

**Early ovariectomy reveals the non-somatic origin of the mammalian anti-A-reactive IgM reflecting developmental malignancy.\*** Peter Arend

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**Abstract.** The germline encoding of a non-immune immunoglobulin M (IgM) molecule in mammals was experimentally documented for the first time as a result of the specifically timed ovariectomy of C57BL/10 mice. The target of this innate antibody involves a trans-species developmental antigen that signifies malignancy when expressed in any non-developmental tissue. Although ovariectomy and castration result in uncontrolled and/or enhanced humoral and cellular immunity involving increased weights of the spleen and thymus with pronounced B and T cell production, the development of mercaptoethanol-sensitive, complement-binding, non-immune anti-A reactivity in murine plasma was not enhanced after an ovariectomy that was performed in C57BL/10 mice prior to the onset of puberty. This non-immune murine anti-A, which is complementary to the trans-species, syngeneic ovarian GalNAc-glycan-bearing glycolipids and distinct from the cross-reactive adaptive anti-A antibody, was strongly down-regulated or did not appear in plasma of animals ovariectomized at the age of 20 days. Thus, contrary to our previous view, this reactivity is unlikely to originate from a somatic, primary immune or autoimmune response. All of the murine tissues expressed the species-specific Forssman reactivity, and further A-like structures were identified in the male and female reproductive and endodermal organs by reaction with innate human anti-A antibody, while the murine anti-A was exclusively inhibited by syngeneic ovarian glycolipids. Moreover, the early ovarian tissue, which represents a last evolutionary and/or developmental location, showed a developmental polymorphism characterized by reactivity to the lectin from *Dolichos biflorus* and the agglutinin from *Helix pomatia* indicating the functional involvement of the O-GalNAc-determined mucin-type A-like, Tn and TF (Thomsen-Friedenreich) epitopes that signify malignancy when accumulated and expressed in non-developmental tissues.

**Keywords:** *Helix pomatia* reactivity, non-developmental tissue, O-GalNAc glycosylation, non-somatic, genital ridge, developmental location.

## **Introduction.**

The human anti-A isoagglutinin and the murine anti-A-reactive IgM appear like a response to a common trans-species A-like, ancestral antigen, while the mercaptoethanol-sensitive and complement-binding anti-A reactivity in murine plasma is, in contrast to our first (1, 2) and recently abandoned concept (3, 4), unlikely to originate from a somatic, primary immune or autoimmune response. Although ovariectomy and castrations result in uncontrolled and/or enhanced humoral and cellular immunity, involving increasing weights of spleen and thymus with pronounced B and T cell productions (5 - 6) associated with autoimmune-related bone loss (11), the development of this early murine IgM or IgM-like molecule was not enhanced after ovariectomy performed in C57BL/10 mice prior to the onset of puberty. On the contrary, the innate anti-A reactivity, which is complementary to syngeneic ovarian GalNAc glycan-bearing hydrophilic glycolipids (12) and distinct from cross-reactive, adaptive anti-A (13), appeared strongly retarded or did not appear at all in the plasmas of animals ovariectomized at the age of 20 days (1, 2), Fig.1. While all murine tissues expressed the expected, species-specific Forssman reactivity and further A-like structures were, identified in the male and female reproductive organs and endodermal tissues by innate human anti-A (14), the murine anti-A was exclusively inhibited by syngeneic ovarian glycolipids, Fig. 2. Moreover, these crude glycolipid preparations showed a developmental polymorphism that was revealed by reactions with the lectin from *Dolichos biflorus* (DBA) and the agglutinin from *Helix pomatia* (HPA) (2), which indicated the functional involvement of mucin-type O-GalNAc-determined, serologically A-like, Tn or TF (Thomsen-Friedenreich) epitopes. Indeed, such epitopes occur on both malignant and normal T cells (15) and can involve NK cell populations (16, 17). They are not expressed by mature B cells and are largely restricted to B cell lymphomas and lymphocytic leukemias, and they signify malignancy when expressed by non-developmental tissues of humans (18 - 21) and mammalian animals (22, 23).

### **Cross-specificities between mammalian anti-A / anti-Tn-reactive IgM and invertebrate defense proteins revealing the evolutionary/developmental position of the Tn epitope.**

The “bulky” GalNAc-molecule (24), possessing undefined biophysical properties, is assumed to be preferential in substrate competitions and hexosamine epimerizations (25) and to dominate the mammalian embryogenic stem cell (ESC)-germ cell (GC) transformation. While the role of specific carbohydrate in sperm-egg recognition remains a subject of discussion (26 - 28) the non-somatic process of germ cell maturation is initiated by transient O-GalNAc-glycosylations (29 - 31). These especially occur on polypeptides (32) that express

glycosidic sites on trans-species functional hydrophilic serine- and threonine-residues (33). Up to 20 distinct polypeptide *O*-GalNAc-transferases catalyze the first addition of GalNAc to proteins, which makes this step the most complex and differentially regulated step in protein glycosylation (34 - 36). These developmental or early ontogenic/non-somatic and genetically as-yet-undefined A-like *O*-GalNAc transferase functions dominate the early carbohydrate metabolism of all vertebrates. They are independent of the species or the phenotype and are characterized by extremely short half-lives, resulting in transient “immature” *O*-GalNAc expressions (29) and potentially imply metabolic relationships or are identical with the mucin-type monosaccharide Tn (GalNAc $\alpha$ 1-*O*-Ser/Thr), the precursor of the disaccharide TF or TFD (Thomsen-Friedenreich) antigen (Gal $\beta$ 1-3GalNAc $\alpha$ 1-*O*-Ser/Thr). In healthy organisms, these cryptic A-like structures are revealed by natural anti-Tn or TF antibodies that are part of the anti-glycan moiety occurring in the plasma of animals and humans (37), and there is agreement for decades that anti-Tn antibodies express A-like cross-reactivity that contributes to the level of the natural anti-blood group A antibody or isoagglutinin (38 - 40). Nevertheless, an IgG3 monoclonal antibody, directed to the Tn glycoprotein antigen and not cross-reacting with blood group A antigen, has been demonstrated after immunization of mice with a purified Tn antigen (41), and a similar Ig was alternatively generated through somatic cell hybridization after immunization of mice with a tumor cell line carrying a Tn-specific mucin (42). However, such antibodies reflect specificities, which may not occur *in vivo*, and the use of vaccines and monoclonal antibodies in the diagnostics and in adjuvant immunotherapy of cancer appears to be burdened with conflicting information (43). Finally, natural anti-Tn reactivity shows lower levels in blood group A individuals (44) but contributes to the higher anti-A levels in blood group O(H) (45), while the human ABO blood group IgM isoagglutinins clearly interact with the Tn and/or TF *O*-glycans in cancer (46) and might reflect growth processes in general (2).

The Tn-bearing *O*-glycan, which in mammalian non-developmental tissues represents an aberrant structure, is most likely synthesized by various *O*-GalNAc transferases but is not restricted to higher organisms, and while occurring in the differentiating ovarian tissue of the mouse, it is normally expressed on the surface of the eggs and liver cells of *Schistosoma mansoni*. Sera from patients, who are infected with this worm, express antibody activities against cancerous tissue, while experimentally infected mice develop antibodies against the Tn and TF epitopes (47). Furthermore, the Tn or aberrant A-like *O*-glycosidic structure is, when arising in tissues of vertebrates, in two ways specifically recognized by the immune system of invertebrates and may indicate common ancestral functions. Indeed, the agglutinin from *Helix pomatia* (HPA) has meanwhile been established as tumor cell marker and prognostic indicator

for different human tumor cell lineages (48), while the agglutinin-free hemocyanin (HPH) from the same snail demonstrates strong anti-proliferative effects in murine models of colon carcinoma (49), and the hemocyanin from *Concholepas* inhibits the growth of bladder tumors (50). In addition, GalNAc determinants of the hemocyanin from *Megathura crenulata* (*Key-hole Limpet Hemocyanin*) show cross-reactivity with T cells and induce a potent Th1-dominant immune response (37), while this hemocyanin (KLH) is meanwhile used in adjuvant immunotherapy of several types of cancer (53).

**Non-somatic, trans-species O-GalNAc glycosylations are distinct from somatic, species-intrinsic GalNAc glycosylations.**

Again, the above developmental, non-somatic and genetically as-yet-undefined, A-like O-GalNAc transferases, which occur in any vertebrate ontogeny, are independent from the species or the phenotype. In fact, they have to be differentiated from the species-intrinsic and especially blood group A determining enzyme proteins or functions that are expressed only after formation of the zygote (54). In humans, the somatic A-allelic, alpha 1-3-N-acetylgalactosaminyltransferase T2 and B-allelic, alpha 1,3 galactosyltransferase functions are encoded on chromosome 9, and in epistatic cooperation with the FUT1 and FUT2 transferases encoded on chromosome 19, they accomplish the mature human ABO(H) histo (blood) group epitope formations on red cells, mucocellular cells and plasma proteins. Furthermore, this epistatic cooperation, governed by two chromosomes, guarantees the specificities of the ABO(H) isoagglutinins by glycosidic accommodation and exclusion of phenotype- or self-reactive antibody activities (54).

Till today, the human histo (blood) group ABO isoagglutinins, involving the Tn and TF cross-specificities, are thought to be exclusively induced by environmental, predominantly intestinal, microbial antigens. However, early clinical studies in patients with inflammatory bowel disease indicated that an adaptive enteral immunization as a source of ABO isoagglutinin production might be restricted to the blood group O(H) and to the formation of anti-B-specific 7S (IgG) antibodies, which were not developed in blood group A patients (55). Moreover, C57BL/10 mice, immunized with the lipopolysaccharide of E.coli O86:B7, developed strong anti-A and anti-B reactivities, which could simply be separated from the innate anti-A reactivity (13) identified as a germline-encoded specificity. This murine anti-A reactive IgM looks like a response to a trans-species and phenotype-independent ancestral antigenic spectrum, which seems to be identically reflected by the human anti-A isoagglutinin (1, 12). The result of the early ovariectomy might show that during germ cell maturation, non-somatic transferase activities have potentially been responsible for synthesizing the A-like, trans-species functional GalNAc-glycans that were iden-

tified on hydrophilic ovarian glycolipids and are volatily expressed by ESCs and/or pluripotent stem cells. After completion of germ cell maturation, these volatile “immature” transferase activities obviously undergo rapid depletion, resulting in the loss of the glycan bonds between cell surfaces and complementary proteins, and the ancestral anti-A-reactive IgM, which has developed as a cell adhesion molecule (CAM), is released into the circulation, Fig. 1. In humans, this non-somatic process, also occurring in reproductive tissues and/or in cell renewal events, is assumed to be accompanied by somatic tissue maturation, in which ancestral, trans-species *O*-glycans on cell surfaces and plasma proteins are permanently “replaced” by ABO-phenotypical, predominantly *N*-linked glycans (54).

According to early reports on the effect of immunoglobulin glycosylation on the enhancement and/or inhibition of tumor growth (56) in connection with the recent discoveries of immunoglobulin fucosylation, in which the immunoglobulin G molecule in initiating antibody-dependent cellular cytotoxicity can be increased 50-fold simply by removing the single fucose residue from the Fc glycan (57), the avidity of antibodies appears to be inversely proportional to the degree of glycosylation (54). Strong antibody glycosylation might protect from autoimmunity, however, obviously favors tumor growth. Indeed, blood group A individuals have an increased risk to develop gastric and pancreatic cancer, while a recent report showed a similar risk for lung cancer (58) and current studies indicate that the overall risk to develop cancer might be increased in any so-called none-O blood group (60, 61). Thus, aberrant *O*-GalNAc-glycosylations, which are even recognizable by the immune system of invertebrates and potentially reflect common ancestral, evolutionary and/or developmental functions, most likely occur in volatile, not measurable expressions in any normal human growth but are, depending on the degree and quality of the phenotype-determined glycosylation of immunoglobulins (54), revealed by the release of anti-A reactivity. Consequently, such release appears most pronounced in the plasma blood group O individuals and involves anti-Tn and TF specificities.

### **Evolutionary and developmental relationships between primordial germ cells and lymphomyeloid cell lineages.**

The reproductive and immune systems have long been thought to share a common evolutionary origin. In the evolution and development of amniotes, these systems arise in topographic-anatomical connection and have their common extraembryonic origin in the yolk sac. In lower vertebrates, IgM secretion occurs in neonates and in adults along the genital ridge (61), and even in skates and sharks, which are some of the oldest non-mammalian vertebrates, the development of the lymphomyeloid system and the ovarian and testicular tissues occurs in the

epigonal organs (62, 63). In birds, the shutdown of somatic B cell functions requires the early removal of the avian bursa of *Fabricius* anlage (64), which develops near the cranial part of the genital ridge and is not anatomically formed in mammals. The mammalian ovary develops from the caudal end of the genital ridge and represents the last evolutionary and developmental location. Unknown numbers and qualities of lymphomyloid cell populations and/or progenitors of the IgM-secreting B cells that occur in the peritoneal cavity of the mammalian female are likely to pass through the ovary and emerge through the fimbriae at the posterior tubal infundibulum, and thus the genetic relationships between B-lymphocytes and GC have been debated for decades. Although the adult mammalian ovary seems to be devoid of definitive germline stem cells and the specific “ovarian reserve” is limited (65), the ovary is considered to be its own type of organism (3), that may generate the complete offspring genome not only in phylogenetically lower species but even in man, where parthenogenetic potential remains (66, 67). Moreover, the cellular traffic in oogenesis involves innumerable intercellular connections and is dominated by those between SC populations and oocytes. These cells have the potential to transform into each other (68, 69) or to differentiate into any somatic tissue-intrinsic cell (70). In males, an IL-4-inducible gene comes to be expressed on differentiating lymphocytes and on spermatozoa (71), while classical V-(D) rearrangements have recently been located in the mouse epididymis (72). The B lymphocyte-induced maturation protein-1 (Blimp-1) is expressed in migrating PGCs as well (73), and membrane receptors that are complementary to each other induce cell divisions in the case of interactions (74). In addition, the lymphocyte antigen 75 (Ly75/CD205) (75), which is expressed on mitotic germ cells in rainbow trout and Atlantic salmon (76), contributes to speculations about the involvement of germ cell maturation, for example, in the production of the anti-A hemagglutinin in *Salmo trutta* (77). Nevertheless, due to different and complex migratory pathways, the developmental relationships involving PGCs, ESCs and LMC are not yet completely understood. The production of the IgM molecule, *per se*, is not restricted to B cells; immunoglobulins also occur in chicken eggs (78), and an IgM-like glycoprotein was isolated from the oocytes of salmon (79). Furthermore, the sporadic production of functional IgM has been demonstrated in normal and malignant human epithelial cells (80). Finally, according to Jerne, “*Germ cells of an animal carry a set of v-genes determining the combining sites of antibodies directed against a complete set of a certain class of histocompatibility antigens of the species to which this animal belongs,*” (81) and there is speculation that the genetic information that governs the IgM polymerization process is primarily divided between germ cell precursors and LMC.

## Conclusions.

Intriguingly, most ovarian and testicular tumors in humans appear to be B cell lymphomas (82 - 84) or develop as germ cell tumors together with non-Hodgkin lymphoma cells (85), while a primary ovarian tumor has been detected in a single lymph node (86). Moreover, the microenvironment of germ cell tumors harbors a prominent antigen-driven humoral response (87). Thus, it is speculated that the evolutionary and/or developmental mystery of the relationship between germ cells and B lymphocytes might become explainable sometime in the molecular biology of B cell tumors. However, such an explanation may also reside in the topographically and molecularly connected synthesis of the trans-species, serologically A-like GalNAc epitope and its complementary, ancestral protein or IgM molecule arising in differentiating mammalian ovarian tissue. As outlined above, the mammalian ovary represents a last evolutionary and/or developmental location and exhibits developmental polymorphism on the basis of DBA and HPA reactivity (2), suggesting the production of mucin-type O-GalNAc-determined, serologically A-like, Tn and TF epitopes that signify malignancy when accumulated and expressed in non-developmental tissue. Nevertheless, these aberrant glycosylations, which are recognized by the defense proteins of invertebrates and potentially involve common ancestral, evolutionary and/or developmental functions, most likely occur in volatile, not measurable expressions in any normal human growth and are, depending on the degree and quality of the phenotype-determined glycosylation of immunoglobulins (54), revealed by the release of anti-A reactive IgM. Consequently, the human “natural” or innate anti-A isoagglutinin, involving cross-reactive anti-Tn and Thomsen-Friedenreich (TF) specificities and reflecting transient developmental malignancy, emerges from these early steps of growth and occurs most pronounced in the plasma of blood group O individuals.

Abbreviations. Ig = immunoglobulin; IgM = immunoglobulin M; GC = germ cells; SC = stem cells; ESC = embryonic stem cells; PGC = primordial germ cells; LMC = lymphomyloid cell lineages; DBA = *Dilochos biflorus* lectin; HPA = *Helix pomatia* agglutinin; NK = natural killer cells.

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Early ovariectomy unmasking the non-somatic  
origin of murine anti-A-reactive IgM

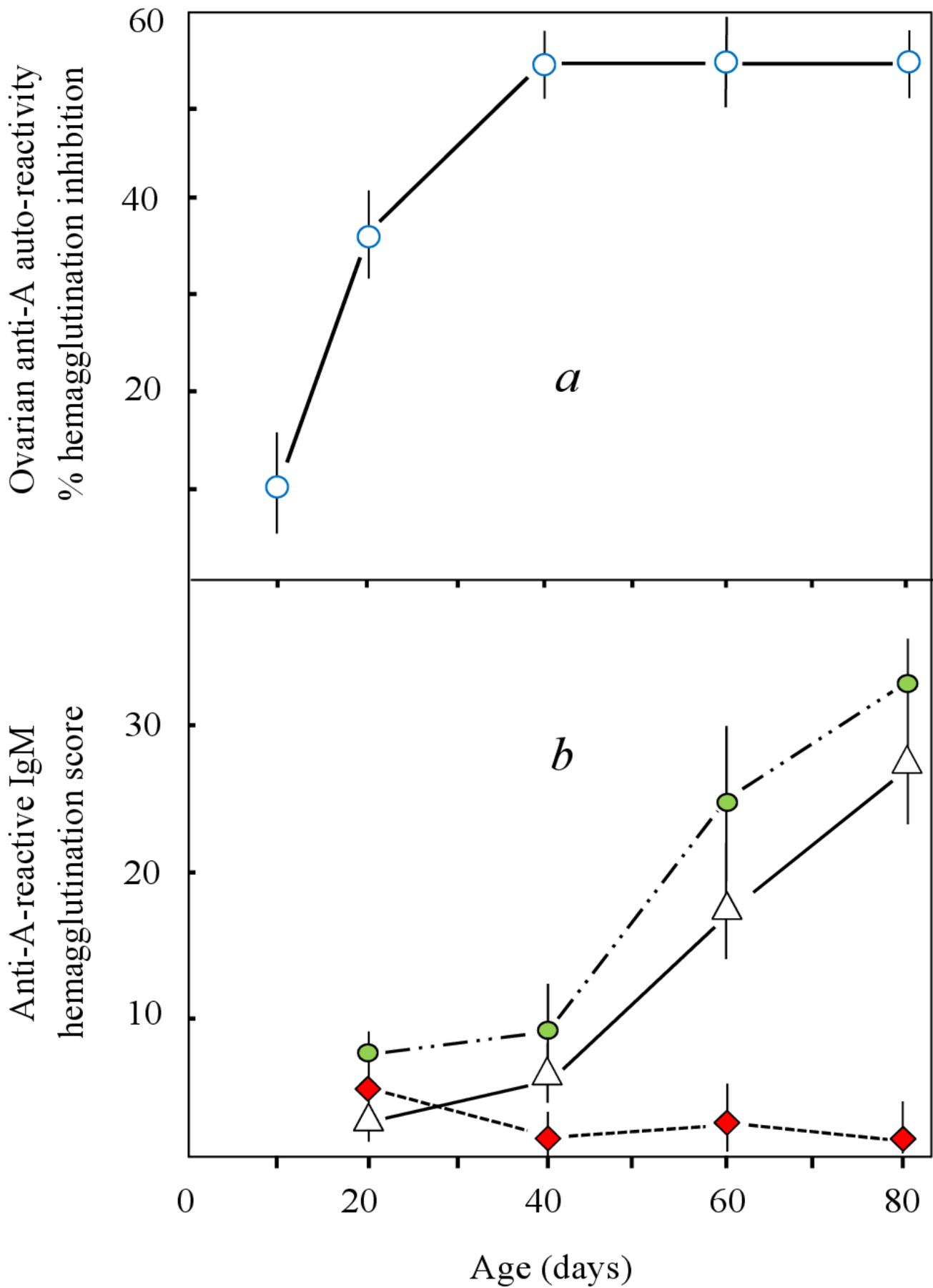


Fig. 1.

**Fig. 1.**

a) Growth-related appearance of auto-reactive, non-somatic GalNAc glycan-bearing hydrophilic glycolipids in the differentiating ovarian tissue with peak levels occurring at puberty: 

b) Subsequently arising, complementary, innate anti-A-like IgM reactivity:

 serum of untreated (non-operated) animals;

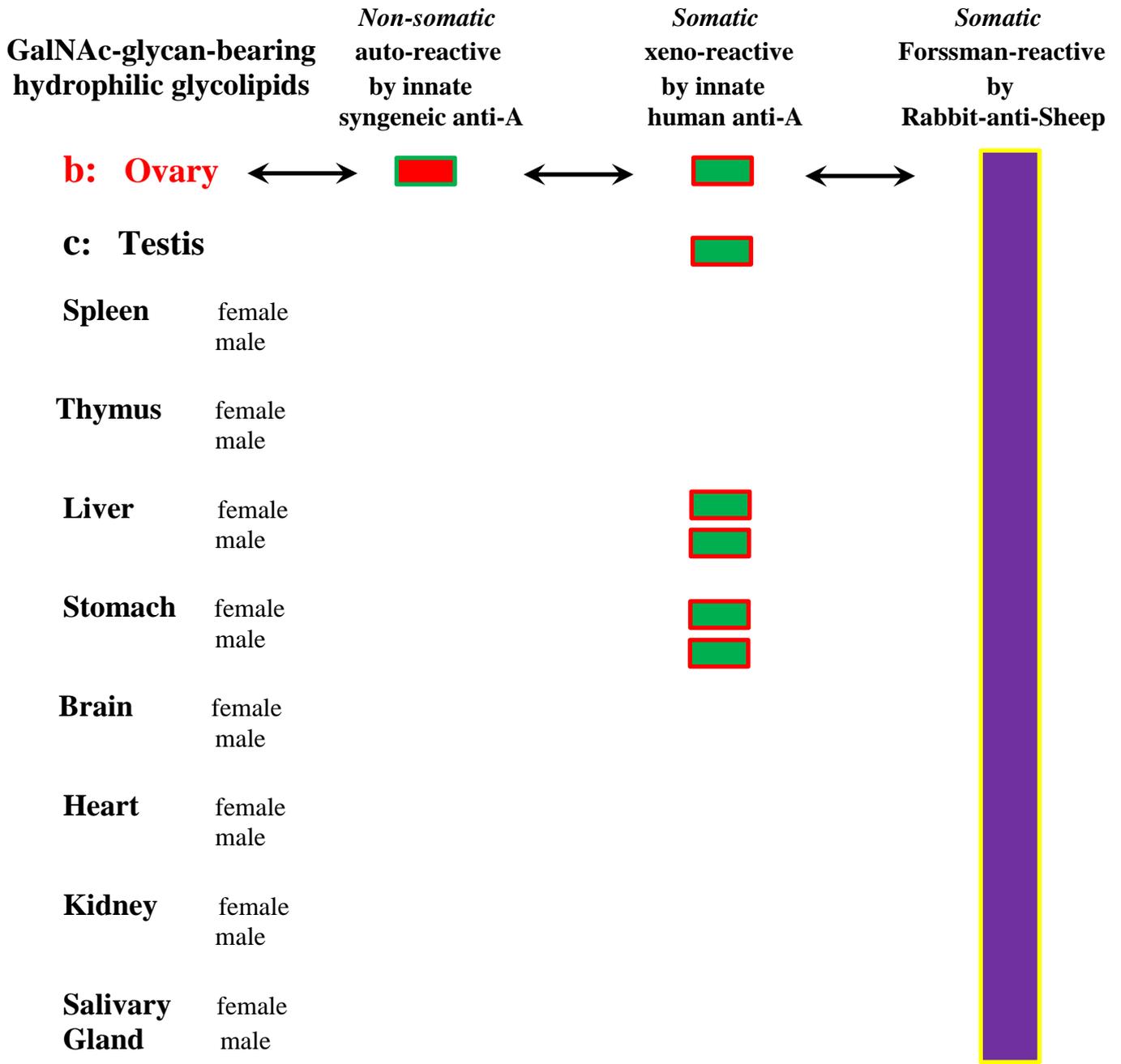
 sham-operated;

 ovariectomized.

Figure reconstructed from (2), Arend, P. and Nijssen, J., 1977 *Nature*, 269, 255–257, cited in *Immunobiology* 221 (2016) 116–127, <http://dx.doi.org/10.1016/j.imbio.2015.07.003>

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# Non-somatic, auto-reactive A-like ovarian determinant distinct from somatic, xeno-reactive A-like structures



**Ovary**

a) = phosphate-buffered saline

Fig. 2

## Fig. 2

Distribution of auto-reactive, non-somatic and xeno-reactive, somatic GalNAc glycan-bearing, hydrophilic glycolipids in C57BL/10 murine ovarian and non-reproductive tissues. While all murine tissues express the species-typical Forssman reactivity and other xeno-reactive A-like structures are, by innate human anti-A, identified in male and female reproductive and endodermal organs, the murine anti-A is exclusively inhibited by syngeneic ovarian glycolipids. The photo was taken during the hemolysis-inhibition experiments as described in Arend, P. 1980, *Immunobiology* 156, 410–417; cited in Arend, 2014, *Immunobiology* 219; 285–29; doi:10.1016/j.imbio.2013.10.01. PMID:26433867