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Abstract

Background: IgA-vasculitis (IgAV) encompasses a systemic form involving kidneys, gut, skin or joints, and a skin-limited form. One characteristic feature of systemic IgAV is deposition of galactose-deficient IgA1 (GD-IgA1) in kidneys (as in IgA-nephropathy). The relevance of GD-IgA1 for cutaneous vasculitis is unknown.

Objective: We investigated if GD-IgA1 is deposited perivascularly in systemic and also skin-limited IgAV, and if its serum levels differ between both forms.

Methods: In a case control study, deposition of GD-IgA1 was analysed immunohistochemically by KM55-antibody in skin biopsies from 12 patients with skin-limited and 4 with systemic IgAV. GD-IgA1-levels were compared by ELISA in sera from 15 patients each with skin-limited and systemic IgAV and from 11 healthy subjects.

Results: All biopsies from systemic, but also from skin-limited IgAV revealed perivascular GD-IgA1-deposition. The average GD-IgA1-level in serum was significantly higher in systemic than in skin-limited IgAV, despite overlap between both groups.

Limitations: Although high GD-IgA1-levels may be predictive of systemic IgAV, patient numbers were too low to determine cut-off values for systemic versus skin-limited IgAV.

Conclusion: While perivascular GD-IgA1-deposition is a prerequisite for systemic and skin-limited IgAV, high GD-IgA1-levels in some patients with systemic IgAV suggest a dose-dependent effect of GD-IgA1 in IgAV.

Capsule summary

- Both systemic and skin-limited Immunoglobulin A vasculitis (IgAV) show perivascular galactose-deficient IgA1 (GD-IgA1) deposition.
- Average GD-IgA1-level in serum was significantly higher in systemic than in skin-limited IgAV, suggesting a dose-dependent effect of GD-IgA1 in IgAV

Introduction

IgA vasculitis (IgAV) is characterized by inflammation of small blood vessels (histologically features of leukocytoclastic vasculitis) and IgA deposition primarily around postcapillary venules. The clinical hallmark of IgAV is palpable purpura with predilection for the lower limbs [1]. Systemic IgAV, formerly known as Henoch-Schönlein purpura (HSP), involves internal organs such as the kidneys (causing mesangioproliferative IgA nephritits), gut or joints in addition to the skin [2]. Vasculitic involvement of the kidneys is referred to as **IgAVN** (IgA Vasculitis with Nephritis). Systemic IgAV occurs more often in children than in adults, but renal involvement (IgAVN) is less likely to resolve in adults [3–6].

There is also a **skin-limited form of IgAV**, without systemic vasculitis, as recently defined in the interdisciplinary nomenclature of cutaneous vasculitides (D-CHCC 2012 [1], an addendum to the Chapel Hill Consensus Conference Nomenclature of Vasculitides CHCC 2012 [7]). Skin-limited IgAV does not usually progress into systemic IgAV and is more often seen by dermatologists than systemic IgAV, but there are no studies yet specifically addressing the respective incidence of skin-limited versus systemic IgAV. The reasons for this restriction to the skin are not known, leading to the question whether skin-limited IgAV is an entity with distinct pathophysiological features or if it is a variant which shares most of its pathophysiological features with systemic IgAV except for clinically detectable manifestation in systemic organs.

IgA exists in two isoforms IgA1 and IgA2 and as monomers or J chain containing polymers [8]. In serum the majority of the IgA is IgA1 monomer (90%). Histologically and pathophysiologically, IgA

nephritis in IgAV (IgAVN) resembles IgA nephropathy (IgAN). Both are characterized by the deposition of poorly *O*-galactosylated IgA1 (GD-IgA1) [9] and probably share further common pathomechanisms [10]. Changes in IgA1 *O*-glycosylation occur through dysregulation of post-translational *O*-glycosylation in IgA committed antibody secreting cells, predominantly in the mucosa. Both genetic and mucosal-microbial interactions have been implicated in this dysregulation [11]. In both IgAN and IgAVN, elevated average levels of GD-IgA1 are found in serum [12] and urine [13], and GD-IgA1 is deposited in glomerular capillary walls and the mesangium [14].

Until recently it has not been possible to study whether GD-IgA1 is deposited also in cutaneous vessels in IgAV. This analysis can now be performed by virtue of a recently described monoclonal antibody KM55 that specifically recognizes the galactose-deficient IgA1 hinge [15]. Since skin-limited IgAV does not present with signs of nephritis, we wondered if this marked and clinically relevant restriction to the skin could be related to lower serum levels or absence of deposited GD-IgA1.

We therefore investigated if GD-IgA1 can be detected in skin samples from IgAV patients and, secondly, if there is a difference between skin-limited IgAV and systemic IgAV with regard to a) deposition of GD-IgA1 in skin tissue and b) serum levels of GD-IgA1.

Patients and Methods

Patients: Adult patients presenting with a diagnosis of IgAV to the Department of Dermatology, Münster between 2016 and 2018 were enrolled in this study for analysis of skin and serum samples (mean age of 49.6 years, 67% female). The diagnosis was based on biopsy-proven leukocytoclastic vasculitis, deposition of IgA on immunofluorescence staining and clinical appearance of palpable purpura on dependent parts of the body, i.e. with a characteristic predilection for the lower limbs.

A diagnosis of skin-limited (instead of systemic) IgAV was made when 1) urinalysis showed no proteinuria, no dysmorphic erythrocytes and no erythrocyte casts and eGFR was >90 ml/min/1.73m^2 (no renal biopsy was indicated or performed in these cases) and when there was 2) no abdominal

discomfort and no positive faecal occult blood or signs of vasculitis on colonoscopy, 3) no arthritis and 4) no sign of disturbance of the CNS.

A diagnosis of systemic IgAV with renal involvement (IgAVN) was made when patients had either biopsy proven renal involvement with mesangial IgA deposits or more than 3g per 24h protein excretion and a GFR < 90 ml/min/1.73m^2. All study subjects signed an informed consent document approved by the Ethical Committee of the University Hospital of Münster, Germany, study protocol record 2016-243-f-S and the study was part of ClinicalTrials.gov identifier: NCT01815190.

Skin biopsy: Biopsies were obtained from early vasculitic lesions, i.e. lesions which showed partially blanching, slightly palpable round purpura from 12 patients with skin-limited IgAV, and from 4 patients with systemic IgAV and renal involvement (IgAVN). One biopsy was obtained from each sample site for cryo- and formalin-fixation. In addition we obtained biopsies from the clinically uninvolved skin in one patient with skin limited and one with systemic IgA vasculitis. We used renal tissue samples from two of these cases as a positive control for KM55 antibody staining [15] and confirmed glomerular deposition of GD-IgA1 in both patients (Fig. 1A).

As a negative control we used 3 lesional skin samples from patients with the histologic diagnosis of leucocytoclastic vasculitis but no perivascular IgA deposition and from 2 IgA-negative samples of clinically uninvolved skin (Fig. 1E).

Serum samples: Serum GD-IgA1 levels were measured in 15 patients with skin-limited IgAV and for comparison in 15 gender-matched adult patients with systemic IgAVN (with renal biopsy proven renal IgAVN and skin involvement) and 11 age and gender matched healthy subjects. Serum samples from healthy subjects and IgAVN patients were obtained from the Glomerular Disease Archive of the Mayer IgA Nephropathy Laboratory, University of Leicester.

Immunofluorescence staining of skin and renal biopsies

Immunofluorescent staining for GD-IgA1 in renal biopsies followed the previously published protocol [15]. Staining for GD-IgA1 in skin biopsies was performed using an adjusted protocol based on the lectin-independent method using KM55 antibody on paraffin sections as previously described [15]. In brief, paraffin-embedded sections were deparaffinized by decreasing ethanol concentrations.

Antigen retrieval was performed at room temperature for 2 hours using bacterial protease (0.05% subtilisin A, Sigma-Aldrich). After blocking of nonspecific binding sites (Protein Block, Dako), sections were incubated with KM55 (100 µg/ml) at 37°C for 60 minutes and a secondary antibody Alexa Fluor 555-conjugated goat anti-rat IgG antibody (Thermo Scientific). For staining of IgA we used anti-human-IgA-FITC (Jackson Immunoresearch) and for visualization of nuclei we used DAPI. For staining of IgG, IgM and C3 we used FITC-labeled anti-human-IgG, IgM (both from Dianova) and C3 (Dako) on corresponding cryopreserved samples. All samples were analyzed using an Axio Observer Z1 microscope (Zeiss, Jena) at 20x magnification.

Measurement of serum GD-IgA1: Serum GD-IgA1 concentrations were measured using a KM55 ELISA assay kit (Immuno-Biological Laboratories, IBL), according to the manufacturer's instructions.

Statistical analysis: Statistical analysis was performed with GraphPad Prism 7.04. Differences among disease groups and healthy subjects were assessed by ANOVA, followed by Bonferroni correction for multiple comparisons. Data are expressed as mean $\pm$ SEM. P values of <0.05 were considered to be statistically significant.

Results

Cutaneous deposition of GD-IgA1 in skin limited and systemic IgA Vasculitis

Immunohistochemical staining with the anti GD-IgA1 antibody (KM55) revealed that the 12 patients with skin-limited IgAV along with the 4 patients with systemic IgAV had perivascular deposition of GD-IgA1, co-localizing with deposition of IgA (Fig. 1B-D). C3 was detected immunohistochemically in corresponding cryo-preserved sections of 10 of the 16 IgA-positive skin samples (in 7 with skin-limited and 3 with systemic IgAV), indicating activation of complement, but additional deposition of IgG was observed in only 2 samples from skin-limited IgAV and additional IgM in 1 sample from skin-limited IgAV. We also observed positive staining for GD-IgA1 and IgA in two biopsy specimens of clinically uninvolved skin from one patient with skin limited and one with systemic IgA Vasculitis (both specimens were also positive for C3, and additional weak positivity for IgG was noted in the

sample from systemic IgAV). As expected, there was positive staining for GD-IgA1 with the KM55 antibody in the renal biopsies from two patients with IgAVN (Fig. 1A), both renal biopsies were also positive for C3, and one had additional IgM deposition. Further confirming specificity of the KM55 antibody, the lesional skin from patients with a histologic diagnosis of leucocytoclastic vasculitis but no perivascular IgA deposition (e.g. cryoglobulinaemic vasculitis) and from IgA-negative samples from clinically uninvolved skin did not contain GD-IgA1 (Fig. 1E).

Serum GD-IgA1 levels in skin limited and systemic IgA Vasculitis

Serum GD-IgA1 levels were measured using a commercial KM55-based ELISA (Figure 2). Patients with systemic IgAVN had significantly higher serum GD-IgA1 levels (mean 15.57µg/ml, SEM $\pm$ 3.19 µg/ml, N = 15) than patients with skin-limited IgAV (mean 7.87µg/ml, SEM $\pm$ 1.13 µg/ml, N=15, P=0.0499) and healthy subjects (mean 5.39 µg/ml, SEM $\pm$ 0.96 µg/ml, N = 11, P=0.0125). It is important to acknowledge that the serum GD-IgA1 levels in several patients with systemic IgAV were similar or only marginally raised compared to levels from patients with skin-limited IgAV or healthy subjects (Figure 2). There was no significant difference in serum GD-IgA1 levels between healthy subjects and patients with skin-limited IgAV, although the mean was slightly higher in skin-limited IgAV.

Discussion

Our results reveal that skin lesions in both systemic IgAV and skin-limited IgAV contain deposits of poorly *O*-galactosylated IgA1 (GD-IgA1) associated with the endothelium of postcapillary venules, similar to those seen in the glomerular capillary walls and mesangium in IgAN and IgAVN. Thus, deposition of GD-IgA1 is one prerequisite for IgAV, and restriction of vasculitis to the skin in skin-limited IgAV is not due to absence of GD-IgA1. Presence of dermal GD-IgA1 deposition alone does not, therefore, predict renal involvement or severity of IgAV. However, we did find quantitative differences in the serum levels of GD-IgA1 between systemic and skin-limited IgAV with systemic IgAV patients having a significantly higher average serum level of GD-IgA1 than those patients with the skin-limited form of IgAV.

We report serum GD-IgA1 levels in precisely phenotyped IgAV patients with systemic and skin-limited IgAV using a specific monoclonal antibody (KM55) based ELISA. Previous studies in adult and pediatric IgAV that have reported serum GD-IgA1 levels have used a lectin-based assay which is known to vary between laboratories [12, 16, 17]. In one study in children with IgAV with and without nephritis no significant difference in the median serum GD-IgA1 levels was seen at the onset of IgAV [18], while Berthelot et al found i) higher serum levels of GD-IgA1 in 60 adult patients with IgAVN when compared with 25 patients with skin-limited IgAV (they did not explicitly use this term and definition), and ii) slightly, but not significantly increased serum levels in skin-limited IgAV compared to healthy subjects (as in our study) [12]. Similarly, two other studies reported that IgA1 from 24 and 33 children with renal involvement showed significantly higher lectin binding (indicating high GD-IgA1 levels) than IgA1 from 22 and 17 children lacking renal involvement [16, 17], while lectin binding of IgA1 from children with IgAV without renal involvement did not differ from healthy subjects [16]. It is noteworthy that despite the significantly higher average serum GD-IgA1 levels, many individual patients with IgAVN had no raised levels (compared to IgAV or healthy subjects) [17, 18]. Our cohorts of skin-limited IgAV, systemic IgAVN and healthy subjects also had overlapping serum GD-IgA1 concentrations in the lower range values. Yet, only in the cohort of systemic IgAVN did we find concentrations of 20µg/ml and higher. It is tempting to speculate that as serum levels of GD-IgA1 increase it may tip the balance in susceptible patients from a skin-limited disease to systemic IgAV and whether a threshold of 20µg/ml could be used in the future to indicate systemic IgAV. Concentrations below this threshold do not allow a clear designation of skin-limited or systemic IgAV. Therefore, it will be interesting to perform standardized measurements of serum GD-IgA1 using the KM55 ELISA (in place of lectin binding assays) in large cohorts of IgAV patients to determine if there is a threshold or cut-off value above which systemic vasculitis can be confidently diagnosed (high predictive value).

When we related serum levels of GD-IgA1 with total IgA levels in our cohorts GD-IgA1 was only about 0.2% of the total serum IgA, consistent with the widely held belief that the pathogenic fraction of

serum IgA is limited to a highly specific subset of circulating IgA molecules. This is similar to previous studies which have also reported that higher GD-IgA1 levels are associated with worse renal outcomes and are independent of total IgA levels [15, 17, 19].

Deposition of GD-IgA1 in cutaneous blood vessels appears to be mandatory, but alone is not sufficient to induce vascular damage, as clinically uninvolved skin from IgAV patients also showed GD-IgA1 deposits and since in previous studies (and reflected by our unpublished data) IgA deposition is often found in biopsies from clinically uninvolved skin from patients with IgAV [20–24]. The dermal deposition of GD-IgA1 in both skin-limited and systemic IgAV and increased serum GD-IgA1 levels in IgAVN suggests that skin-limited IgAV, systemic IgAV and IgAN (which is not associated with cutaneous vasculitis) may be variants of the same disease.

After we have provided evidence that Gd-IgA1 is deposited at vessels of systemic and skin-limited IgAV, we believe it is reasonable to extrapolate the IgAN multi-hit hypothesis [25–28] to IgAV as shown in Figure 3. We detected co-deposition of IgG (as in IgAN) in 2 cases of skin-limited IgAV consistent with the presence of IgA-IgG immune complexes [29]. In those cases with no IgG co-deposition it is likely that these patients developed IgA1 autoantibodies to GD-IgA1 (as has been described in IgAN) which cannot be definitively identified with current staining techniques. Immune complex deposition leads to activation of neutrophils in the vessel wall, which is one of the initiating steps in vascular injury [30, 31]. Immune complex, rather than monomeric IgA, deposition is essential for this process because only IgA immune complexes are capable of cross-linking neutrophil FcαRI and thus of inducing production of reactive oxygen species, degranulation, cytokine secretion and release of neutrophil extracellular traps (NETs) [30, 32]. The additional factors that lead to aberrant activation of transmigrating neutrophils and vessel damage are not completely known.

The presence of perivascular C3 in 63**%** of IgA-positive skin samples indicates that complement activation plays a part in the IgA-induced tissue injury both in the skin and at systemic sites in IgAV,

similarly as in IgAN where it is thought to contribute to the formation and nephritogenic activities of the complexes.

A limitation of our study is the low number of skin biopsies and serum samples from skin-limited IgAV and systemic IgAVN patients. Yet, based on our provisional data it will now be possible to undertake longitudinal studies on more diverse and larger patient cohorts to evaluate the prognostic utility of serum GD-IgA1 levels and its predictive value for renal involvement in IgAV.

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~~*Our results confirm that higher serum GD-IgA1 levels are associated with, and may be causally related to, renal involvement in IgA vasculitis (IgAVN). It is tempting to speculate that as serum levels of GD-IgA1 increase it may tip the balance in susceptible patients from a skin-limited disease to systemic IgAV.*~~

~~*Hit 1. Changes in IgA1 O-glycosylation are required, but not sufficient to induce vessel wall injury [29].*~~
~~*Hit 2. The production of autoantibodies (IgA or IgG) recognizing GD-IgA1.*~~

~~*Hit 3. In susceptible individuals the IgA-IgG and IgA-IgA immune complexes deposit in blood vessel walls. One, but not the only, reason for immune complex deposition along cutaneous postcapillary venules may be the slow blood flow in dilated vessels of the lower legs. IgA*~~

~~*Hit 4. A subset of these individuals has additional susceptibility factors which result in immune complex deposition in additional vascular beds –intestinal/pulmonary/renal resulting in systemic IgAV.)*~~

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Legends to Figures

Figure 1

Immunohistochemical staining of renal and skin biopsy tissue for IgA and GD-IgA1. **A** glomerular staining demonstrating dual positivity for IgA and GD-IgA1 deposited at mesangial and capillary wall (DAPI staining omitted) in a case of systemic IgAV with nephritis. **B** Dual positivity for IgA and GD-IgA1 deposition in cutaneous postcapillary venules in a case of systemic IgAV with nephritis. **C** and **D** Dual positivity for IgA and GD-IgA1 deposition in cutaneous postcapillary venules in two representative cases of skin-limited IgAV. **E** Absence of IgA and GD-IgA1 deposition in a case of non-IgA mediated leukocytoclastic vasculitis.

Figure 2

Serum GD-IgA1 concentrations. Serum GD-IgA1 concentrations were measured in µg/ml using a KM55 ELISA assay kit in patients with skin-limited IgAV (n=15), systemic IgAV (n=15) and healthy subjects (n=11). Serum levels of GD-IgA1 were significantly higher in systemic IgAV compared to skin-limited IgAV ($p<0.05$) and healthy subjects ($p<0.05$). The difference in GD-IgA1 levels between healthy subjects and skin-limited IgAV was not significant.

Figure 3

Simplified scheme of the multi-hit-hypothesis for deposition of immune complexes in skin-limited IgAV, systemic IgAV and IgAN.

Synthesis of GD-IgA1 (hit 1), which is recognized by circulating anti-glycan autoantibodies (IgG or IgA1) (hit 2), resulting in formation of large pathogenic circulating immune complexes (hit 3). The target antigen(s) of GD-IgA1 and possibly other components of these immune complexes are unknown. Under certain circumstances they deposit at walls of postcapillary venules in skin and – especially when present at higher serum levels – in glomerular capillary walls and mesangium (hit 4). They activate complement and neutrophils, but the exact processes leading to ensuing vascular or renal injury (hit 5) are not completely known yet.

Figure 1

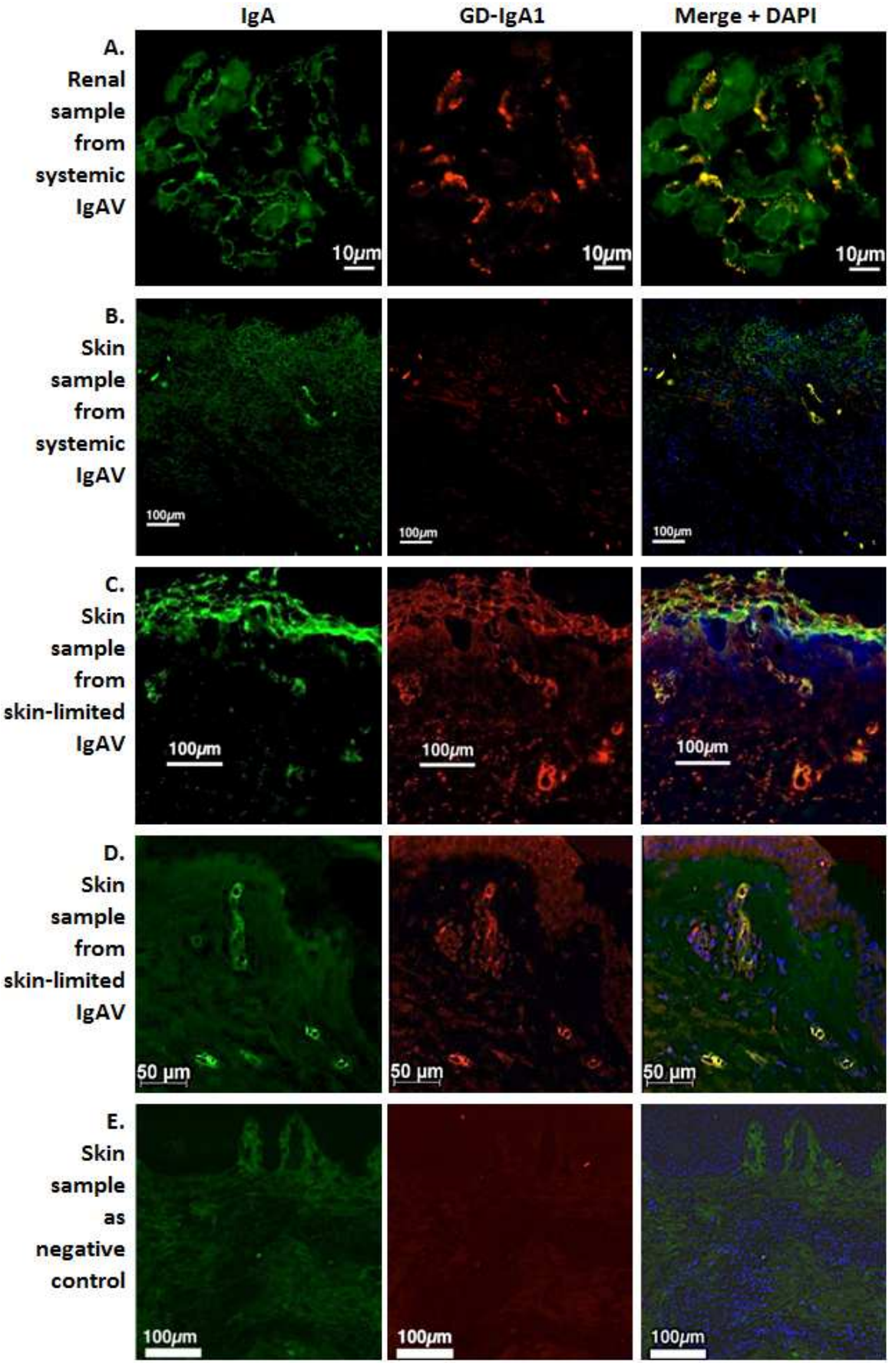

IgA
GD-IgA1
Merge + DAPI
A. Renal sample from systemic IgAV
B. Skin sample from systemic IgAV
C. Skin sample from skin-limited IgAV
D. Skin sample from skin-limited IgAV
E. Skin sample as negative control
10μm
100μm
50 μm

Figure 2

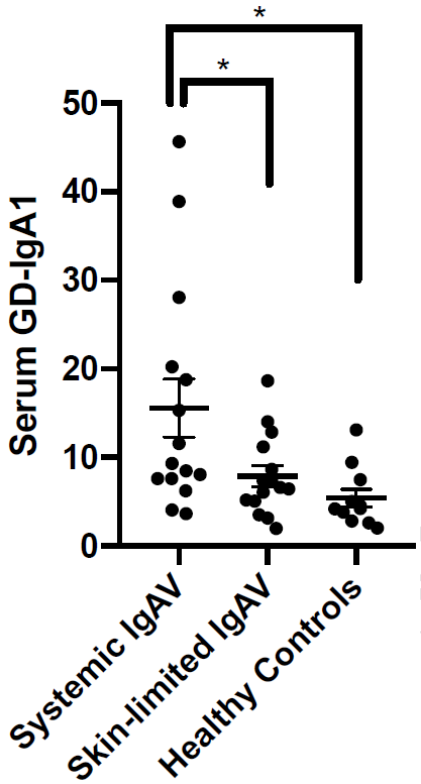
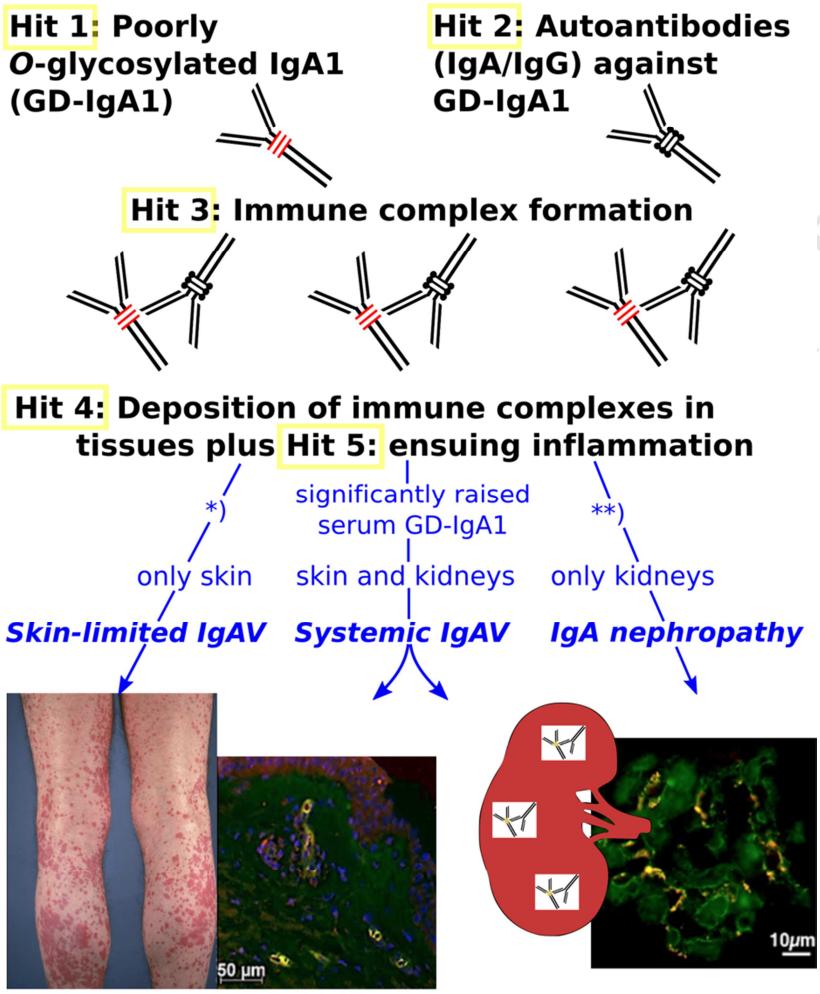
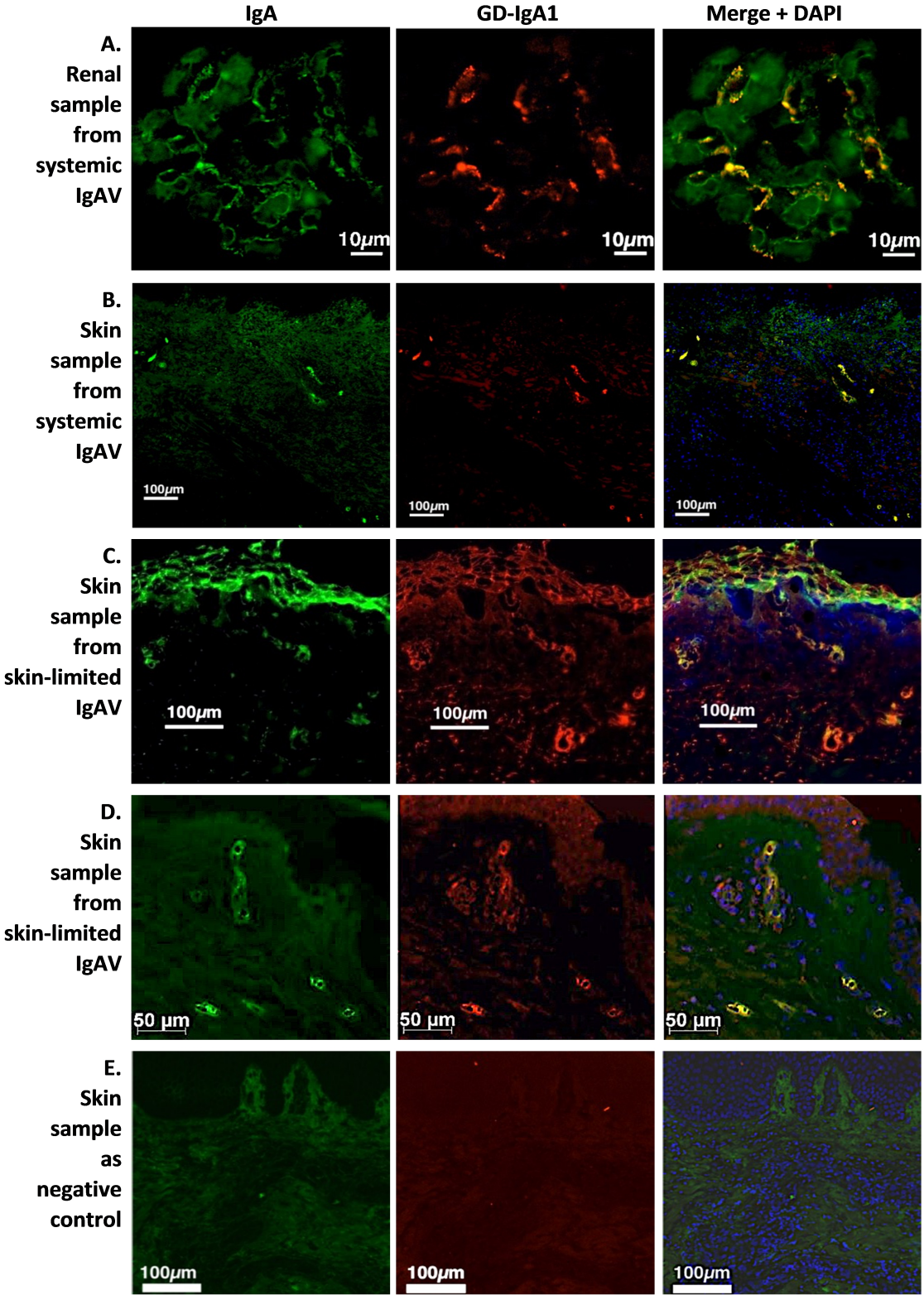


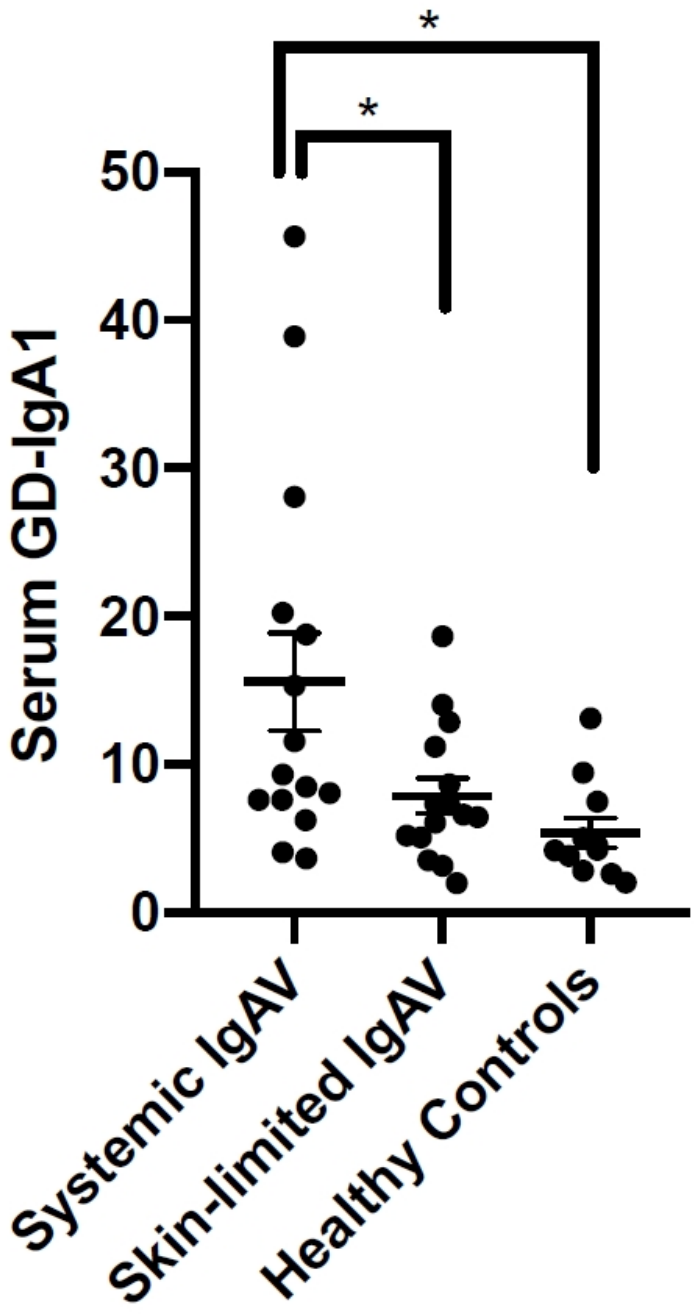
Figure 3

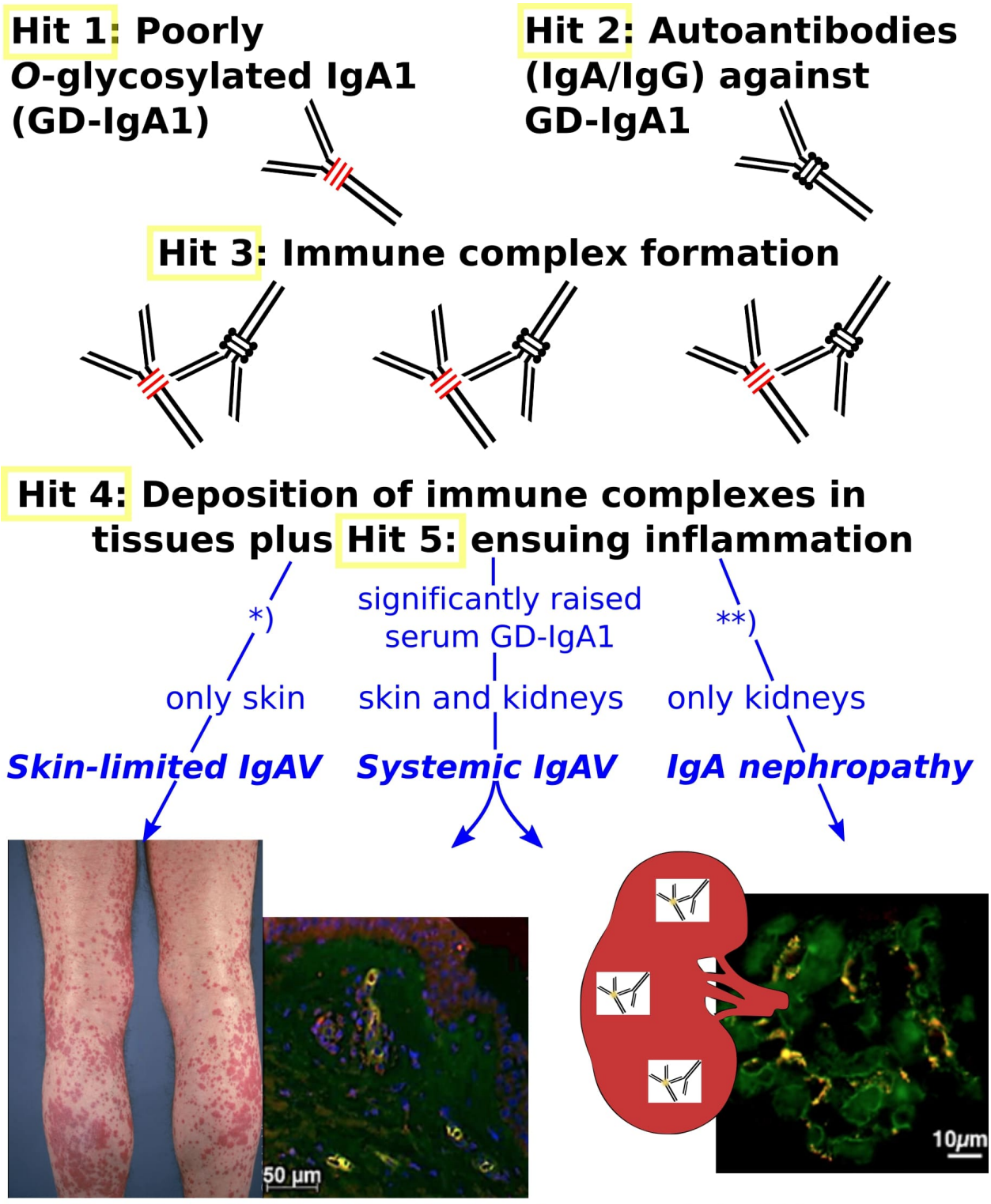


*) Significantly lower levels than in systemic IgAV but higher levels albeit not significantly than in healthy subjects

**) Significantly higher levels than in healthy subjects (lectin-based assays)


IgA
GD-IgA1
Merge + DAPI
A. Renal sample from systemic IgAV
B. Skin sample from systemic IgAV
C. Skin sample from skin-limited IgAV
D. Skin sample from skin-limited IgAV
E. Skin sample as negative control
10μm
100μm
50 μm


*
*
50
40
30
20
10
0
Serum GD-IgA1
Systemic IgAV
Skin-limited IgAV
Healthy Controls



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