



Review

Differential diagnosis of membranous nephropathy with autoantibodies to phospholipase A2 receptor 1 [☆]

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ABSTRACT

Membranous nephropathy (MN) accounts for most cases of the nephrotic syndrome in adults. Recently, studies on the underlying pathomechanisms led to the identification of the podocyte M-type receptor for secretory phospholipase A2 (PLA₂R1) as a target antigen of circulating autoantibodies.

Autoantibodies to PLA₂R1 may not only play a role in the development of primary MN, but also serve as a marker for diagnosis, disease activity and therapy monitoring. Antibody detection is crucial to discriminate between patients with primary MN and those with a secondary form of the disease, as both forms require different diagnostic approaches and treatment strategies. Standardized test systems based on recombinant PLA₂R1 allow for the sensitive and specific analysis of anti-PLA₂R1 autoantibodies. Further research into pathogenic mechanisms and other disease markers can pave the way for improved patient care.

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1. Introduction: membranous nephropathy

1.1. Classification and clinical presentation

Membranous nephropathy (MN) belongs to a family of kidney diseases that is associated with inflammation and hypersensitivity of the glomeruli (glomerulitis) and/or interstitium (nephritis) of the kidney. This is in contrast to tubulopathies, where mainly the renal tubules are affected. The characteristic hallmark of MN is a subepithelial *in situ* deposition of immune complexes at the glomerular basement membrane (GBM) [1]. Since the thickened GBM can be seen by light microscopy in later stages of illness, this disease was initially termed “membranous glomerulonephritis” [2].

Due to the formation of the immune deposits, the permeability of the capillary loops is altered, thus leading to proteinuria and frequently to a nephrotic syndrome. The latter is mainly characterized by proteinuria (>3.5 g of protein in 24 h urine), hypoalbuminemia (<3 g/dl), hyperlipidemia and edema [3]. Accounting for about 20% of cases, MN represents one of the most common causes of nephrotic syndrome [1]. Although spontaneous remission occurs in approximately one-third of MN patients, a similar amount of patients develops end-stage renal failure within ten years [4]. The remaining third of the patients present with a persistent proteinuria. The reported annual incidence of MN amounts to 5–10 cases per million population in northern Europe [5].

Approximately, 20–30% of MN arises as a consequence of different diseases or drug intoxication and are classified as ‘secondary’ MN. They have to be differentiated from ‘primary’ MN (pMN), for which an underlying secondary disease is unknown. pMN can be further subdivided into cases positive for autoantibodies to phospholipase A2 receptor 1 (anti-PLA₂R1) and ‘idiopathic’ cases (without detectable antibodies and not connected to another disease as mentioned above) (Table 1) [6].

This discrimination is of great clinical importance as diagnostic procedures and therapeutic strategies completely differ between both forms of the disease [7]. While in the secondary MN therapy focuses on the underlying disease, patients with pMN are mostly treated with an immunosuppressive strategy. Thus, the correct diagnosis avoids unnecessary drug exposure or extensive diagnostic procedures.

1.2. History

The term ‘membranous glomerulonephritis’ (MGN) was coined by E. Bell in 1946 [8]. By that time, it was not an independent disease but rather belonged to a family of kidney diseases called Bright’s disease Type II, along with other conditions such as membranoproliferative glomerulonephritis, minimal change disease and focal/segmental glomerulosclerosis. In 1957, D. Jones described MGN as a distinct clinicopathologic entity [9]. He established a special stain with periodic acid-Schiff–silver methenamine (Jones’ stain) and was able to show (i) alterations in the basement membrane structure and (ii) thickening of the capillary wall. Subsequently, Mellors et al. identified the thickening to be caused by (iii) subepithelially located electron-dense deposits,

containing immunoglobulin G (IgG) [10]. These three features are still the hallmarks of MN histology.

2. Autoimmune background

2.1. Heymann nephritis: podocyte antigens

In 1959, W. Heymann developed a rat model that mimics the pathogenic features of an MN (active Heymann nephritis) by immunizing rats with a crude kidney extract plus complete Freund’s adjuvant [11]. The subepithelial deposits were induced by fractions of renal brush-border membrane rather than by glomerular extracts. Therefore, it was believed that the deposits were circulating immune complexes consisting of brush-border-related antigens and their corresponding antibodies which were trapped in the glomeruli [1].

This initial assumption was disproved by the introduction of a passive model in the 1980s. By injecting anti-brush-border antibodies into rats, immune deposits occurred in the glomeruli within minutes, leading to proteinuria four to seven days later [12,13]. Additionally, experiments based on *in vitro* and *ex vivo* perfused kidney systems showed that the antibodies bound to a target antigen located on podocytes, thus indicating an *in situ* formation of the immune complexes [12–14].

The primary autoantigen in both active and passive Heymann nephritis was finally identified as the transmembrane protein megalin (also known as low-density lipoprotein (LDL) receptor-related protein 2, LRP-2). Megalin is a 600 kDa endocytic receptor of the LDL receptor family, that is localized within clathrin-coated pits in the brush-border of proximal tubules and at the base and the sides of podocyte foot processes [15–17].

Although the rat model of Heymann nephritis has added a major contribution to our current understanding of the pathomechanisms in MN, there is no evidence that megalin, which also occurs in humans, is involved in the human disease as well.

2.2. Alloimmune neonatal membranous nephropathy: neutral endopeptidase

In 2002, the group of P. Ronco was the first to describe a human counterpart to passive Heymann nephritis in a case of neonatal MN, which was associated with antibodies to neutral endopeptidase (NEP) [18,19].

Here, a mother congenitally deficient for NEP developed IgG1 and IgG4 antibodies, when the antigen was expressed by the fetus. As the antibodies are able to permeate through the placenta, they can bind to native NEP of the fetus, thus leading to an MN at birth or at a later time point. Based on these experiments, Kerjaschki postulated that (i) there is a pivotal role for podocytes and their membrane-associated proteins as targets for circulating antibodies as well as deposit-formation and that (ii) there is a common pathogenic pathway of glomerular immune-complex diseases caused by complement activation [20].

2.3. Major target antigen: phospholipase A2 receptor 1

For many years the search for a target antigen in human MN had been unsuccessful, since it was impossible to identify anti-podocyte antibodies in the blood, podocyte antigens in immune deposits and specific reactivity in eluates from the kidneys of patients with MN [1]. This was most likely due to the low titers of circulating antibodies that require highly sensitive detection methods. In 2009, Beck and coworkers solved the problem by using a Western blot based on a glycoprotein-enriched glomerular extract as the source of antigen [21]. The Western blot was performed under non-reducing conditions and enabled the identification of a 185 kDa protein in 70% of serum samples from patients with primary, but not with secondary forms of MN. Reactivity to this protein persisted after N-deglycosylation resulting in a 145 kDa band, but disappeared after the use of reducing agents, suggesting that the conformation of one or more epitopes in the molecule was

Table 1
Causes of membranous nephropathy.

Primary membranous nephropathy
– Anti-PLA ₂ R1 autoantibodies
– Idiopathic (no detectable autoantibodies or not yet identified secondary cause)
Secondary membranous nephropathy (for a complete list see KDIGO [7])
– Infection-associated (e.g. hepatitis B virus, hepatitis C virus, syphilis, malaria)
– Disease-associated (e.g. rheumatoid arthritis, Sjögren’s syndrome, bullous pemphigoid)
– Drug/toxin-induced (e.g. nonsteroidal anti-inflammatory drugs, gold, mercury, penicillamine)
– Malignancy-associated (carcinomas and non-carcinomas)
– Miscellaneous (e.g. sickle cell disease, Guillain-Barre syndrome)

KDIGO: “Kidney Disease: Improving Global Outcome”.

dependent on disulfide bonds [21]. The target antigen, the M-type phospholipase A2 receptor 1 (PLA₂R1), was subsequently identified via mass spectrometry of the reactive Western blot band.

PLA₂R1 is a type 1 transmembrane receptor that is expressed by podocytes. Besides a short membrane-spanning intracellular domain, the receptor consists of a long extracellular domain with a cysteine-rich head, a fibronectin type II-like repeat domain and eight repeated carbohydrate-recognition domains [22].

PLA₂R1 is one of four members of the mannose receptor family [23]. All of them have a conserved domain structure and share common characteristics such as constitutive endocytic recycling at the plasma membrane [24] which may provide a constant source of accessible PLA₂R1 at the podocyte membrane for immune-complex formation [23]. Furthermore, the receptors exist in both an extended and a bent conformation conferring distinct ligand binding and oligomerization capacities [25]. Since the anti-PLA₂R1 autoantibodies in patients with pMN only recognize a conformation-dependent target epitope [21], it is assumed that antibody-binding might only occur in one of these two configurations. Therefore, pMN might represent an autoimmune conformational disease ('conformeropathy'), such as Goodpasture syndrome [1]. The biological function of PLA₂R1 in podocytes is currently unknown.

2.4. Other autoantigens

In 2010, M. Prunotto et al. analyzed serum and glomeruli of patients suffering from pMN [26]. With different proteomic methods they identified specific autoantibodies to superoxide dismutase 2 (SOD2) and aldose reductase (AR). These two antigens are, unlike NEP and PLA₂R1, cytosolic proteins that are generally not expressed on podocyte membranes. It is postulated that translocation of the proteins to the podocyte surface upon oxidative stress might play a role for the exposition of immunogenic epitopes [27]. Both antibody-specificities are not exclusive for MN, and are only seen in 28–50% (SOD2) and 25–34% (AR) of the cases [28,29]. Additionally, the group of G.M. Ghiggeri discovered antibodies against α -enolase as a potential marker for pMN [30], but this protein has been reported as a target of autoantibodies in other diseases as well [31]. Besides cDNA screens which delivered potential hits [32], more than 20 targets have been found in mice models of Heymann nephritis which are still under investigation [33,34]. Hence, the quest for new anti-podocytic antibodies is ongoing.

3. Pathogenesis

Although the exact mechanisms causing pMN are still under investigation, different studies show a strong relationship between antibody levels to podocytic proteins and disease activity. Since the discovery of anti-PLA₂R1 autoantibodies by Beck et al. in 2009, there has been strong evidence that they are a key player in the pathogenesis [21]. For example, autoantibodies to PLA₂R1 can not only be eluted from kidney tissue of pMN patients, but also colocalize with PLA₂R1 in the glomeruli [21]. Therefore, it is widely accepted that upon binding of circulating autoantibodies to PLA₂R1 on podocytes, subepithelial deposits are formed *in situ*, leading to complement activation and a cascade of events subsequent to the nephrotic syndrome (in most of the cases) [37]. Interestingly, autoantibodies to PLA₂R1 have shown to be mainly of the IgG4 subclass, which is regarded as being unable to activate the complement pathway [38,39]. Nevertheless, since the terminal complement component C5b-9 is detectable in glomeruli and urine of pMN patients [40,41], there is strong evidence for the involvement of the complement system. As most patients with MN have low or undetectable levels of C1q, the classical complement pathway can be excluded. Therefore, either the alternative or the lectin pathway seems to be predominantly involved [42]. This hypothesis is supported by the notion, that mannose-binding lectin can be detected in glomeruli of MN patients [43].

Despite the efforts so far, the etiology that leads to the described pathogenesis is still unclear. It is assumed that podocyte injury, a

conformational change in PLA₂R1 induced by unknown factors or molecular mimicry by exogenous antigens could be involved [1]. Furthermore, stimulation of the relevant autoreactive B-cell clones as well as B cell epitope spreading might contribute to the pathogenic condition.

4. Serological diagnosis

In order to use anti-PLA₂R1 autoantibodies for the diagnosis, classification and monitoring of pMN, validated test systems with high sensitivity and specificity are necessary. Initially, Beck and coworkers detected anti-PLA₂R1 autoantibodies by applying a Western blot after non-reducing SDS-PAGE [21]. However, this method is not suitable for most diagnostic laboratories and for the analysis of large sample numbers. In order to overcome these limitations, a recombinant cell-based indirect immunofluorescence assay (RC-IFA) has been developed that uses the human cell line HEK293 overexpressing the full-length human PLA₂R1 as substrate [44]. The PLA₂R1-positive cells are arranged in a Biochip-format in conjunction with "mock"-transfected HEK293 cells in one incubation field (Fig. 1) [45]. Using this assay, antibodies to PLA₂R1 could be detected with maximal specificity (100%) and with a prevalence of 52% in a cohort of pMN patients, several of which were undergoing immunosuppressive therapy (Rituximab) [44]. This RC-IFA mosaic enables the determination of anti-PLA₂R1 autoantibody titers during the monitoring of patients. For a more precise determination of antibody concentrations (U/ml), Hofstra et al. introduced an in-house enzyme-linked immunosorbent assay (ELISA), based on a recombinantly produced extracellular domain of PLA₂R1 as the solid surface [46]. Almost in parallel, a standardized, commercially available ELISA was developed using the same principle [47]. In a large cohort of clinically well-characterized patients with primary or secondary MN, this ELISA revealed a very high sensitivity with respect to RC-IFA (96.5%) at a set specificity of 99.9% [47]. Accordingly, this assay provides a very accurate and quantitative method for the assessment of anti-PLA₂R1 autoantibodies that is highly suitable for routine diagnostic purposes.

5. Anti-PLA₂R1 autoantibodies as a marker for diagnosis and monitoring

5.1. Differential diagnosis

In several studies anti-PLA₂R1 autoantibodies have shown to be a pathognomonic marker for pMN (Table 2). Initially, Beck et al. could demonstrate that these autoantibodies were exclusively found in patients with pMN (70%), but not in secondary forms of the disease [21]. Furthermore, Gunnarsson and coworkers could also confirm the absence of anti-PLA₂R1 autoantibodies in a European cohort of SLE patients suffering from membranous lupus nephritis [48].

In a recent study, however, one of 20 Chinese patients with membranous lupus nephritis as well as a minor number of patients with MN secondary to hepatitis B or cancer was anti-PLA₂R1 positive [49]. Hence, the occurrence of anti-PLA₂R1 autoantibodies in secondary forms of MN is assumed to be either sporadic, based on ethnic differences or due to a coincidental parallel presence of pMN. Therefore, it should not be excluded that anti-PLA₂R1 autoantibodies are present in a few cases of MN deemed secondary because of a known other disease.

5.2. Disease activity and course

Anti-PLA₂R1 autoantibodies are not only a specific marker for the differential diagnosis of MN but also for its immunologic activity, as shown by several independent groups [36,50,51]. According to the studies by Hofstra et al. the levels of circulating autoantibodies to PLA₂R1 strongly correlated (73%) with the clinical disease activity, as measured by level of proteinuria [36]. Furthermore, anti-PLA₂R1 autoantibodies are a good marker for predicting the course of the disease. In a group of 90 patients with biopsy-proven MN and 90-month follow-up a

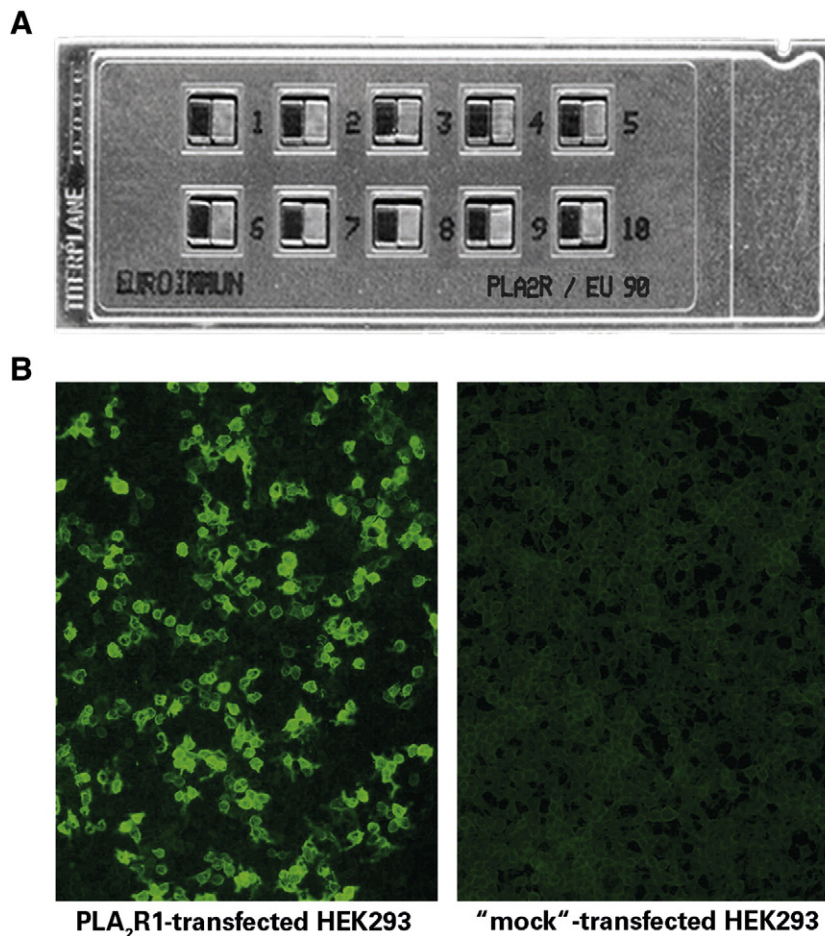


Fig. 1. Recombinant cell-based indirect immunofluorescence assay (RC-IFA) for the determination of autoantibodies to PLA₂R1. (A) Microscope slide with ten reaction fields, each containing two Biochips with PLA₂R1- or “mock”-transfected HEK293 cells forming a mosaic. (B) PLA₂R1-RC-IFA after incubation with an anti-PLA₂R1 positive serum.

clear correlation was observed between the anti-PLA₂R1 titer and the clinical outcome [51]. Moreover, Hofstra and coworkers reported that renal failure tended to occur less frequently at low titers, whereas, more importantly and statistically significant, spontaneous remissions were associated very rarely with high anti-PLA₂R1 titers [46]. In the same study, patients with high antibody titers required an immunosuppressive therapy more often than those presenting with low titers.

Additionally, antibody levels allowed predictions concerning the response to immunosuppressive therapy, defined as time from the start of therapy until remission [46]. A median of five, seven or ten months was observed for low, medium or high classified anti-PLA₂R1 titers, respectively.

5.3. Therapy monitoring

Several studies provided evidence that patients undergoing immunosuppressive therapy show a decline of anti-PLA₂R1 titers, and that the autoantibodies reappear during a relapse [35,44,52]. Characteristically, an increase in the antibody titers preceded a rise in proteinuria, while a decrease in antibody levels was followed by a fall in proteinuria [52]. According to Beck and Salant, residual proteinuria (2–3 g/24 h urine) can still be detected at a point, where autoantibody levels already reached zero [2]. They observed that, in patients with complete remission in response to immunosuppressive therapy, anti-PLA₂R1 titers become undetectable months before the proteinuria is completely resolved. Based on the correlation between clinical and immunological disease activity, anti-PLA₂R1 titers can be used for therapy monitoring in patients with pMN, thus facilitating an early decision concerning the choice of therapeutic strategies according to the “Kidney Disease: Improving Global Outcome” (KDIGO) glomerulonephritis work group [2,7,51,53].

5.4. Risk estimation

In up to 40% of patients, pMN recurs after renal transplantation [54]. The risk for recurrence is higher, when anti-PLA₂R1 autoantibodies are

Table 2

Prevalence of anti-PLA₂R1 autoantibodies in membranous nephropathy (modified and updated from Hofstra [57]).

Author (year)	Patients (n)	Anti-PLA ₂ R1 positive (n; %)	Assay
Beck (2009) [21]	37	26 (70)	WB
Kanigicherla (2011)	40	29 (73)	ELISA
Debiec (2011) [58]	42	24 (57)	IFA
Hoxha (2011) [44]	100	52 (52)	IFA*
Beck (2011) [35]	14	12 (86)	WB
Beck (2011)	35	25 (71)	WB
Bruschi (2011) [30]	24	14 (58)	WB
Hofstra (2011) [36]	18	14 (78)	WB
Qin (2011) [49]	60	49 (82)	WB
Hofstra (2012) [46]	117	79 (74)	ELISA/IFA
Hoxha (2012) [50]	88	61 (69)	IFA*
Behnert (2013) [59]	165	85 (52)	ELISA/IFA*
Kanigicherla (2013) [51]	40	30 (75)	ELISA
Cossey (2013) [60]	22	10 (45)	IFA
Larsen (2013) [61]	85	64 (75)	IFA
Svobodova (2013) [62]	65	45 (69)	IFA*

ELISA: enzyme-linked immunosorbent assay; IFA: immunofluorescence assay; WB: Western blot.

* Standardized method.

detected prior to the transplantation [52]. Based on these findings, anti-PLA₂R1 titers can be used to estimate, if or to which extent an immunosuppressive therapy after kidney transplantation is mandatory in order to prevent a recurrence of MN. Two studies analyzed the role of anti-PLA₂R1 autoantibodies in kidney transplantation and in the differentiation of recurrent *versus de novo* MN [55,56]. In the study of Debiec et al. five out of ten patients with recurrent pMN were anti-PLA₂R1 positive while all *de novo* cases were negative [55]. In addition, Larsen and Walker reported that autoantibodies to PLA₂R1 could be detected in 83% of recurrent cases whereas only one *de novo* case (8%) was anti-PLA₂R1 positive [56]. Thus, anti-PLA₂R1 autoantibodies seem to play a pivotal role in the recurrence of MN after transplantation, but not in the mechanisms resulting in *de novo* MN.

6. Outlook

The exact pathomechanisms leading to pMN are still widely unknown. However, the identification of anti-PLA₂R1 autoantibodies by Beck et al. in 2009 [21] evidenced an association between these autoantibodies and disease pathogenesis. Since then, MN diagnosis, prognosis and therapy decisions have changed. Today, anti-PLA₂R1 autoantibodies are accepted as a pathognomonic marker for pMN, thus enabling a differential diagnosis of the disease. In addition, the antibody titers have a highly predictive value concerning disease activity and course, therapy monitoring and risk estimation. Hence, anti-PLA₂R1 autoantibodies appear as a promising and adequate theranostic marker for pMN as well.

Nevertheless, the prevalence of anti-PLA₂R1 autoantibodies in patients with pMN differs between cohorts depending on immunosuppressive therapy, time intervals between biopsy and serum sampling, and the usage of non-standardized test systems. Additionally, it should be kept in mind that one-third of pMN patients undergo spontaneous remission, accompanied by a decrease in autoantibodies to a level, which might not be detectable anymore. In order to obtain reliable prevalence data, prospective studies with large cohorts of patients are needed. Moreover, the usage of standardized assays is highly recommended. Recently, two standardized test systems (a recombinant cell-based indirect immunofluorescence assay and an ELISA) have been developed and proved highly specific for pMN. As supposed by the KDIGO, further research should determine the potential role of additional biomarkers [7]. These frame conditions can help to mark a new route to diagnosis and treatment of pMN.

Take-home messages

- The identification of anti-PLA₂R1 in patients with primary membranous nephropathy (pMN) evidenced an association between organ-specific autoantibodies and the pathogenesis of the disease.
- Anti-PLA₂R1 is a pathognomonic marker for pMN and enables a differential diagnosis of the disease. Besides predictions regarding disease activity and course, the antibody titers allow for therapy monitoring as well as estimations concerning the risk of recurrence after transplantation. Therefore, anti-PLA₂R1 has a promising theranostic value.
- Standardized test systems (recombinant cell-based indirect immunofluorescence assay and ELISA) allow for the quantification of anti-PLA₂R1 autoantibodies, hence leading to a change in MN diagnosis, prognosis and therapy decision.

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Autism-specific maternal autoantibodies recognize critical proteins in developing brain

Autism spectrum disorders (ASDs) are thought to be neurodevelopmental in origin. **Braunschweig D, et al. (Transl Psychiatry 2013;3:e277)** demonstrate that lactate dehydrogenase A and B (LDH), cypin, stress-induced phosphoprotein 1 (STIP1), collapsin response mediator proteins 1 and 2 (CRMP1, CRMP2) and Y-box-binding protein to comprise the seven primary antigens of maternal autoantibody-related autism. Exclusive reactivity to specific antigen combinations was noted in 23% of mothers of ASD children and only 1% of controls. ASD children from mothers with specific reactivity to LDH, STIP1 and CRMP1 and/or cypin (7% vs 0% in controls; $P < 0.0002$; odds ratios of 24.2 (95% confidence interval: 1.45–405)) had elevated stereotypical behaviors compared with ASD children from mothers lacking these antibodies. The authors describe the first panel of clinically significant biomarkers with over 99% specificity for autism risk thereby advancing our understanding of the etiologic mechanisms and therapeutic possibilities for autism.