCR-AAB. Therefore, we expect that BC 007 treatment will most likely have the same benefit for patients as that seen after IA. To further pave the path of BC 007 to patients, presently specifically thought to patients with DCM, all relevant preclinical investigations to prepare the BC 007' phase 1 clinical trial have been successfully completed. In summary, BC 007 showed a very favorable safety profile. No morphological, histological or other side effects were noted in 14 day toxicology studies at concentrations of up to 90 and 100 mg/kg b.w. in dogs and rats. Safety pharmacology studies with the same dosages showed no neurological, pulmonary or cardiologic side effects. The hERG channel was not affected by BC 007. In a secondary pharmacological safety screen, at concentrations of 10 μM, BC 007 did not show any relevant interaction with 44 targets. The phase1 clinical trial of BC 007 in currently ongoing (ClinicalTrials.gov: NCT02955420; (240)). Among the interim results, it is most important that BC 007 was well tolerated and without clinically relevant adverse events. GPCR-AAB neutralization has been seen 24 h after BC 007 infusion of 18.75 mg/min for 40 min in 5/6 volunteers.

For the future, the important question will be: can the proposed benefit of BC 007 for GPCR-AAB-associated diseases of the cardiovascular system be transferred to patients with the other diseases indicated in Table 1; e.g. such as thromboangitis obliterans, dementia and Alzheimer's disease, prostate hyperplasia, and disorders presenting with signs typical for postural orthostatic tachycardia syndrome (POTS), chronic regional pain syndrome (CRPS), and fatigue syndrome.

5. CONCLUSION

Therapeutic plasma exchange, extra-corporeal immunoadsorption, and concepts for *in vivo* neutralization were studied with respect to their application in patients suffering from diseases with an autoimmune background based on the presence of GPCR-AAB. This was mainly demonstrated in patients with DCM and β1-AAB. At this time, and among these

treatment options, only IA using peptides or proteins to bind IgG and also GPCR-AAB resulted in a clearly evidenced long-term benefit. Treatment strategies for the *in vivo* attack of GPCR-AAB, such as IVIG, drug-dependent immunosuppression, and the *in vivo* binding and neutralization of GPCR-AAB would be superior with respect to patients' burden, cost and logistics. Whereas IVIG and immunosuppression related to patient benefit are confusing, strategies using peptides or aptamers for the neutralization of GPCR-AAB tested in animals were hopeful. Only the aptamer concept that is directed to the neutralization of several GPCR-AAB in parallel is currently under extensive investigation.

The translation of therapies for GPCR-AAB reduction such as immunoadsorption and - probably more importantly in the future – aptamer-dependent GPCR-AAB *in vivo* neutralization into other functional autoantibody diseases, e.g. such as scleroderma, pulmonary hypertension, chronic fatigue syndrome, POTS, diabetes mellitus and even dementia might be a very long way away. However, considering the prior work in autoimmunity-compromised heart failure patients, specifically those with DCM, the immunoadsorption but even more the aptamer treatment concept should kept in mind for all other functional autoantibody diseases.

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