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„Investigation of beta-lactoglobulin presence in cattle tissue"

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Abstract

In recent years, the lipocalin beta-lactoglobulin (BLG) has been shown to influence the human immune system depending on its loading status either towards immunogenic tolerance or allergic sensitization. Moreover, our working group could identify the protein in bovine urine of both sexes indicating that the protein is not solely present in bovine milk. In this work, the presence of BLG in different bovine organs was investigated. Immunohistochemistry, ELISA, western blot and RT-PCR were performed on bovine udder, kidney, adrenal gland and testis. All investigated organs contained BLG on a protein level. The here presented results show that BLG is not unique to the bovine mammary tissue. Although the original biological function of BLG remains elusive, our findings indicate a function beyond that of an amino acid source for the calf.

Zusammenfassung

Untersuchungen der letzten Jahre am Lipocalin beta-Lactoglobulin (BLG) haben gezeigt, dass das Protein das Immunsystem beeinflusst, wobei es abhängig von seinem Ladungszustand sowohl allergische Sensibilisierung forcieren oder davor schützen kann. Die Identifizierung von BLG in bovinem Urin beider Geschlechter durch unsere Arbeitsgruppe indiziert, dass dieses Protein nicht nur in der Kuhmilch präsent ist. In der hier durchgeführten Arbeit wurden unterschiedliche bovine Organe auf BLG Präsenz untersucht. Hierfür wurden Immunhistochemische Färbungen, ELISA, Western Blot und RT-PCR an bovinem Euter, Nieren, Nebennieren und Hoden durchgeführt. Es konnte gezeigt werden, dass BLG kein exklusives Protein des Milchdrüsengewebes ist. Obwohl die genaue biologische Funktion von BLG weiterhin ungelöst ist, deuten die hier präsentierten Ergebnisse auf eine Funktion hin, die jener einer Aminosäurequelle für das Kalb übersteigen.

Content

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1. Introduction

1.1 A brief history of milk

The production of milk is a unique ability within the animal kingdom and consequently the name-giving trait for all mammals. This highly nutritious body fluid is secreted from the mammary tissue and is produced by female mammalian species to feed their respective off-spring. Although mammalian milk is very similar among all mammals, its composition and nutritional value varies as it is designed to meet the specific need of the neonate of the respective species.[1] Principally milk is composed of water, lipids, lactose (sugar), proteins (caseins, whey proteins, immunoglobulins) as well as vitamins, hormones, enzymes, minerals. The amount of these general components differs enormously among all mammals as the content of lipids, proteins and lactose varies between 2 to 55%, 1 to 20% and 0-10%, respectively. Additionally, milk composition of a lactating female changes over time.[1]

Generally, female mammals only produce milk for a short period of time and correspondingly their off-spring loses the ability to digest (unprocessed) milk over time. This is attributed to the discontinued production of lactase, an intestinal enzyme enabling the processing of the main and unique sugar found in milk – lactose. However, this only applies to 65% of all human individuals. The remaining 35%, fortunate carrier of a single nucleotide polymorphism which can be found predominantly in Europe, are able to continue to consume milk into adulthood.[2]

Since milk is intended to supply a neonate with essentially everything before it can switch to a different food source, mankind has harvested milk of other species and incorporate it (as well as derivates such as cheese, butter or yoghurt) into its meal plan. The consumption of dairy precedes written history and in consideration of the aforementioned “35%”, has even driven genetic adaptations in humankind. Today it is assumed that humans have consumed dairy of ruminant species such as goats, cattle, donkeys and horse for over 10000 years.[2]

Since it is not in the intention of the cow to feed a human with its milk, cattle milk differs from human breast milk in various ways. A dominant difference between human´s milk and cow´s milk is the total absence of the major whey protein β-lactoglobulin (BLG).[1,3,4]

1.2 Beta-lactoglobulin (BLG)

Milk proteins have been studied for centuries dating back to the early nineteenth century. Today milk proteins are one of the most thoroughly investigated proteins as milk´s protein composition has the highest influence on its properties.[1] Generally milk proteins are divided into two sections: caseins and whey proteins. In bovine milk whey proteins make up about 20% of total milk proteins

which mainly consists of α-lactalbumin, serum albumin and β-lactoglobulin, the latter represents about 50% of the total whey fraction.[1,3]

Since BLG was originally found in the milk of ruminants such as cattle, buffalo, sheep and goat and is completely absent in human milk, it was assumed to be specific for ruminant´s milk. Today, however, the presence of BLG has been confirmed in an array of other animals as shown in table 1. Although BLG is not present in the milk of all mammals, it has been described in milks of the eldest existing mammals such as the platypus which belongs to the monotremes. Additionally, it can be found in the milks of many marsupials, indicating that BLG is not a unique feature of ruminant species, but rather as old as mammalians themselves and most likely has been lost in some species.[5]

Table 1 Species with or without BLG in their milk. Data on BLG in milk has been gathered on a considerable number of animals. If available, ProteinID of Uniprot is stated, if not stated otherwise mRNA/gene sequence is derived from NCBI database. * https://www.ebi.ac.uk/ena/data/view/, ** unreviewed, *** promotor region/partial cds

	Species	Protein	Gene	mRNA
Species secreting BLG in milk	Cow (Bos taurus, B. javanicus, B. grunniens, B. indicus)	P02754	280838	NM_173929.3
	Bears (Ursus americanus, U. maritimus, U. arctos horribilis, U. arctos yesoensis, U. arctos middendorf fi, U. malayanus)[6]	XP_008702521	103675264	XM_008704299.1
	Bison (Bison bison)[6]			
	Brush-tailed possum (Trichosurus vulpecula)[7]	Q29146		AAA93180.1*
	Buffalo (Bubalus arnee, B. bubalis)[6]	P02755		
	Caribou (Rangifer arcticus)[6]			
	Cat (Felis catus)[6]	P33687 P21664 P33688		
	Dog (Canis familiaris)[6]	P33685 P33686		
	Dolphin (Tursiops truncatus)[6,8]	Q7M2T0* Q7M2T1*		
	Echidna (Tachyglossus aculeatus)[6,9]	AJD87461.1**	KM491792.1***	
	Eland (Taurotragus oryx)[6]			
	European elk (Alces alces L.)[6]			
	Fallow deer (Dama dama)[6]			
	Fur seals (Callorhinus ursinus, Arctocephalus gazella, A. pusillus doriferus, A. tropicalis)[6]	W5QN41* W5QNH4*		
	Giant panda (Ailuropoda melanoleuca)[6]			
	Giraffe (Giraffa camelopardalis)[6,10]			
	Goat (Capra hircus)[6]	P02756		
	Grey kangaroo (Macropus giganteus, M. rufus, M. eugenii)[6,11]	P11944	AH001251.2*	
	Horses (Equus caballus, E. quagga, E. asinus)[6]	P02758 P07380 (horse LGB2)		
	Macaque (Macaca fascicularis)[6]			
	Manatee (Trichechus manatus latirostris)[6]			
	Mouflon (Ovis aries musimon)[6]	P67975		
	Musk ox (Ovibos moschatus)[6]			
	Okapi (Okapia johnstoni)[6]			

	Peccary (Pecari tajacu)[6]			
	Pig (Sus scrofa domestica)[6]	P04119		
	Platypus (Ornithorhynchus anatinus)[6]			
	Pronghorn antelope (Antilocapra americana)[6]			
	Red deer (Cervus elaphus L.)[6]			
	Reindeer (Rangifer tarandus L.)[6,12]	Q00P86**	AH015144.2*	AH015144.2*
	Rhinoceros (Diceros bicornis)[6]			
	Rhinoceros (Rhinoceros unicornis)[6]			
	Sheep (Ovis aries, O. ammon musimon)[6]	P67976		
	Tammar wallaby (Notamacropus eugenii)[11]	Q29614		AAA31597.1*
	White-tailed deer (Odocoileus virginianus)[6]			
	Yellow baboon (Papio hamadryas)[6]			
Species with BLG free milk	Camel (Camelus dromedarius)[6]			
	Guinea pig (Cavia porcellus)[6]			
	Human (Homo sapiens)[6]			
	Llama (Lama glama L.)[6]			
	Mouse (Mus musculus)[6]			
	Rabbit (Oryctolagus cuniculus)[6]			
	Rat (Rattus norvegicus)[6]			

1.2.1 LGB – Beta lactoglobulin genes

The gene encoding bovine beta lactoglobulin (LGB) is encoded on chromosome 11 (11q28) and is about 6700 bp long consisting of 7 exons. Several polymorphisms are known for bovine LGB. In cattle the most common variants A and B have already been described in the 1950ties[13] and since then 10 additional variants have been described (C, D, E, F, G, W, H, I, J, X). Variant A and B differ at position 64 and 118, where variant A encodes for aspartic acid and valine, while variant B encodes for glycine and alanine.[14] BLG alleles are regulated co-dominantly causing simultaneous presence of different variants in milk of heterozygote individuals.[15] The expression of the variants has significant influences on the physiochemical properties of milk. Statistically the most frequent genotype of bovine LGB is AB (49.5-66.6%) followed by AA (18.2-26.4) and BB (15.2-24.1%).[14]

Genetic variants also exist in other ruminants such as sheep and goats with a high similarity (~95%) between bovine, ovine and goat BLG.[14] Additionally, cattle possess an inactive pseudogene. Interestingly, while some species have different variants of the beta-lactoglobulin gene (LGB), many non-ruminant species possess distinct genes altogether. Moreover, these LGB sequences differ among each other considerably. In dolphins, dogs, cats, donkeys, horses and pigs more than one active LGB gene have been discovered. Cats possess three LGB genes (I, II, III), dogs have two (LGB, III) like horses (I, II) and donkeys (I, II).[13]

The synthesis of milk proteins by the mammary gland is generally positively regulated by insulin, prolactin, glucocorticoids and thyroid hormones, while progesterone acts as a suppressor. Several

nuclear factors have been described such as the mammary gland factor, milk protein-binding-factor, pregnancy-specific mammary nuclear factor and mammary cell-activating factor.[16]

1.2.2 BLG protein structure

BLG is a small extracellular protein consisting of 162 amino acids (depending on the variant) with the size of about 18 kDA (bos taurus, monomeric). Under physiological conditions ruminants' BLG forms dimers (pH 5.5-7.5). Spectrometric analysis revealed that the protein consists predominantly of β-sheets (50%), about 15% α-helixes and 30% random coils.[15] The protein is very compact and nearly spherical (figure 1[17]). BLG consist of an 8 stranded β-barrel. This β-barrel is formed by two four-stranded anti-parallel β-sheets (A-D and E-H). While the loops connecting the sheets on the open end of the barrel are long, those on the other end connecting BC, DE and FG are short and less flexible. In its native form BLG is generally very resistant to proteolysis, rendering it less valuable as a nutrition source in milk.[1,15] Moreover, the protein is able to bind small hydrophobic molecules within its central calix such as vitamin D, vitamin A or quercetin.[3,4,18] The amount of binding studies for BLG listed in literature have reached excessive numbers.

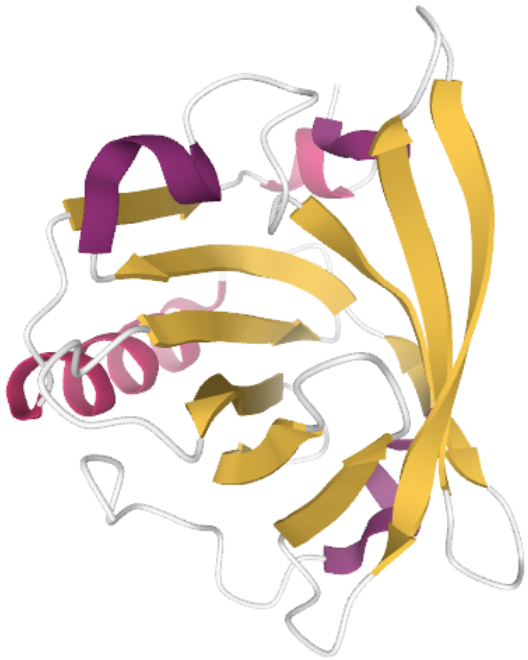

Figure 1 Protein structure of BLG. BLG of ruminant species generally forms under physiological condition dimers. In the here shown picture (PDB ID: 3NPO) the monomeric structure of bovine BLG is shown.

1.2.3 BLG function

Although BLG was isolated more than 80 years ago, the protein specific function remains elusive.[19] Until now no specific receptor for BLG has been found in cattle and scientists were able to produce viable BLG knock-out cows[20]. Although many argue that BLG serves as an amino acid source for the calf, the protein is generally resistant to acid and pepsin.[13] In some studies BLG has been shown to have antimicrobial effects against mastitis caused by bacteria.[21] BLG has been shown to enhance retinol uptake in the small intestine and therefore a similar function to the retinol binding protein (RBP) has been suggested. Yet BLG has higher affinities for vitamin D_3. The closest related human protein to BLG on the other hand is the retinol-binding protein glycodelin (also known as PP14). Glycodelin is expressed during the first trimester of human pregnancy.[15] Although many attempts have been made to ascribe a function to BLG up to this day, no satisfactory explanation has been found.

1.3. Lipocalins

BLG is a small extracellular molecule which can bind a wide variety of small hydrophobic molecules into an intramolecular pocket, thus shielding and transporting those molecules.[15] As more and more molecular structures of proteins were investigated, many proteins with similar structures and abilities to BLG have been identified. This finally led in the late 1980ties to the introduction of the term lipocalin to describe proteins which can bind lipophilic substances within a pocket-like structure minimizing contact of the ligand to the environment.[15,22] Besides BLG, as one of this family´s oldest members, the lipocalin protein family is comprised of many proteins originating from bacteria, arthropods, plants, invertebrates, vertebrates to mammalians.[23]

Lipocalins, which first have been thought to be solely transporter proteins for small and lipophilic molecules, are present in many species and interestingly despite a generally low amino acid sequence identity of only 20 – 30%, they all share short characteristic conserved sequence motifs[24]. By these motifs lipocalins can be divided into the kernel lipocalins and the outlier lipocalins. BLG belongs to the kernel lipocalins which all share three conserved sequence motifs and compose the bigger subset of the lipocalin family. The outlier lipocalins only share one conserved sequence motif.[24,25]

Although lipocalins have low sequence conservation they exhibit high structural similarities and commonly consist of a β-barrel made of a single 8-stranded hydrogen-bonded anti-parallel beta-sheets[25]. Additionally to this structural similarities, lipocalins also share other common properties such as binding a range of small hydrophobic molecules, forming complexes with soluble macromolecules as well as binding to specific cell-surface receptors.[24] Originally classified as

transporter molecules, nowadays lipocalins have been found to participate in many other important biological functions. For example, they play a role in the transportation of retinol, olfaction and pheromones as well as the synthesis of prostaglandins, regulation of cell homoeostasis and modulation of immunogenic response. This demonstrates that lipocalins have several other functions than the mere transportation of endogenous or exogenous compounds.

1.3.1 Lipocalins can modulate the immune system

Lipocalins have been studied in humans and animals alike. In context of disease it has been shown that lipocalins interfere with the immune system in various ways. For example, in humans and dogs, lipocalins are elevated and released in response to inflammation, infection and sepsis. Furthermore, in humans lipocalins such as LCN2 are elevated in cancer and are linked to metastasis. Lipocalins also get overexpressed in the brain in cardiovascular inflammation and elevated levels in the brain are also linked to depression.[25] Since many lipocalins change their plasma level during the acute phase response, many of them are regarded as biomarkers for an array of diseases.[24,25] For example lipocalins AGP, NGAL, PP14 are elevated while RBP is decreased at the beginning of an immune response.[24] Additionally, it is generally believed that these acute phase proteins have an anti-inflammatory function resulting in protection of inflammatory tissue damage and the transportation of immune factors. Apart from this function many lipocalins are well known allergens which is also true for BLG, also called “Bos d 5” in context of allergic diseases. Interestingly, no special structural, functional or physicochemical property of lipocalin allergens explain their allergenicity.[25] As the tolerogenic versus the allergenic capabilities of lipocalins are important for a subsequent immune response, the immune reactions will further be described here.

1.4. The immune system and allergies

Our body is confronted daily with huge amounts of different microbes, viruses, fungi and parasites. While most of them are not immediately dangerous to the healthy individual, they surely need to be kept in check. This is accomplished by our immune systems, which can be divided into two fundamental units working together. The first unit is the innate immune system which acts when the first defence line, which consists of the physical barrier (skin, mucosa, epithelia…), is crossed by a potential pathogen. An important part of the innate immune system is the complement system which consists of solvent plasma proteins mainly produced in the liver (p61) and antibodies (part of the adaptive immune system). The complement system can either further activate the innate immune system or additionally activate the adaptive immune system. While the innate immune system has the ability to react fast, in the long run it is less efficient compared to the adaptive immune system which can be adapted to fight many different presented pathogens specifically

and furthermore is able to memorize them. This enables the adaptive immune system to act fast upon re-encounter and to prevent the onset of disease by eliminating the threat before any damage is done.

The adaptive immune system consists of B-lymphocytes and T- lymphocytes. Both cell types are able to identify and bind specific antigens. This is done by so-called antigen receptors which are expressed by both B- and T-lymphocytes, however they differ in their structure and functional abilities. Secreted B-cell antigen receptors are also called immunoglobulins or antibodies and are produced by B-plasma cells.[26]

Each antibody consists of an Fc-region (fragment crystallization) and a variable region. There are only five different types of Fc-region isotypes while there is a seemingly infinite number of variable regions. An antibody consists of 2 heavy chains and 2 light chains which together have a Y-like shape enabling the binding of 2 antigens per antibody. While the variable region binds antigens, the constant Fc-Region acts as an effector deciding with which immune cell to interact. The five classes of immunoglobulins in humans are: IgM, IgD, IgG, IgA and IgE. Additionally, in humans IgG can be divided into IgG1, IgG2, IgG3 and IgG4 (numeration according to prevalence in a decreasing manner) as well as IgA which can be divided into IgA1 and IgA2.[26]

IgM is the biggest produced antibody, as it is secreted by B-lymphocytes as a pentamer and therefore only present in the blood. It is also the first antibody produced during an immune reaction and its presence indicates a fresh infection. The smaller IgG which is produced during an immune reaction is also present in the blood but can additionally enter the extracellular matrix. Both Ig-Isotypes can activate the classical complement pathway. While IgA is also present in the blood stream, it is active in mucosal tissues, such as intestine, lung, tears, etc. It is also secreted into the maternal milk. IgA and IgE cannot activate the complement system. While IgA can also form dimers, IgG as well as IgE always remain monomeric.[26]

1.4.1 Immunoglobulin E

Each isotype has its specific function and distribution within the human body. Originally, the production of IgE in humans is a response to a parasitic infection. Nowadays, at least in most western countries, an increased concentration of IgE in serum serves as an indicator for the presence of allergic disease and as such is here discussed in more detail. IgE is monomeric and small which allows it to travel from the blood into the surrounding tissue. However, usually the concentration of IgE in the blood or extracellular fluid is low. IgE is mainly receptor-bound to mast cells or basophiles sitting beneath the skin, in the mucosa or in the connective tissue of blood

vessels. If mast cell bound IgE is cross-linked by an antigen, a release of chemical mediators of the mast cells such as histamine takes place, which leads to local defence reactions.[26]

While bacteria are bound by IgG, which mediates the phagocytosis and thereby eliminates the bacterium, phagocytosis is not possible with bigger pathogens such as parasitic helminths. These parasites are simply too big and therefore are fought in a different way. Here the parasite is attacked by eosinophils and/or mast cells together with IgE. Therefore, in diagnostics elevated levels of eosinophils and IgE in the blood indicate a parasitosis. Moreover, in the intestine, mast cells can locally accumulate during an intestinal parasitosis with helminths. IgE, mast cells, basophiles and eosinophils are also important for the defence against blood-sucking insects. Elevated levels can also be found in atopic and allergic individuals where antigen binding of IgE leads to allergic reactions.[26] The role of IgE in allergies is elaborated in the next chapter.

1.4.2 Types of allergies

In the case of allergies, the adaptive immune system hyper-reacts to normally harmless body foreign and non-pathogenic antigens such as pollen, animal dander or house dust mites. This misguided response is commonly known as allergic reaction. This hypersensitivity of the immune system has been already studied in the 1960ties and categorised into 4 different groups in 1963 by the scientists Gell and Coombs. These groups are defined by the involved antibodies as well as the specific parts of the immune system (table 2). Although this classification system still is able to categorize the underlying mechanism, in the recent past it has been shown that these types can be overlapping with each other and in many patients more than one type of hypersensitivity is causing the displayed allergic symptoms.[26,27]

Table 2 Types of hypersensitivities after Gell and Coombs. This system allows the categorisation of the immunogenic processes underlying allergic symptoms. In about 28% of all cases more than one hypersensitivity is present.[26,27]

Type	Mechanism	Prevalence
Hypersensitivity Type 1 immediate hypersensitivity	induced by IgE-Ab and antibody cross-linking	48%
Hypersensitivity Type 2 antibody-mediated cytotoxicity	activation of the complement system by IgG-Ab	6%
Hypersensitivity Type 3 immune complex	IgG mediated stimulation of FcR expressing effector cells	10%
Hypersensitivity Type 4 delayed hypersensitivity	T-lymphocyte-mediated reaction	18%

Nevertheless, this system is still used today and in the context of this work the focus lies on type I hypersensitivity which is also the most prevalent one. This kind of hypersensitivity is mediated by the binding of IgE to the high-affinity receptor FcεRI expressed on mast cells and basophils. When the bound IgE-antibodies get crosslinked by a subsequent antigen-encounter, this leads to activation of cells. The activated mast cells then release mediators leading to the allergic reaction.

Preceding allergy to a specific antigen, the immune system needs to be sensitized to the allergen (figure 2). The primary sensitization occurs when an atopic person is exposed to an allergen such as BLG and the allergen passes the mucosal barrier where it is taken up by antigen-presenting cells such as dendritic cells or B cells. The APC then presents the antigen to naïve T cells which differentiates into a Th2 cell. These Th2 cells release cytokines such as interleukin (IL) 4 and IL13 inducing a class switching of B-cells to IgE producing plasma cells (sensitization phase).

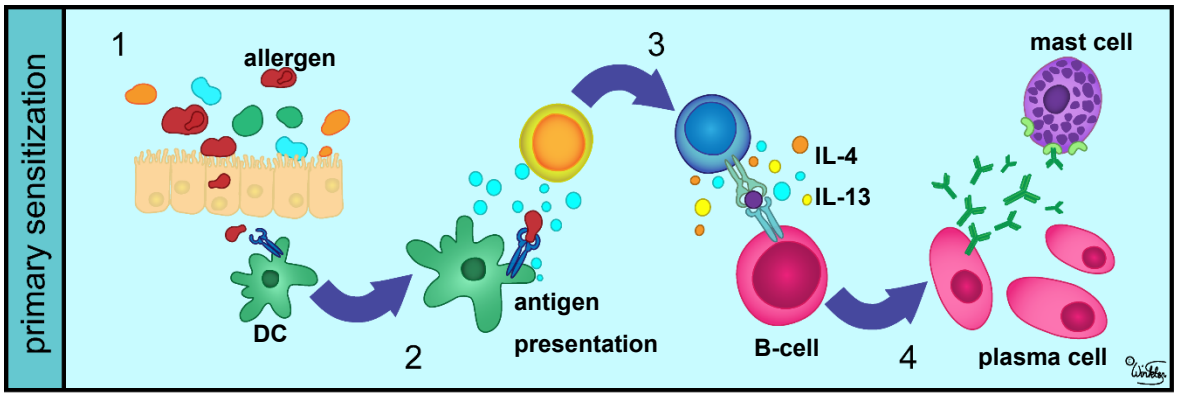

Figure 2 Primary sensitization. Primary sensitization occurs when the immune system of an atopic person is confronted with an allergen which is taken up by dendritic cells (1). These dendritic cells (DC) imprint a naive T-cell (2) leading to differentiation into a Th2 cell. The Th2 cell binds a B cell (3) which starts to produce IgE. The B cell transforms into a plasma cell. The by the B cell produced IgE antibodies then bind to the FcεRI receptor of mast cells or basophiles (4).

The FcεRI-receptor of these IgE then binds to the surface of mast cells. This first sensitization to allergen remains symptom free, however, upon re-exposure the allergen is bound by the IgE receptors on the mast cell surface resulting in IgE cross-linkage followed by degranulation of the mast cells (release of histamine, tryptase, cathepsin B etc.) which leads to more or less severe symptoms for the affected person (effector phase, figure 3). Allergic reactions can manifest in allergic rhinitis, conjunctivitis, asthma, atopic eczema, nausea, etc. or in its most severe form anaphylaxis.[26,28]

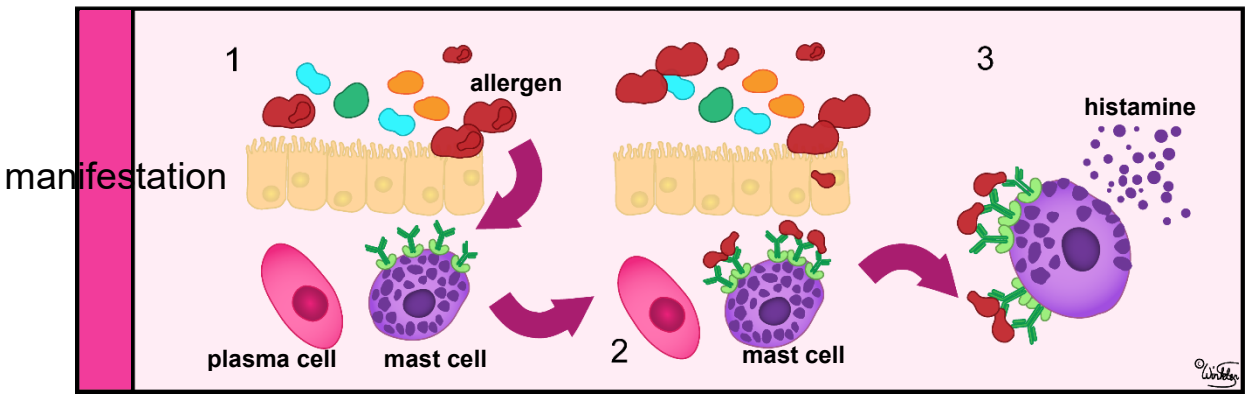

Figure 3 Manifestation of hypersensitivity type I. After the primary sensitization to an allergen (1) the atopic person first exhibits allergic reactions upon second encounter of the allergen (2). Mast cells release their granules containing several cytokines, toxic mediators, chemokines and enzymes upon cross linking of IgE antibodies due to antigen binding on its surface (3).

1.4.3 Hygiene hypothesis

Although allergic reactions are well understood, what exactly drives the immune system to become atopic and develop hypersensitivities towards otherwise harmless compounds is not entirely understood yet, but interesting observations have been made throughout the decades. For example, children of atopic parents have a 40 – 60% risk of developing an IgE mediated allergy while this risk is as low as 10% if no parent is atopic. This shows that there is a genetic aspect to allergic diseases. Since we are not solely the product of our genes, but also of our surroundings, it should not be surprising that the development of allergies also depends on the environment.[26]

That the environment plays a crucial role in the onset of allergic diseases has been first discussed in 1989 by the British scientist Strachan who has studied hay fever prevalence in British families. His paper established the colloquially named “Hygiene hypothesis” which postulates that infection protects against atopy.[29] This hypothesis is especially in coherence with observed epidemiological distribution of allergy prevalence. While in the western world the numbers for atopic people are increasing, little reports can be found in the Middle east or Africa. According to the hygiene hypothesis this can be ascribed to the modern western lifestyle with its tremendous improvements in hygiene and infection management reducing exposure to potentially infectious agents. And indeed, in parallel in the last 50 to 60 years allergy prevalence has increased drastically in the industrial countries.[26]

In this context it has been observed in recent years, that growing up in rural areas and especially on farms, prevents the development of atopy and asthma. Especially growing up on farms, prenatal farm exposure through the mother, close contact with farm animals and raw milk

consumption are correlating with decreased risk of atopy and development of allergic diseases, yet not all farms are equally protective. Today the protective effect of farming is primarily ascribed to the present microbiome and high endotoxin exposure through farm dust.[30–33]

1.4.4 Lipocalins as allergens

Interestingly the protective effects of farming have been shown to be especially high with children in close contact with farm animals as well as consumption of raw cow´s milk. Animals themselves, however, are releasing allergens and today many people have allergic reactions to one or more animals. In a cohort study of 696 school children more than one third (n = 259) were sensitized to at least one animal. With the rising number in pet owners the question whether animal proximity provides protection or increase the risk of atopy and allergy is intriguing and hotly debated.[25]

A closer look on animal allergens reveals that the most important allergens belong to one protein family – lipocalins (table 3). In consideration of the already mentioned immunomodulatory capabilities this is not entirely surprising. These lipocalins derive from fur, shed skin, saliva and urine. Although these lipocalins are responsible for the majority of animal allergies, lipocalins in general have only few T cell epitopes which additionally are suboptimal and allergen-specific CD4+ cells are scarce in peripheral blood.[23] Generally, there is no molecular, structural, physio-chemical explanation for the allergenicity of mammalian lipocalins.[24,25]

Table 3 Mammalian lipocalin allergens. Many important mammalian allergens have been identified as lipocalins. In this list important allergens are shown with their origin source.

Allergen	Animal source	Found in
Bos d 2	cow	dander
Bos d 5	cow	milk
Can f 1	dog	saliva, dander
Can f 2	dog	saliva, dander
Can f 4	dog	saliva, dander
Can f 6	dog	
Cav p 1	guinea-pig	dander, urine
Cav p 2	guinea-pig	dander, tears
Cav p 3	guinea-pig	saliva
Cav p 6	guinea-pig	
Equ c 1	horse	dander, saliva, urine
Equ c 2	horse	dander
Fel d 4	cat	saliva
Fel d 7	cat	saliva
Mus m 1	mouse	urine, dander
Ory c 1	rabbit	dander, saliva
Ory c 4	rabbit	
Rat n 1	rat	urine

Lipocalin, ligands and allergies

Recently, epidemiological studies have linked allergy with iron-deficiency, with allergic patients exhibiting a higher degree of iron deficiency than non-allergic and low iron status at birth increasing the risk of developing allergies in later live.[34] The lipocalins' highly

conserved fold allows binding of siderophores, which bind irons. Via this means, lipocalins may act bacteriostatic by withdrawing iron from microbes, a trait which has been investigated on BLG (Bos d 5) as a protection against mastitis-causing bacteria in cows.[21,25,34] Both structural as well as in silica analysis revealed that BLG can bind iron via siderophores (catechol-based flavonoid – quercetin, myricetin, luteolin).

Additionally, depending on BLGs loading status (holo: iron-siderophore bound, apo: empty) the protein induced different immunological reactions. While apo-BLG promotes inflammation and Th2 cells response resulting in elevated levels of CD4+ cells as well as IL13 and IFNγ release, holo-BLG has an immunosuppressive effect. This was also proven in an in vivo mouse model[35]. Moreover, a similar relationship was found between BLG and retinoic acid binding.[3,17,25]

BLG and cow´s milk allergy

Since cow´s milk is the first introduced foreign food to infants, cow´s milk is the most important source of allergens with about 7.5 % of all infants developing an allergic response to cow´s milk within their first year of life. The dominant allergen in those children besides casein (Bos d 8) is BLG inducing the highest IgE-mediated reactions. Milk allergy in childhood is associated with increased risk of developing allergies and asthma in later live even though cow´s milk allergy in 85-90 % of all cases is outgrown.[3,18,36,37] On top of that cow´s BLG has been detected in maternal breast milk after mothers ingested cow´s milk and can be detected in human breast milk for up to 7 days after consumption of one glass of milk.[36] Therefore, mothers of allergic infants have to control their intake of cow´s milk as the BLG transpiring into their breast milk can cause allergic reaction in their children.

Therefore, although BLG in children is associated with allergies, depending on its loading status it can be also beneficial for its immune system as the consumption of unpasteurized milk and contact with barn animals is associated with a reduced risk of atopy, asthma and allergy. Recently our working group has identified BLG also in the urine of cattle independent of sex, and investigations on stable dust collected in cow sheds has revealed considerable BLG amounts indicating that the protein might also be inhaled by people in proximity of the animals and stables, and thus might also play a role in the protective effect of farms.

Until now, only the mammary gland (i.e.: udder) of cows was recognized and described as BLG production site. However, the excretion via urine indicated that there may be additional BLG production sites in other bovine organs, which we aimed to investigate here.

1.5 Bovine organs

In order to identify additional BLG production sites, a closer look on the individual bovine organs is crucial.

1.5.1 Bovine udder

Topology

The mammary gland is a unique skin gland only found in mammals. There it forms the most important and biggest modified skin gland. Originally this tissue derived from modified sweat glands which now produce milk. While also present in the male, the mammary gland is only fully developed in the female under the influence of specific hormones. Size, amount, structure and location vary among species. While the mammary gland in carnivores and pigs is located at the breast and / or the stomach wall, that of ruminants and horses is located in the loin region.[38]

The mammary gland generally consists of several mammary complexes (figure 4). Each mammary complex consists of a corpus mammae and a teat (*papilla mammae*). The size of the corpus mammae depends both on species and individual as well as on the lactation status. Generally, the mammary tissue is a highly adaptive tissue. In cows the mammary gland consists of 2 mammary complexes for each body half which are attached to the *rego inguinalis*. One mammary complex can be divided into 3 major parts. Those are the alveolar gland tissue, milk ducts (*ductus lactiferi*) and the udder cistern (*sinus lactifer*).[38]

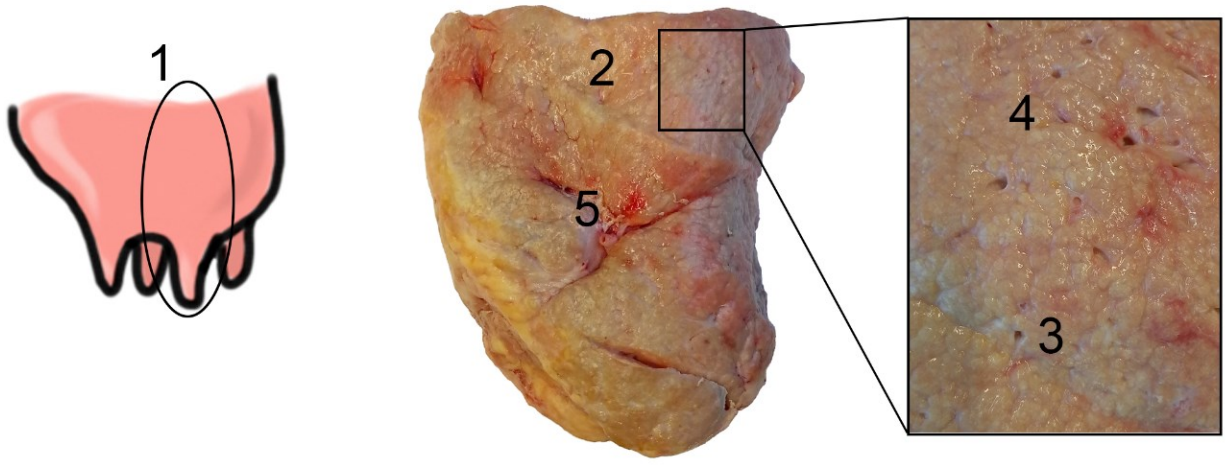

Figure 4 Bovine udder. 1 – schema of the mammary gland with one of four mammary complexes (corpus mammae with teat) encircled, 2 – corpus mammae, 3 – small milk duct within the alveolar milk producing glandular tissue (4) and big milk ducts (5).

Morphology and function of the udder

The corpus mammae consist of epithelial glandular tissue as well as connective and fat tissue (figure 5). Within the corpus mammae the smallest unit is the alveolar milk producing glandular tissue which forms lobes. These lobes are surrounded by connective tissue and fat. Milk produced in the alveolar tissue is led through small ducts which gather into the *ductus lactiferi*. Several of this big *ducuts lactiferi* lead into the *sinus lactifer* where the milk is gathered. The cistern can be divided into the upper part – *pars glandularis sinus lactiver,* which still lies in the corpus mammae and the lower part – *pars papillaris sinus lactifer,* which is in the teat of the udder. The teat and the *ductus pappilaris* (in cows in contrast to humans there is only 1 canal through which milk leaves the teat)[39] are covered with multi-layered keratinised mucosal tissue. The udder is generally highly innerved as well as supplied with big arteries and big veins. Between the *glandular sinus lactifer* and the *papillaris sinus lactifer* is the so called Fürstenberg-vein ring which surrounds the upper part of the teat.[38]

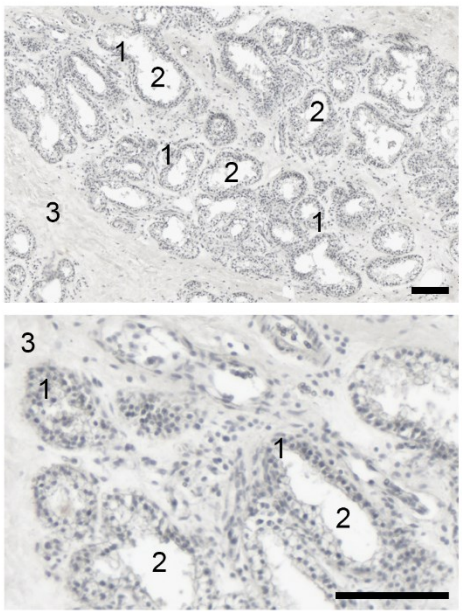

Figure 5 Haematoxylin stained bovine udder tissue. 1 – milk producing alveolar gland epithelium, 2 – small milk ducts, 3 – connective tissue surrounding the lobe, scale bar = 100 µm.

1.5.2 Bovine testis

Topology

The male reproductive systems (figure 6) consist of the testis, the seminal ducts, the epididymis and the penis. The testis is the tissue where the male reproductive cells, the sperm, is produced, which is the homologue to the female ovaries. Like their female counterpart, the testis are formed in pairs, however during embryonal development they travel from the abdominal cavity into the scrotum. The testis are egg-shaped and are surrounded by an intricate vascular and membrane system. The scrotum consists of two pockets each harbouring one testicle, and consists of three layers: the epidermis, *tunica dartos* and *fascia spermatica externa*.

The testis itself is enclosed by a 1-2 mm thick connective tissue capsule (tunica albuginea) which is interspersed with the major testicular arteries and veins. Moreover, the tunica albuginea exerts pressure on the testis making any kind of enlargement a painful endeavour. Other connective tissues are *septula testis,* which divides the testis parenchym into pyramid-shaped lopes (*lobuli testis*) and the *mediastinum testis,* which is in the middle of the testis. Each lobuli testis contains two to five *tubuli seminiferi contorti* which are responsible for the production of male germ cells.

The epithelia of this ducts consist the *Sertoli cells* and the germinating epithelial cells, which differentiate into the sperm cells (figure 7). The seminal ducts gather then in the middle of the testicle in the rete testis, which is surrounded by the *mediastinum testis*. From the rete testis eight to twelve ducts (*ductuli efferentes testis*) break through the tunica albuginea and lead into the head of the epididymis (*caput epididymidis*).[38]

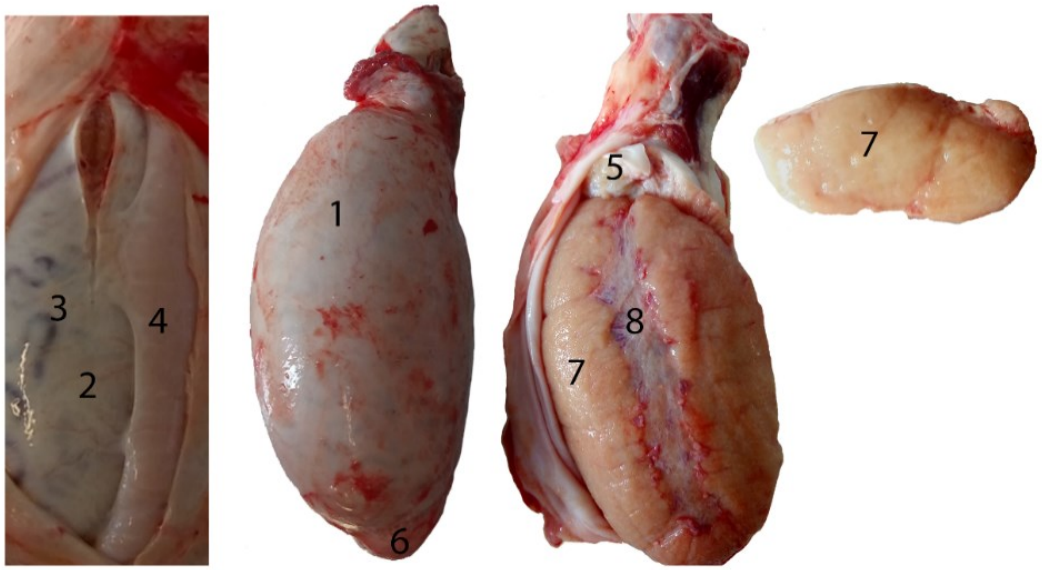

Figure 6 bovine testis (18-month-old bull). 1 – epichorium, 2 – tunica albuinea, 3 – ateria testicularis, 4 – corpus epididymidis, 5 – caput epididymidis, 6 – cauda epididymidis, 7 - testis parenchym, 8 - rete testis

Morphology and function of the testis

The testis functions both as an exocrine as well as endocrine organ. In the testis highly differentiated gametes are formed.[40] This is done in the *tubulus seimiferus contortus,* whose twined structure is dominating in the testis parenchyma. The epithelia of this ducts consist of male reproductive cells and the Sertoli cells. The germ cells divide, undergo meiosis and differentiate into semen cells in these ducts.[39] The Sertoli cells are high-prismatic epithelial cells with multilobed nuclei which have diverse functions[40]. Those functions include support, nutrition and transport of the germ cells from basal to apical. They also form the blood-testis barrier and they phagocytose the cytoplasm of the spermatids before they release them into the lumen of the *tubulus seimiferus contortus*. They have receptors for FSH (follicle stimulation hormone) and testosterone and produce the peptide hormone inhibin which inhibits FSH. They also regulate the testosterone level of the ducts by secretion of androgen binding proteins.[39] Sertoli cells are directly correlating with the amount of sperm production. Sertoli cells massively proliferate in the foetus, and in bulls proliferation stops around the age of 6 months. Depending on breed, puberty in bulls is reached on average after 42 weeks.[40] Spermatogenesis starts around 20 weeks.[41] The germ cells undergo

several differentiating steps before they are released in the *tubulus seimiferus contortus.* These states are spermatogonia, primary spermatocyte, secondary spermatocyte, spermatid and spermatozoa. Between the seminal ducts is interstitial testis tissue. This tissue consists of the *lamina propria,* made of myofibroblasts, surrounding the seminal ducts and causing rhythmic contraction. In the remaining interstitial tissue is loose connective tissue containing blood and lymph vessel as well as nerves.[39]

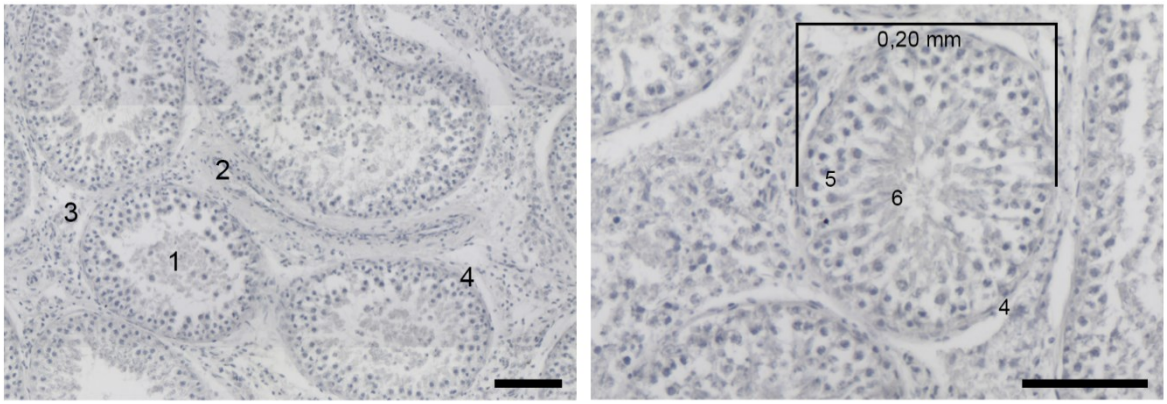

Figure 7 Haematoxylin stained testis tissue. 1 – lumen of tubuli seminiferi contorti, 2 – blood vessel, 3 – interstitial tissue containing Leydig cells, 4 – lamina propria, 5 – spermatocyte, 6 – spermatids. Scale bars = 100 µm

An important cell type of the interstitium are the big Leydig-cells which are responsible for the endocrine function of the testis[40]. They have big nuclei which are pushed to the border by a big smooth endoplasmic reticulum.[39] Leydig-cells produce the major sex steroids and their number and functional capacity is established during puberty and hardly changes thereafter.

They massively proliferate and maturate in the 5 to 28 weeks old bull. The mature testis consist of about 10% Leydig cells and even less Sertoli cells of the total cell volume.[41]

1.5.3 Bovine kidney

The kidney is important for many body functions as it controls water and mineral homeostasis in such a manner that extracellular space volume and osmolarity stays constant. It regulates the acid/alkaline balance and excretes end products of protein degradation, nitrate, creatine and uric acid. The kidney also produces hormones such as erythropoietin, angiotensin II, calcitriol and prostaglandins and therefore has an additional endocrine function. The kidneys also influence the arterial blood pressure.[38,39]

Topology of bovine kidney

Within the domestic animals, cattle have a unique kidney structure, as dog, cat, small ruminants (sheep, goat), pig as well as human have an even surface, whereas cattle kidneys have clearly visible furrows which separate the kidney lopes. Some kidney lopes, however, might also be

merged. This kind of total division of kidney lopes (figure 8) is otherwise only present in marine mammals.[38]

In the living animal the kidney is protected by an *adipose capsula* and surrounded by the collagen rich *capsula fibrosa.* The tissue of each lope is divided into the *cortex renis* and medulla renis. The cortex consists of *pars convoluta* and *pars radiata*. The medulla consists of the *zona externa* harbouring the *basis pyramidis* and the *zona intera* with *papilla renalis*. The medulla zones have a pyramid like formation and border the *sinus renalis*. The tip of the papilla renalis reaches into the *calix renalis,* which is a goblet-shaped opening and in the cattle leads into the *ureter* as there is no kidney pelvis like in human.[38] The kidney is extremely well supplied with blood which is provided by the *ateria renalis* and many small blood vessels present in the kidney tissue. These small blood vessels are used to filter the blood. The blood then leaves the kidney via the *venera renalis*.[38,39]

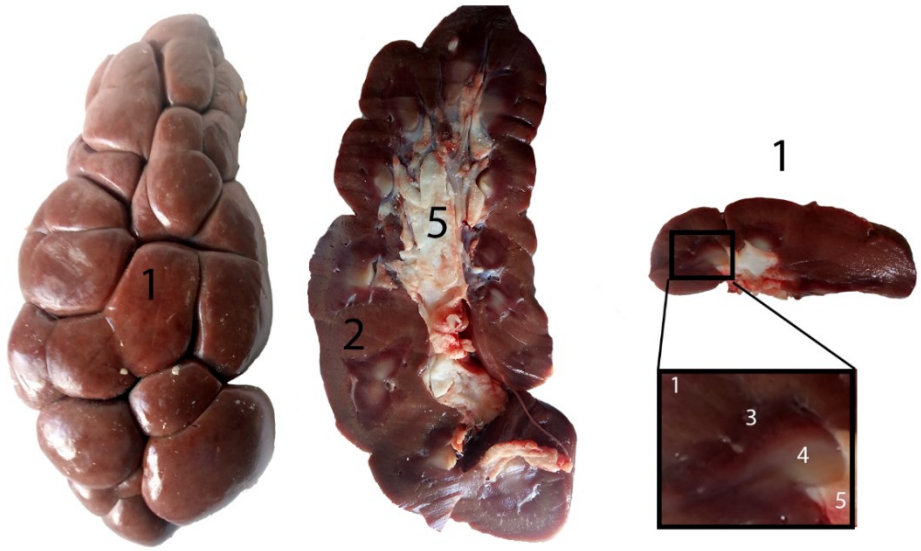

Figure 8 bovine kidney (18-month-old bull). 1 – lobus retinalis, 2 – cortex renis, 3 – zona externa, 4 – zona interna with papilla renalis, 5 – pelvis retinalis.

Morphology and function of the kidney

To fulfil its function as a detoxing organ the kidney filters the blood and gathers waste material in urine. This is done by a structure called nephron and a fine capillary system. In the kidney cortex the capillaries form bundle-like structures which are surrounded by a capsule and are called the glomeruli (figure 9 -1). Into the glomerulus leads the *ateriolae afferents* which divides into a fine loop forming a capillary network. Then this network merges again back together into the *ateriolae efferentes*, which has additionally the ability to vary its tonus. The nephron consists of renal corpuscles and unbranched renal tubuli. The renal corpuscle is built by the Bowman´s capsule including the inner and out layer, the glomerulus and the mesangium. The mesangium cells are

claiming the space between the capillary system of the glomeruli. These cells produce special extracellular matrix which consist of microfibrils, collagen type IV, V and VI, proteoglycans and fibronectin. These cells are necessary for stabilization of the high inner pressure of the glomeruli. In figure 9 a renal corpuscle is shown with the glomeruli as well some tubuli (figure 9 –2/ –3). The glomeruli´s endothelia are fenestrated without diaphragms with a width of 70 -100 nm. This fenestration allows water, ions, small proteins, minerals, ect. to pass, but holds back blood cells. The Bowman´s capsule consists of an inner visceral and an outer parietal layer. The inner layer surrounds the glomerulus while the outer layer builds the border of the capsule space. Both are monolayer and the inner layer cells differentiate into podocytes and with their star-like shape they engulf the capillaries. Between the podocytes stays a small gap which is then covered with an extracellular layer. All these structures of the renal corpuscle build the blood-urine barrier.[38,39]

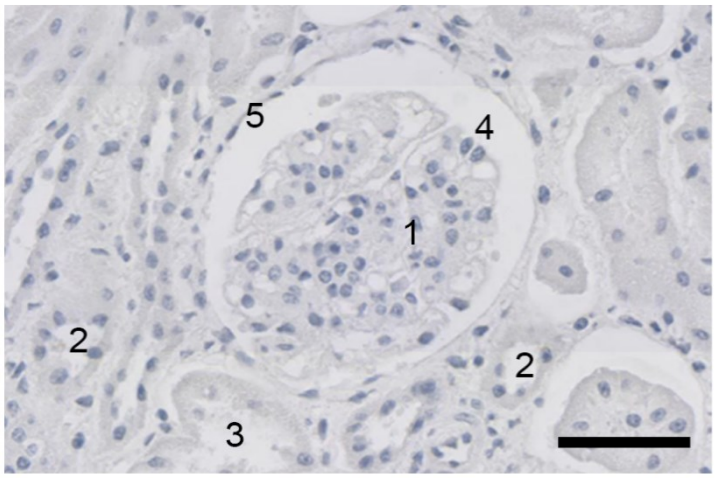

Figure 9 Renal corpuscle. *In this haematoxylin stained bovine bull kidney the renal corpuscle and its compartments are clearly visible. 1 – glomerulus, 2 – proximal tubulus, 3 – distal tubulus, 4 – capsule space, 5 – inner layer of bowman´s capsule.*

The *tubuli renalis*, varying for species, can be divided into 3 to 4 segments. These segments are the *tubulus conterus proximalis*, *ansa nephroni* (Henle's loop) and the *tubulus contortus distalis*. Following the capsula space of the renal corpuscle the primary urine is lead into the *tubuls conterus proximalis* which leads down towards the kidney medulla. There the primary urine flows into the *ansa nephroni* which consists of a downward part, a turn (usually in the medualla) and an upward part leading towards the kidney cortex. The next segment, the *tubulus contortus distalis* leads back into the cortex to the renal corpuscle and then into the collecting duct of the kidney. One collecting duct is shared between several nephrons.[38,39]

1.5.4 Bovine adrenal gland

Topology

The adrenal gland is an endocrine gland, which is present pairwise in all mammals. It consists of two tissue complexes which can be divided into the cortex (figure 10) and medulla (figure 10 - 2).[38] While in humans the adrenal gland sits on the upper fat capsule of the kidney[39], in cattle it is not immediately connected to the kidney. The adrenal gland is in close proximity to the *aorta*

abdominals and *vena cava caudalis*. The medial border of the left adrenal gland is in neighbourhood of the aorta and in cattle the right adrenal gland is merged with *vena cava caudalis* membrane. This connection between the *vena cava caudalis* and the right adrenal gland is unique for cattle within the domestic animals. Generally, the female´s adrenal glands are bigger than the male´s or castrate's counterparts. Additionally, pregnancy and lactation leads to an enlargement.[42] In cattle the left and the right adrenal gland have different shapes. The right one has the form of a "9" (figure 10) and the left one resembles a heart. The adrenal glands are extremely well connected to the blood streams as the big arteries *a. abdominalis*, *a. renalis*, a. abdominalis cranialis and *a. phrenica caudalis* are in close proximity.[38] Arteries of the adrenal gland form radial cortex arteries. In the medulla, jugular veins are able to hold back blood controlling the blood flow within the organ. These veins gather into a central vein (figure 10 - 3) which releases the venous blood filled with hormones into the blood stream of the body.[38]

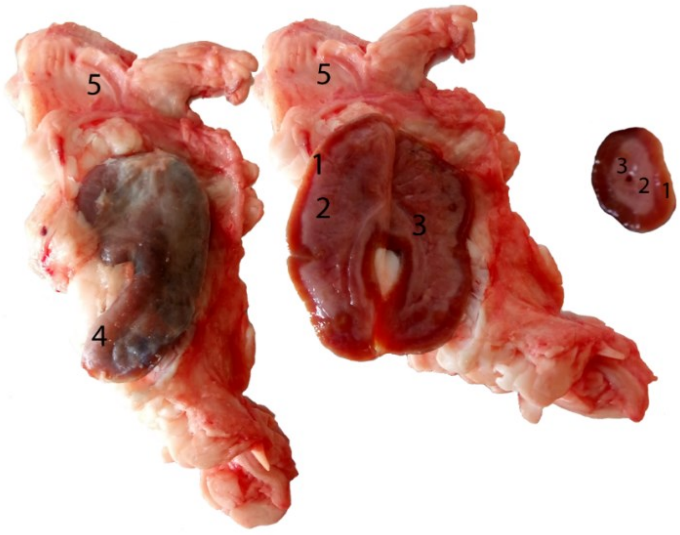

Figure 10 Right adrenal gland (18-month-old bull): 1 – cortex suprarenalis, 2 – medulla suprarenalis, 3 – venera centralis, 4 – capsula suprarenalis, 5 – adipose tissue.

Adrenal gland morphology and function

The adrenal gland overall consists of two tissues – cortex and medulla. These two tissues derive from two different developmental parts: The cortex derives from the mesoderm while the medulla derives from the ectoderm.[38,39] In lower chordates these two parts are actually separate organs. Since both play a crucial role in stress response and regulation, their co-localisation enables a more efficient coordination of these functions and therefore in higher chordates they form one organ.[39]

Below the capsula sporenalis surrounding the organ is the cortex which can be divided into three layers. These three layers are the *zona glomerulosa, zona fasciculata and zona reticularis*. The

cortex is important for several body functions as the z. glomerulosa produces mineral corticoids which regulate the mineral and water homeostasis. The z. fasciculata regulates mainly the carbohydrate metabolism by glucocorticoid production, but also plays a role in lipid und protein metabolism by supporting glucose production/depletion, lipogenesis or lipolysis. Additionally, glucocorticoids play a crucial role in stress response by suppressing inflammatory and immune reactions, leading to an increased susceptibility to bacteria, fungi and viruses.[43] The most inner layer of the cortex, z. reticularis, produces androgen hormones. The activity of the adrenal gland´s cortex is regulated by the anterior lobe of the pituitary gland through the adrenocorticotrophic hormone (ACTH).[38,39]

Below the z. reticularis is the medulla suprarenalis which produces the hormones adrenalin and nor-adrenalin. In herbivores more adrenalin is produced than nor-adrenal in opposition to carnivores and human. The medulla consists of modified dendritic-less sympathetic nerves which contain catecholamines.[38] These cells are called paraganglia and derive from the neuronal crest. The adrenal gland medulla is the biggest paraganglia in the body. Catecholamines also affect haemodynamic balance by influencing vasoconstriction, vasodilatation or by directly affecting heart activity. The catecholamine adrenalin is produced in so-called A-cells which are situated at the periphery of the medulla. Noradrenalin producing cells (N-cells) can be found in the central parts of the medulla.[43] The jugular veins within the medulla can regulate the blood flow in the organ.[38,39]

2. Aim and research question

Lipocalins such as β-lactoglobulin are well-known human allergens, yet studies have shown that β-lactoglobulin does not always influence the human immune system in a negative manner. Now evidence has been gathered by our working group in the past years demonstrating that BLG exposure of the right loading state can even prevent the onset of allergic sensitization towards other allergens.[3] Yet an immuno-protective effect is just another attribute assigned to the already wide array of proposed functions of BLG. 80 years after its isolation the true function of BLG remains elusive and according to literature BLG expression has been primarily ascribed to the female and its mammary tissue. Accordingly, only milk would qualify as a source of exposure for mankind.

Recently our research group, however, could detect this protein in the stable dust of both dairy farms as well as cattle farms only inhabited by male individuals. By conducting investigation on bovine urine our working group could detect BLG in the urine of both genders revealing a possible way of environmental BLG exposure[44]. These findings highly indicate (i) that BLG is present in the male individual, (ii) that BLG is not only secreted via milk, and (iii) therefore there are presumably other tissues in addition to the udder producing the protein.

The aim of the here conducted experiments was to investigate whether BLG is also present in organs other than the bovine udder. We set out to check BLG presence in different bovine tissues and thereby reveal additional BLG-harbouring organs. To do so, a method for immunohistochemical staining of BLG in cow tissue was established and different bovine tissues were tested for BLG presence by WB, ELISA, IHC and PCR.

Organs of interest were the udder due to its already verified BLG expression, the bovine kidney since BLG is present in bovine urine, bovine adrenal gland as a hormone-producing organ and bull testis (figure 11).

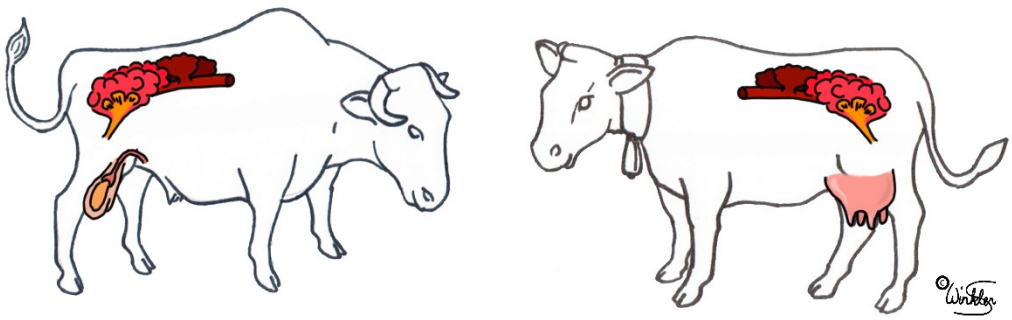

Figure 11 bovine organs. Schematic depiction of tested bovine organs in the female as well as male cattle.

3. Materials and Methods

3.1 Materials

3.1.1 Animal-derived samples

The different animal tissue samples (table 4) were collected from the slaughterhouse, butcher or kindly donated by the University Clinic for Ruminants (University of Veterinary Medicine, Vienna) from animals which had to be euthanized.

Table 4 Animal register. Samples derived from 19 cows, 7 bulls, 1 ox, 1 horse, 2 chicken and 1 human. Saliva samples were collected in the cow stables of the Veterinarian University of Vienna or on farms in Lower Austria.

Animal Nr.	Species	Organs	Usage for methods
A1	Cow	udder, kidney, adrenal gland	IHC, RT-PCR
A2	Bull	testis, kidney, adrenal gland	IHC, Protein extracts, RT-PCR
A3	Cow	udder	Protein extract, RT-PCR
A4	Cow	kidney	Protein extract, RT-PCR
A5	Chicken	ovarian duct, kidney, liver, spleen	IHC, RT-PCR
A6	Chicken	liver	Protein extracts
A7	Cow	kidney, adrenal gland, liver	RT-PCR
A8	Cow	blood	ELISA
A9	Cow	saliva – CS1	ELISA
A10	Cow	saliva – CS2	ELISA
A11	Cow	saliva – CS3	ELISA
A12	Ox	saliva – CS4	ELISA
A13	Bull	saliva – BS1	ELISA
A14	Horse	saliva – HoS1	ELISA
A15	Cow	saliva – CS4	ELISA
A16	Cow	saliva – CS5	ELISA
A17	Cow	saliva – CS6	ELISA
A18	Cow	saliva – CS7	ELISA
A19	Cow	saliva – CS8	ELISA
A20	Cow	saliva – CS9	ELISA
A21	Cow	saliva – CS10	ELISA
A22	Cow	saliva – CS11	ELISA

A23	Cow	saliva – CS12	ELISA
A24	Cow	saliva – CS13	ELISA
A25	Cow	saliva – CS14	ELISA
A26	Bull	saliva – CS1	ELISA
A27	Bull	saliva – CS2	ELISA
A28	Bull	saliva – CS3	ELISA
A29	Bull	saliva – CS4	ELISA
A30	Bull	saliva – CS5	ELISA
A31	Bull	saliva – CS6	ELISA
A32	Calf (f)	saliva – CaS1	ELISA
A33	Calf (f)	saliva – CaS2	ELISA
A34	Calf (m)	saliva – CaS3	ELISA
A35	Human (f)	saliva – HuS1	ELISA

3.1.2 General buffers

10x gel electrophoresis buffer

- TRIS (250 mM), TRIZMA base, Sigma-Aldrich, T1503-1kg
- Glycin (1.92 M), BIOFROXX, 1154KG005
- SDS (1%) SIGMA-Aldrich, 74255

10x TBS:

- TRIS (0.5 M), TRIZMA base, Sigma-Aldrich, T1503-1kg
- NaCl (1.5 M), Sodium Chloride, Sigma, 71382-1kg

10x Transfer buffer (pH 7.4):

- TRIS (250 M), TRIZMA base, Sigma-Aldrich, T1503-1kg
- Glycine (1.92 M), BIOFROXX, 1154KG005
- Methanol (20%), Sigma-Aldrich, 322415

Blocking buffer:

- 1x TBS (IHC) / 1x TBS-T (western blot, ELISA)
- 5% Bovine Serum Albumin (IHC) / 1%BSA (western blot, ELISA), Sigma-Aldrich, A3912-100G

Water:

- MilliQ water (MQ): Milli-Q® Advantage A10 Water Purification System, Merck, Z00Q0V0WW

3.1.3 Immunohistochemistry

General equipment:

- Embedding cassettes, Roth, E478.1
- Object slides, Thermo Scientific, Superfrost Plus, J1800AMNZ
- Coverslips, vwr international, #631-0146
- Mounting medium, sigma, F4680-25ML

Material for fixation:

- HOPE Fixation kit, DCS Inoovative Diagnostik-Systeme, HL001R500
    - HOPE®I Solution
    - HOPE®II Solution
- Acetone, lab-honeywell, #32201-2.5L
- Low melting paraffin, Sanova, ATS-200850 #1253890

Material for deparaffination:

- Isopropanol, lab-honeywell, #33539-1L
- 70% Acetone
- MQ

Material for immunostaining:

- 0.5% H_2O_2, Sigma, H1009-100ML
- Dako pen, Dako, S2002
- 1x TBS
- Bovine Serum Albumin, Sigma-Aldrich, A3912-100G
- Antibodies:
    - 1st antibody: Anti-Lactoglobulin beta antibody, abcam, ab112893
    - 1st antibody: Anti-Ovalbumin, merck, AB1225-100UL
    - 2nd antibody: Goat Anti-Rabbit IgG H&L (HRP), abcam, ab205718
- Detection kit: HRP/DAB detection IHC Kit, abcam, ab80437
- Haematoxylin, Merck, 109249

3.1.4 Protein extracts

Protein extraction solutions:

- Extraction buffer
    - K_2HPO_4 (10 mM), Sigma Aldrich, P2081-600G
    - KH_2PO_4 (1 M), VWR, 436053H

- EDTA (2 mM)

Upon use addition of:

- DIECA (1 mM), Sigma-Aldrich, 228680-80-100G
- NaN_3 (3 mM), Sigma-Aldrich, 199931
- 1 cOmplete™ tab per 0,5 L, Roche Diagnostics, 11697498001
- Dialysis buffer
  - K_2HPO_4 (10 mM), Sigma Aldrich, P2081-600G
  - KH_2PO_4 (1 M), VWR, 436053H
  - NaN_3 (3 mM), Sigma-Aldrich, 199931

ELISA

- Bovine Beta-Lactoglobulin Elisa Quantitation Set, Bethyl Laboratories Inc., Cat. No. E10-125
  - Affinity purified Rabbit anti-Bovine Beta-Lactoglobulin Coating Antibody A10-125A, 1 ml at 1 mg/ml
  - Bovine Beta-Lactoglobulin Calibrator (RC10-125-8), 0.1 ml
  - HRP Conjugated Rabbit anti-Bovine Beta-Lactoglobulin Detection Antibody A10-125P, 0.1 ml at 1 mg/ml
- Microplate washer, 5165010, ThermoFisher SCIENTIFIC, serial no.: 888-1381B
- Plate reader: infinite M200 PRO, 30050303, TECAN Austria GmbH, serial no.: 1301004650,
- Nunclon 96 Flat Bottom Transparent Polystyrol flat bottom plate, ThermoFisher SCIENTIFIC, Cat. No 26962
- Tween, Sigma-Aldrich, P1754
- 1x TBS-T (0.05% Tween)

Western blot

- SDS-Page-Gel 18%
  - 30% Acrylamide, AppliChem Panreac, A36260500
  - Lower buffer (pH 8.8)
    - SDS (0.4%), SIGMA-Aldrich, 74255
    - TRIS (1.5 M), TRIZMA base, Sigma-Aldrich, T1503-1kg
    - MQ
  - Upper buffer (pH 6.5)
    - SDS (0.4%), SIGMA-Aldrich, 74255
    - TRIS (0.5 M), TRIZMA base, Sigma-Aldrich, T1503-1kg

    - MQ
  - TEMED, Sigma-Aldrich, T7024-50ML
  - 10% APS, Sigma-Aldrich, A3678
- β-Lactoglobulin from bovine milk, Sigma, L0130-1G, Aliquot: 2 mg/ml (BLG)
- Protein ladder: Page RulerTM, Thermo Scientific, Prod.#26616 (Marker)
- 4x Laemmli sample buffer, Bio Rad, Prod.#1610747
  - MilliQ water: Milli-Q® Advantage A10 Water Purification System, Z00Q0V0WW, Merck
- Antibodies:
  - 1st antibody: Anti-Lactoglobulin beta antibody, abcam, ab112893
  - 2nd antibody: Goat Anti-Rabbit IgG H&L (HRP), abcam, ab205718
- Nitrocellulose Blotting Membrane, GE Healthcare, 10600001
- Bovine Serum Albumin, Sigma-Aldrich, A3912-100G
- Whatman filter paper, neoLab, 2-4327
- Clarity™ Western ECL Substrate, BioRad, #170-5061
- Tween, Sigma-Aldrich, P1754
- 1x TBS-T (0.05% Tween)

3.1.5 Reverse transcription PCR

Table 5 PCR-Primers: PCR was conducted with listed BLG primer and 2 housekeeping genes as controls (ACTB, GAPDH).

	Manu-facturer	forward primer (5´-3´)	reverse primer (5´-3´)	amplicon size [bp]	genome product size [bp]
GAPDH	Sigma -Aldrich	CCTGCCCGTTCGACAGATAG	TGAAGTCGCAGGAGACAACC	914	3834
ACTB		GAGCGGGAAATCGTCCGTGAC	GTGTTGGCGTAGAGGTCCTTGC	278	367
LGB		CCCCCCTGAGAGTGTATGTGGAG	TGGGTTGGGTTGAAGGACAGCCG	355	3014

- Trizol, Sigma Aldrich, 4500234862
- RNAse away spray, Molecular Bioproducts, #7002
- iScript™ cDNA Synthesis Kit, Biorad, #170-8891
- Direct-zol™ RNA MiniPrep, Zymo Research, R2052
- Quick-RNA™ FFPE Kit, Zymo Research, R1008

- 100mM dNTPs Set, invirtogen, 10297-018
- DNase/RNase free water, Hyclone, SH40003.1
- Agarose, BioCat, #AGA500-BCAT
- Primers for PCR (Table 5)

3.1.6 General programs and equipment

- Lyophilisator, Alpha 1-2 LDplus, Christ, 24210
- Wellwash Versa, thermo scientific, microwell washer, 5165010
- ELISA Reader: infinite M200Pro, tecan, 30050303
- ELISA Program: i-control 1.10, tecan, Version: 3.4.2.0
- SoftMax Pro 4.8, IPA 70867-BNTP
- Professional BASIC 96 Gradient Thermocyler, Biometra, 846-070-601
- Nanophotometer Pearl, P 300, IMPLEN, 3753
- ChemiDoc™Touch Imaging System
- Microtome, Leitz 1512
- Microtome blades, thermo scientific, MX35 Premier+
- Microscope: Axio Imager.Z1, Serial-Nr.: 3512001446, Carl Zeiss Microimaging GmbH
- Objective
    - 2,5x: Zeiss, DC- Plan-Neofluar
      Objective 2.5x, Air, 0.075
    - 20x: Zeiss, LD Plan-Neofluar (421350-9970)
      Objective 20x, Air,0,4,7950µm
      Numerical Aperture 0,4
      TL Shutter Opened
      With Extended Focus
      Focus Matrix - 3x3
      Focus Algorithm Autofocus Multi Channels
      Use Focus Plane True
      Exclude outlier points distance 10
      Force Full Focus False
      Channel: Tran, Offset: 0
      Microscope Settings:
      Condenser Aperture: 0,400110843373494
      Lamp Intensity: 7,2080078125
    - 63x: Zeiss Plan-Neufluar (420480-9900)

Objective 63x, Oil,1,25,100µm
Numerical Aperture 1,25
TL Shutter Opened
With Extended Focus
Focus Matrix - 3x3
Focus Algorithm Autofocus Multi Channels
Use Focus Plane True
Exclude outlier points distance 10
Force Full Focus False
Channel: Tran, Offset: 0
Microscope Settings:
Condenser Aperture: 0,9
Lamp Intensity: 8,411328125

- Microscope acquisition camera: PixeLINK PL-B623CF / 623000787
- TissueFax, Tissue Gnostic, TissueFax 6.0.1.0130
- HistoQuest, Tissue Gnostic, HistoQuest 6.0.1.130(x64)

Table 6 Antibody concentrations for IHC, ELISA and western blot. Different antibodies as well as antibody dilutions were used and are summarized in the presented table.

Method	Antibody	concentration
IHC	Anti-Lactoglobulin beta antibody, abcam, ab112893, primary ab	1:1500 / 1:2000
IHC	Anti-Ovalbumin, merck, AB1225-100UL primary ab	1:1500 / 1:2000
IHC	Goat Anti-Rabbit IgG H&L (HRP), abcam, ab205718, secondary ab	1:25000
ELISA	Affinity purified Rabbit anti-Bovine Beta-Lactoglobulin Coating Antibody A10-125A, 1 ml at 1 mg/ml	1:100
ELISA	HRP Conjugated Rabbit anti-Bovine Beta-Lactoglobulin Detection Antibody A10-125P, 0.1 ml at 1 mg/ml	1:100000
western blot	Anti-Lactoglobulin beta antibody, abcam, ab112893	1:10000
western blot	Goat Anti-Rabbit IgG H&L (HRP), abcam, ab205718	1:12500

3.2 Methods

3.2.1 Immunohistochemistry

Immunohistochemistry was used to identify tissues harbouring BLG in cattle of specific sites in both sexes. Moreover, this technique was used to localize BLG within examined samples. The samples for IHC analysis derived from A1 (cow) and A2 (bull). Totally three organs were prepared derived from the cow: udder, kidney and adrenal gland, and three organs were derived from the bull: testis, kidney and adrenal gland.

Sample preparation

All samples were fixed by the HOPE® Fixation System according to the manufacturer: the fresh tissue was sectioned into small pieces (max. size: 8x8x8 mm) and each piece was immersed in a 15 ml tube containing 5 ml ice cold (0-4°C) HOPE®I Solution for 48 hours (manufacturer's recommendation: 12-72 h). Following the initial incubation HOPE®I Solution was discarded and each sample was transferred into an embedding cassette. Thereafter the cassettes were immersed in ice-cold HOPE®II/ acetone solution (100 µl HOPE®II Solution per 100ml acetone) for 2 hours at 4°C. HOPE®II/ acetone solution was replaced by ice-cold acetone 3 times with a respective incubation time of 2 hours at 4°C. This 8-hour dehydration process was followed by overnight incubation in pre-melted low-melting paraffine at 54-55°C. Finally, the tissue was embedded and paraffine tissue blocks were stored at 4°C. Upon sectioning the paraffine block was frozen for 30 minutes at -20°C. Sections were cut by the microtome 3 µm thick and first transferred into a RT water bath and finally stretched at a ~39°C warm water bath. Obtained slides were dried at 37°C overnight and stored at 4°C upon further usage.

Immunohistochemical staining (IHC)

Slides were deparaffinized by 10-minute incubation in warm isopropanol (60°C) two times. Thereafter, the slides were washed with warm isopropanol and rehydrated in cold 70% acetone (0-4°C) 2 x 10 minutes. Slides were washed twice in MQ and additionally incubated in MQ for 5 minutes. Due to the non-crosslinking nature of HOPE® Solution, heat-introduced-epitope-retrieval was neither required nor applied. After rehydration, endogenous peroxidase was blocked by incubation in 0.5% H_2O_2 for 30 minutes and afterwards washed twice in TBS for 5 minutes. Tissue sections on the slides were circled with a Dako Pen and blocked by 5% BSA for 2 hours at RT in a humidified chamber. Afterwards slides were washed twice in TBS for 5 minutes and primary antibody was applied overnight at 4°C (table 6). Thereafter slides were washed 3 times for 2 minutes with TBS at RT and the secondary antibody was applied for 2 hours at RT. Following this, the slides were again washed 3 times in TBS for 2 minutes and meanwhile the chromogen was prepared (30 µl DAB Chromogen + 1.5 ml of DAB Substrate). The DAB was applied to each tissue

section for 3 minutes and washed in the same manner as before. Finally, haematoxylin was applied for 4 minutes to each section and then the slide was washed in water for at least 10 minutes.

TissueFax acquisition

IHC stained samples were acquired by a Zeis Axio ImagerZ.1 and PixeLINK PL- B623CF / 23000787 with the TissueFax application (TissueGnostic). For preview, each slide was scanned with 2.5x objective and the whole tissue was marked for 20x acquisition. 20x picture acquisitions were performed and exact settings are listed under 3.1.6. Additionally, of each stained section a 2x3 mm big field of view (FOV) was added and acquired by 20x and 63x magnification. For acquisition, extended field of focus was used as well as automatic focusing applied, unless the program could not obtain a sharp picture automatically. In this case, pictures were retaken by manual focusing.

HistoQuest analysis

Analysis of acquired 63x pictures was conducted by HistoQuest 6.0.1.0130 software, which measured BLG and haematoxylin area (µm²), mean intensity and standard deviation of mean intensity.

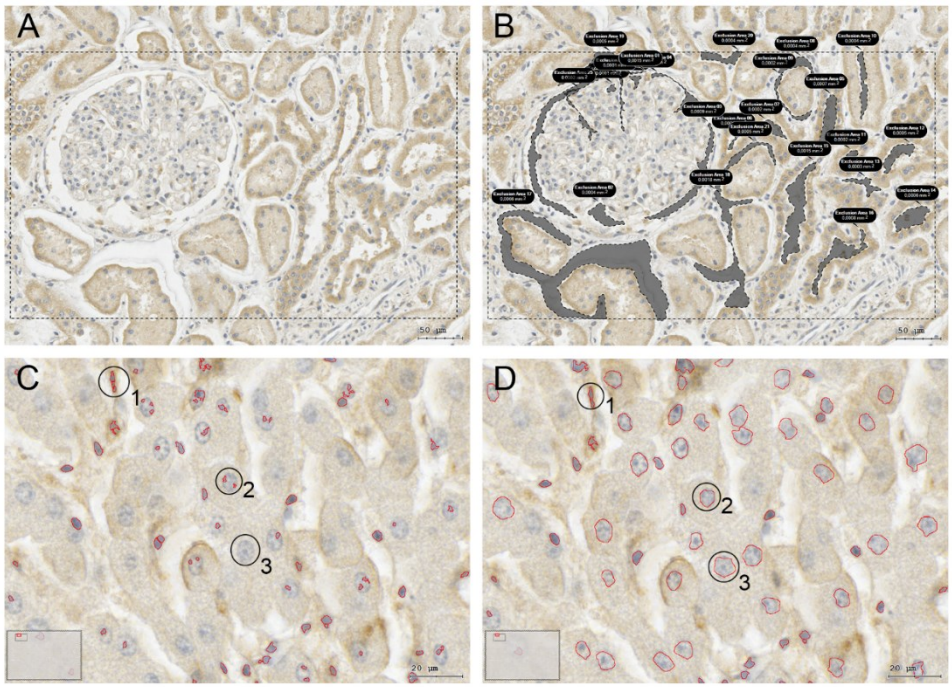

Figure 12 Demonstrative illustration of manual correction of HistoQuest analysis. Manual correction can only be applied to nucleus detection by haematoxylin. A - picture of bovine kidney before exclusion areas were set. This was done to limit false positive detection in empty space. B - picture of bovine kidney with set exclusion areas. C and D – manual correction of bovine adrenal gland tissue: C1/2/3 – fractioned cell nucleus, D1/2/3 – corrected fractured nucleus

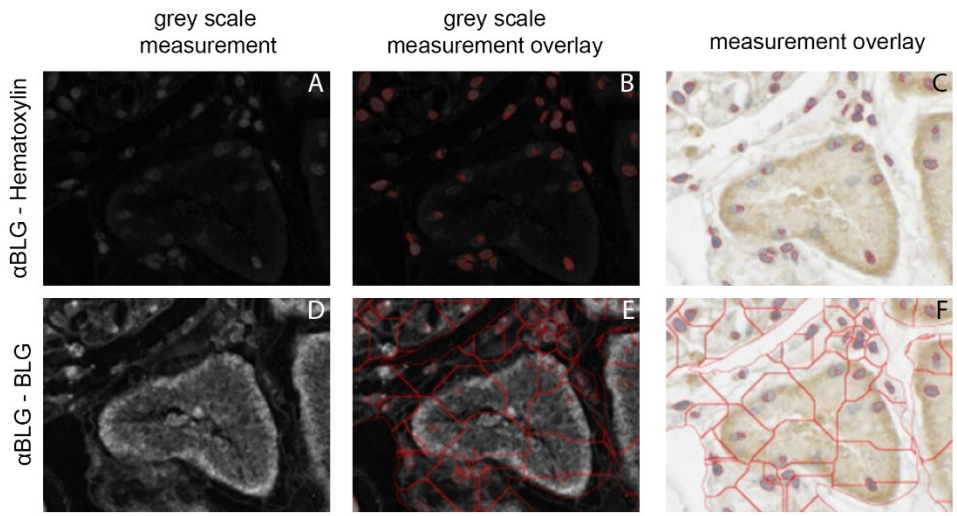

Figure 14 Demonstrative image of detection of DAB and haematoxylin in bovine kidney tissue. Grey scale measurement (A) of haematoxylin, calculated detection of haematoxylin (B) and colour overlay (C). Grey scale measurement (D) of BLG presence, calculated detection based on detected nuclei and BLG signal (E) and colour overlay (F).

In the first step all pictures where subjected to illumination correction as well as the same marker profile setting. The colour of the BLG (original marker) and haematoxylin (master marker) was picked by the auto-detect function, which was adjusted for each tissue organ on the BLG stained acquisition. Then the region of interest (ROI) was set. A standard ROI with 0.15 mm² was added (0.3 mm x 0.5 mm) to all samples and in the BLG-stained ROI exclusion areas were set (excluding all empty space within the ROI), before all samples were analysed. Following automatic detection, backward data for haematoxylin was viewed and manually corrected by either merging falsely set

A

Segmentation Method Parameters

Parameter	Value
Nuclei Size	10
Discrimination Area	5
Discrimination Gray	3
Automatic Background Threshold	Yes
Threshold Range	[25, 200]
Virtual Channel	no
Post Processing Order	Remove, Merge
Remove Labels	Yes
Smaller Than	1,02µm²
Larger Than	33,46µm²
Weaker Than	20
Stronger Than	do not use
Use Merging Rules	Yes
Max Combined Area	33,46µm²
Max Involved Compactness	0,9
Group Max	4
Min Resulted Compactness	0,6

B

Segmentation Method Parameters

Parameter	Value
Use Ring Mask	Yes
Interior Radius	0µm
Exterior Radius	45,43µm
Use Identified Cell Mask	No
Use Nuclei Mask	No
Automatic Background Threshold	Yes
Threshold Range	[5, 255]

Figure 13 Segmentation method parameter settings. In A the segmentation method parameter of HistoQuest analysis for the Master Marker – haematoxylin is presented. In B segmentation method for the Original Marker DAB stained BLG is shown.

multiple nuclei, adding non-detected nuclei, and deleting falsely marked nuclei. An example is shown in figure 12. Afterwards, cut-off values were set on the BLG stained acquisition (25% for both markers) and this cut-off value was copied to the other two controls.
For analysis, HistoQuest requires setting of a marker profile consisting of “segmentation method parameters” for the original marker (DAB-stained BLG) and the master marker (haematoxylin for the nuclei, figure 14). General segmentation setting was “nuclear segmentation”. While the identical segmentation method parameters were applied to all samples, the colour separation parameters were, as already mentioned, picked by the automatic tool for each organ individually.

3.2.2 Protein extracts

For additional confirmation of IHC findings, protein extracts of the following organs were prepared: udder, cow kidney, bull testis, bull kidney and bull adrenal gland. Unfortunately, no cow adrenal gland could be obtained for protein extract production.

The protein extracts were analysed by ELISA, which supplied quantitative BLG information, and by western blot which verified BLG presence qualitatively. To produce the protein extracts tissue was frozen with liquid nitrogen and grinded into a fine powder. The powder was divided into 5 g portions, which were dissolved in 20 ml of extraction buffer, and samples were put on a roller overnight at 4°C. Samples were then transferred into snakeskin-membrane and dialysed against dialysis buffer, which was changed 3 times in 24 hours. After dialysis, samples were frozen at -80°C and then lyophilized. Lyophilized samples were solved in MQ and additionally dialysed against water in the same manner.

ELISA

BLG quantities in produced protein extracts were determined via bovine BLG-specific ELISA kit (Bethyl Laboratories Inc., Cat. No. E10-125) according to manufacturer´s instructions: the 96-well plate was coated with 100 µl coating antibody (1:100) and incubated for 1 hour at RT. Thereafter the plate was washed 5 times with 200 µl TBS-T (general setting for all following wash steps) and blocked for 30 minutes with Blocking Solution at RT. The plate was then washed and 100 µl sample in doublets in the various concentrations (PE: [1:10], [1:25], [1:50], [1:75]; blood: [1:20]) were applied for 1 hour at RT. After incubation the sample was discarded and the plate washed before 100 µl detection antibody (1:100000) was added for 1 hour at RT. Again, the plate was washed and 100 µl TMB was added and colour reaction developed for 10 minutes in the dark. The enzymatic reaction was stopped with 100 µl Stop Solution (H_2SO_4) and the reaction was measured with the plate reader at 450 nm.

Western blot

The western blot technique is a semi-quantitative test for protein presence. The same antibody system was applied as in IHC staining. For western blot analysis proteins are separated by size via gel electrophoresis. For this, 15 µl of different dilutions of samples ([1:2], [1:5], [1:10], [1:25], [1:50], [1:75]) were mixed with 5 µl 4x Laemmli Buffer and incubated for 5 minutes at 95°C. The samples were centrifuged at 14000 rpm for 3 minutes and loaded into a 18% SDS-PAGE, which was immersed in gel electrophoresis buffer, 120 V was applied to the gel electrophoresis chamber and the gel was run until the marker reached the bottom of the gel. Thereafter the gel and a nitrocellulose membrane were incubated for 10 minutes in 1x transfer buffer. A semi-dry sandwich was assembled (bottom to top: 2 Whatman filter papers – nitrocellulose membrane – SDS-PAGE gel – Whatman filter paper). The transfer chamber with the assembly was put into the blotter and the following program was applied twice: BioRad – 1 Mini Gel – Mixed MW, thereafter the SDS-PAGE as well as Whatman filter paper was discarded and the membrane was blocked by 1% BSA in 0.05% TBS-T for 1 hour. The primary antibody was applied overnight (4°C) after the blocked membrane was washed trice in 0.05% TBS-T for 10 minutes. On the following day the blot was washed in the same manner as before described, and the 2nd antibody was applied for 2 hours at RT. The blot was finally washed 4 x with 0.05% TBS-T for 10 minutes and developed by application of ECL substrate for 5 minutes. The fluorescence reaction was detected by the ChemiDoc^{TM}Touch Imaging System in the chemiluminescence mode.

3.2.3 Reverse transcription PCR

RT-PCR was conducted with both Hope-fixed paraffine embedded samples (HFPE) as well as tissue which was frozen shortly after obtaining.

RNA-Extraction from frozen tissue samples

RNA was extracted by Direct-zolTM RNA MiniPrep kit (Zymo Research, R2052) according to manufacturer. Tissue samples were pulverized using liquid nitrogen and a motar. Tissue (100 mg) was solved in 200 µl TRI Reagent by up-and-down pipetting. Following centrifugation at 15 000 rpm for 3 minutes, the supernatant was transferred into a new vial and solved in equal amount of 95-100% EtOH. After mixing the solution was transferred into Zymo-Spin IICR Column and the sample was centrifuged at 15000 rpm for 30 seconds (general setting for centrifugation unless stated otherwise). The flow-through was discarded and 400 µl RNA wash buffer was added to the column and centrifuged. DNA was digested by DNase I (5 µl DNAse I + 75 µl DNA digestion buffer) incubation for 15 minutes at RT. Afterwards 400 µl RNA PreWash Buffer was added and centrifuged. Flow-trough was discarded, the step repeated, 700 µl RNA wash buffer was added and 2 minutes centrifuged. RNA was eluted by addition of 15 µl DNase/RNase free water and

centrifugation. Obtained RNA (amount and quality) was evaluated by NanoDrop and stored at -80°C upon reverse transcription.

RNA-Extraction from HFPE samples

Each sample was cut (4x20µm) into one sterile vial and frozen at -20 °C. Between different samples the microtome was cleaned with EtOH and RNAeasy spray and the blade was changed to avoid cross contamination as well as RNase contamination. Before further processing excess paraffine was removed. RNA was extracted by Quick-RNA™ FFPE Kit (Zymo Research, R1008) according to the manufacturer´s instructions: 4 trimmed 20 µm were put into sterile 2 ml Eppendorf tube and 400 µl were added to the sample for 1 minute at 55°C. The sample was vortexed, the deparaffinization solution was removed and sample was digested by addition of digestion mix (95 µl DNase/RNase-Free water, 95 µl 2x Digestion Buffer, 10 µl Proteinase K) for 1 hour at 55°C followed by 15 minutes at 65°C. Thereafter 600 µl RNA lysis buffer was added, vortexed and centrifuged at maximal speed for 1 hour. Insoluble debris were removed and sample transferred into new 2 ml Eppendorf tube where 1 volume of EtOH (95-100%) was added. The sample was vortexed and transferred into Zymo-Spin™ IICR Column in a collection tube and centrifuged for 30 seconds at 15000 rpm (standard centrifugation setting unless stated otherwise). Flow through was discarded and column was washed with 400 µl RNA Wash Buffer and centrifugation. Following this DNA was digested by application of DNase I (5 µl DNAse I + 75 µl DNA digestion buffer) to the matrix for 15 minutes at RT. Column was washed by 400 µl RNA prep buffer and centrifugation. Then 700 µl RNA wash buffer was added and the column was centrifuged. Before RNA was eluted by addition of 15 µl DNase/RNase free water and centrifugation, the column was washed with 400 µl RNA wash buffer and 2-minute-long centrifugation. Obtained RNA was again evaluated by NanoDrop and stored at -80°C after reverse transcription.

Evaluation of RNA

RNA was evaluated before reverse transcription by NanoDrop, and samples which had an A260/A280 value below 1.80 and A260/A230 below 1.80 (only frozen tissue as remaining paraffine debris distorted the value) were discarded immediately. Remaining samples were analysed via gel electrophoresis.

Reverse Transcription

Extracted RNA from both frozen tissue and HFPE tissue were reverse transcribed with iScript™ cDNA Synthesis Kit (Biorad, #170-889) according to manufacturer. Therefore, 4 µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase and RNA was added in order to produce a sample with a concentration of 40 ng/µl. Nuclease-free water was added to a total volume of 20 µl. The

mix was put into the thermal cycler and following program applied: 5 min 25°C, 30 min 42°C, 5 min 85°C and hold on 4°C.

Primer Sequences

Primer pair sequences for BLG mRNA, ACTB mRNA and GAPDH mRNA were either taken from literature or designed via the primer blast tool of the NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and tested by the bioinformatics web tool: Sequence Manipulation Suite – Version 2 (https://www.bioinformatics.org/sms2/) were the tools "PCR primer stats" (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) and "PCR products" (https://www.bioinformatics.org/sms2/pcr_products.html) were applied to the obtained primer sequences. Primer sequences are listed under 3.1.5.

Table 7 Master Mix for PCR. was conducted by producing a master mix which was divided into aliquots of 45 µl and finally 5 µl sample (40ng/µl) containing totally 200ng cDNA were added.

Component	1x	
5X Green or Colorless GoTaq Reaction Buffer	10	µl
PCR Nucleotide Mix	1	µl
GoTaq G2 DNA Polymerase	0,25	µl
upstream Primer (1:10)	1	µl
downstream Primer (1:10)	1	µl
water	31,75	µl
Template (40ng/ul)	5	µl
total Volume	50	µl

Table 8 PCR set up. PCR of the bovine samples was conducted by running 35 cycles since the amplicons were solely tested in gel electrophoresis and no sequencing or cloning of the amplicons was desired. Therefore, incorrect copies were of no concern.

0.	initial denaturation	2 min	95°C
1. step 2. step 3.step	denaturation annealing extension	30 sec 30 sec 60 sec	95°C 60°C 73°C
4.step	final extension	5 min	73°C
Number of cycles	35		

Polymerase Chain Reaction with reverse transcribed RNA

For conduction of the PCR, master mixes were produced for 2 housekeeping genes (ACTB, GAPDH) and LGB. The master mix was produced (table 7) on ice in such a quantity that each primer pair was tested with cDNA as well with mock control (nuclease-free water) in duplicates. Samples were put into the thermal cycler (applied settings are stated in table 8).

Analysis of PCR-amplicons

For analysis of conducted PCR, an agarose gel was 1.2% cast. The 35 µl of each PCR product was put into a gel-slot and 100 V were applied until the marker reached the end of the gel (5 cm). Additionally, 8 µl ladder were applied. The gel then was analysed with ChemiDoc™Touch Imaging System in the Ethidium Bromide Mode.

4. Results

In the milk industry BLG is a protein of high interest and up to today several books as well as a vast amount of papers has been written. Although BLG has several different genomic genomic variants, their physical characteristics and impact on milk characteristics have been investigated thoroughly, its original purpose and function still remains elusive. Interestingly, after a thorough search of the NCBI´s PubMed, immunohistochemical investigations have last been conducted in the 1970ties[45] where only udder tissue has been included. Reports of BLG expression and presence in other tissues is, at least on PubMed, entirely missing. Since we have found BLG also in bovine urine independent of sex, it is highly unlikely that the protein is only expressed in the udder. In the here presented experiments we demonstrate additional organs harbouring BLG and therefore reveal possible new production sites.

4.1 Immunohistochemical staining of BLG in cattle tissue

In the first part of the here conducted investigation a method for immunohistochemical staining of BLG in bovine tissue was established. Several different antibody concentrations were tested on bovine mammary tissue, as the presence of BLG in this organ has already been confirmed by fluorescence immunohistochemistry in the 1970ties[45].

For our initial testing of the method, cow udder tissue was stained with different combinations of antibody concentrations. From these pre-experiments we concluded that an antibody combination of 1:1500 to 1:2000 of primary antibody dilution and 1:25000 secondary antibody dilution was suitable for further testing in other bovine tissues. Udder tissue samples stained with different antibody concentrations are presented in figure 15.

Following the establishment of the staining method, tissues of both sexes were tested. Organs of interest, beside the udder, were bovine kidneys, adrenal glands as well as testis. Kidney tissue was chosen due to BLG presence in bovine urine. The adrenal gland was chosen for its function as an endocrine organ and bull testis was chosen as a gender-specific organ.

IHC was conducted as described in 3.2.1 and representative acquisitions are shown in figure 16.

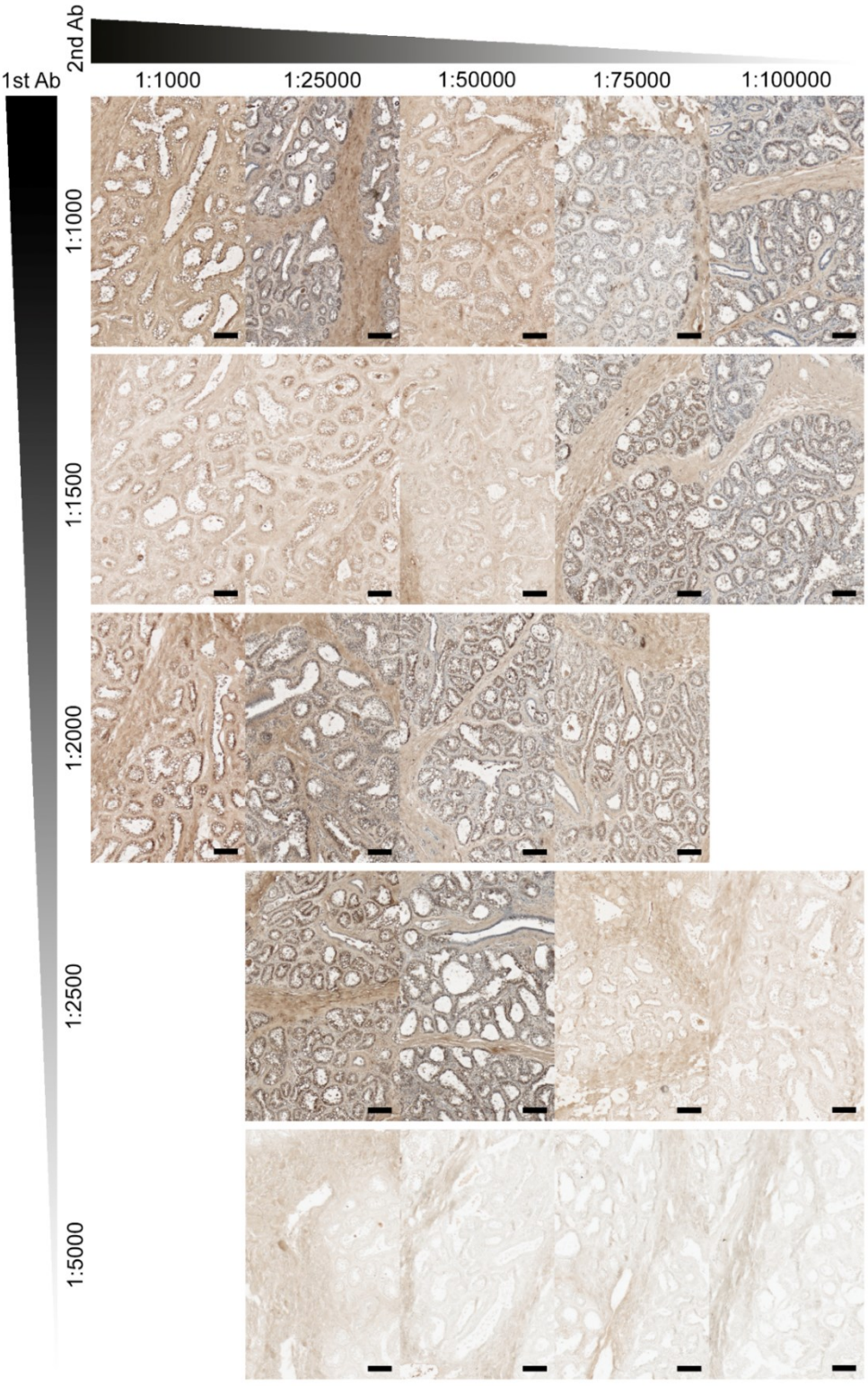

Figure 15 Differnet antibody concentrations on bovine udder. Primary as well as the secondary antibody was applied to the already confirmed BLG containing tissue in various combinations. Several antibody combinations yielded acceptable results. Since udder tissue is a highly BLG positive tissue a dilution of 1:1500 to 1:2000 for the primary antibody combined with 1:25000 concentration of the secondary antibody was deemed suitable for staining the other tissues. Scalebars = 100 µm

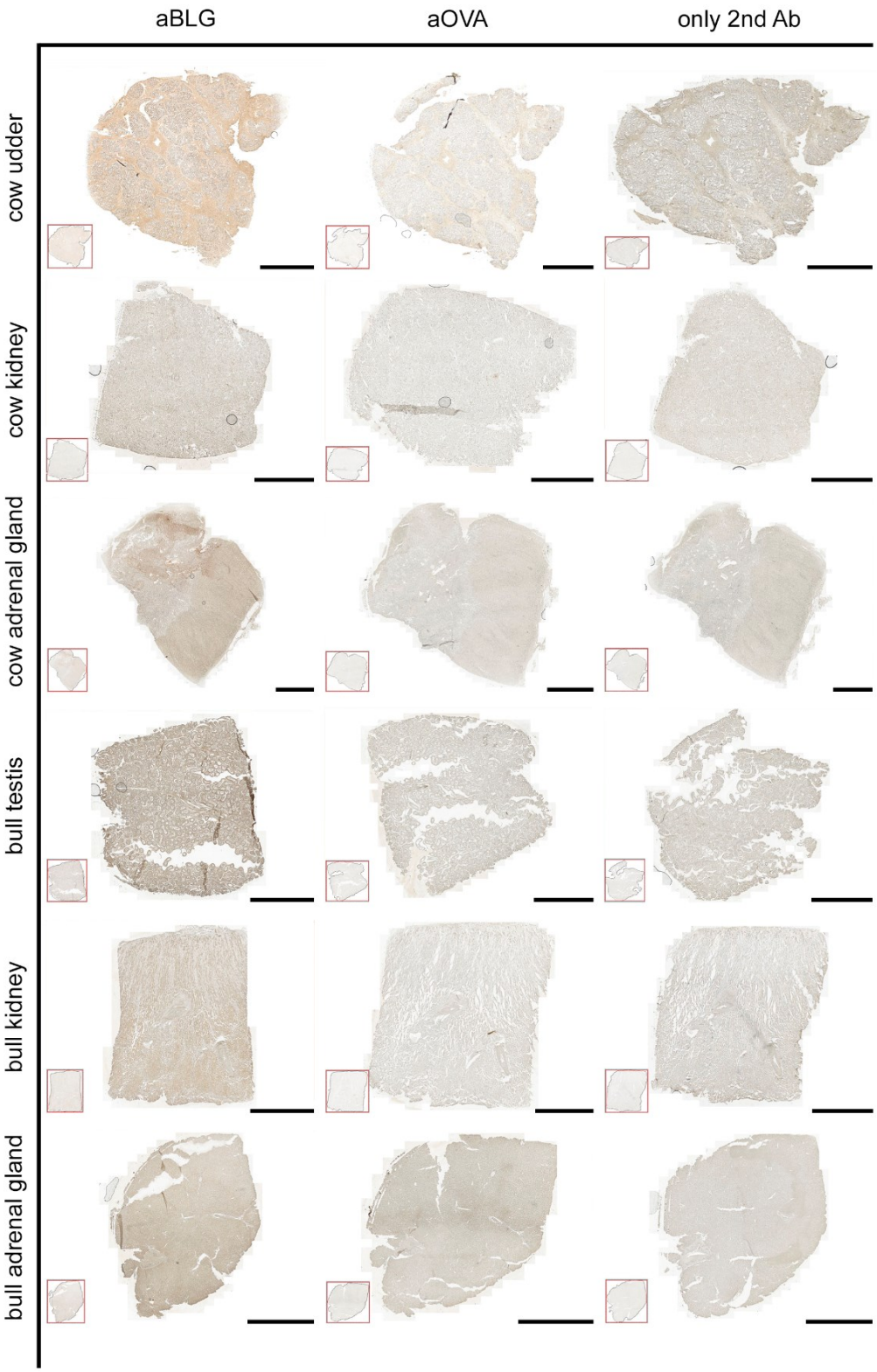

Figure 16 Tissue overview. Application of TissueFax to IHC allows scanning of whole tissue samples in a semi-automatic manner. Demonstrative computed scans of all tested tissues are shown next to their controls. Here shown scans were stained with 1:1500 primary Ab concentration (3-minute DAB development) and 5 minutes with haematoxylin. Scans were generated of 2.5x acquisition. Scalebars = 1 mm.

4.1.1 Immunohistochemical staining of BLG in cow mammary tissue

Cow udder tissue was stained as described in detail in 3.2.1 with the antibody concentrations established in the pre-experiments (4.1).

HistoQuest analysis

The immunohistochemical staining with αBLG detected the protein in the bovine udder as expected. Following the setting of ROI (0.15 mm², 63x acquisition) in all three samples (αBLG, αOVA, only 2ndAb) the marker colours were chosen (figure 19) with the autodetect tool. HistoQuest analysis of chosen regions of the cow udder sample revealed in the αBLG sample 568 cells, in αOVA sample 529 cells and in only 2nd antibody 503 cells as haematoxylin positive. In αBLG 431 were BLG positive which translates to a total of 75.88%, in αOVA 0 cells (0%) and in only 2ndantibody 0 (0%) produced a positive BLG signal. These findings are represented in figure 17.

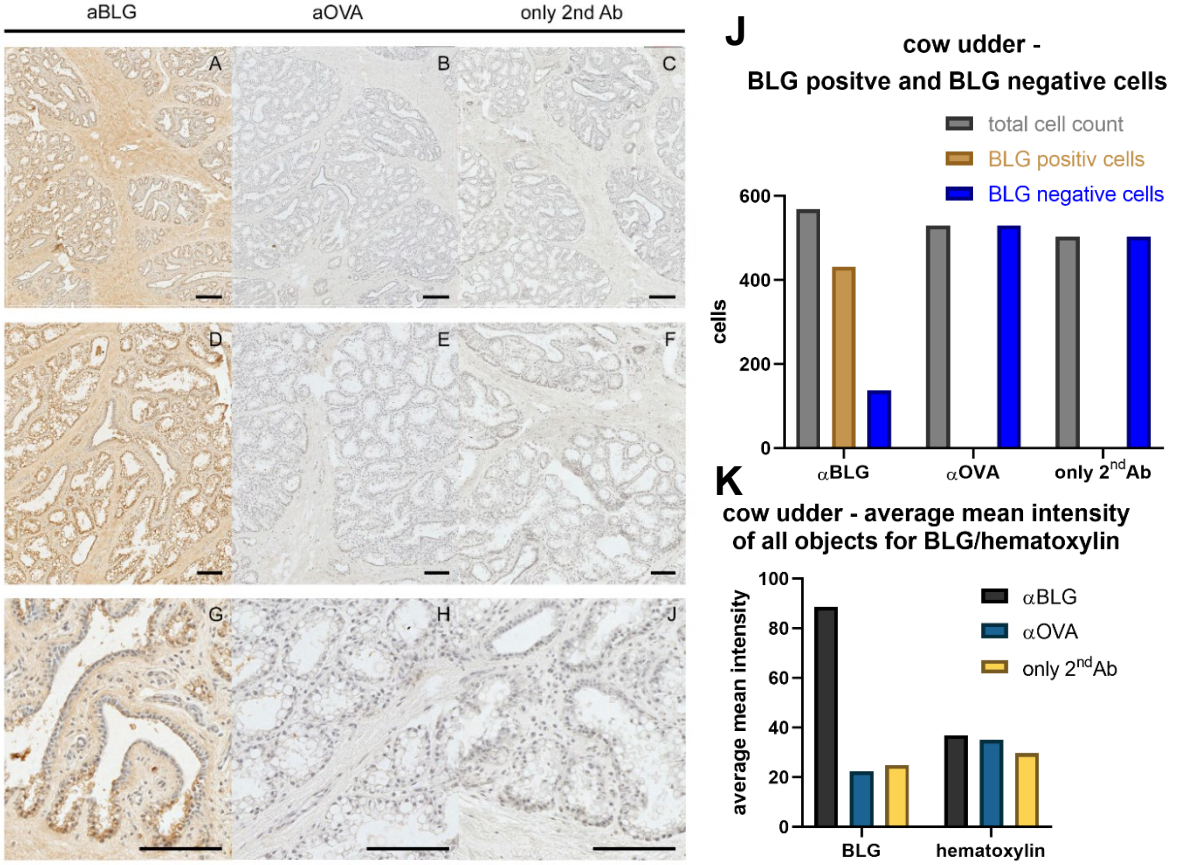

Figure 17 Immunohistochemical analysis of bull testis for BLG. A, D, G: αBLG - DAB (1:1500, 2nd Ab: 1:25000) and Haematoxylin stained cow udder tissues. B, E, H: αOVA - DAB (1:1500, 2nd Ab: 1:25000) and Haematoxylin stained cow udder tissues. C, F, J: only 2nd Ab (1:25000) and Haematoxylin stained cow udder tissues. A - C: scale bar = 200 µm, D-J: scale bar = 100 µm. J: HistoQuest analysis of 0,15 mm² big ROI (region of interest) for BLG positive cells shows that no BLG is present in negative controls (αOVA, only 2nd Ab). K: Average mean intensity measured by HistoQuest for BLG and Haematoxylin staining in selected ROI. ROI for J, K derives from 1:2000 primer Ab and 1:25000 secondary Ab stained samples.

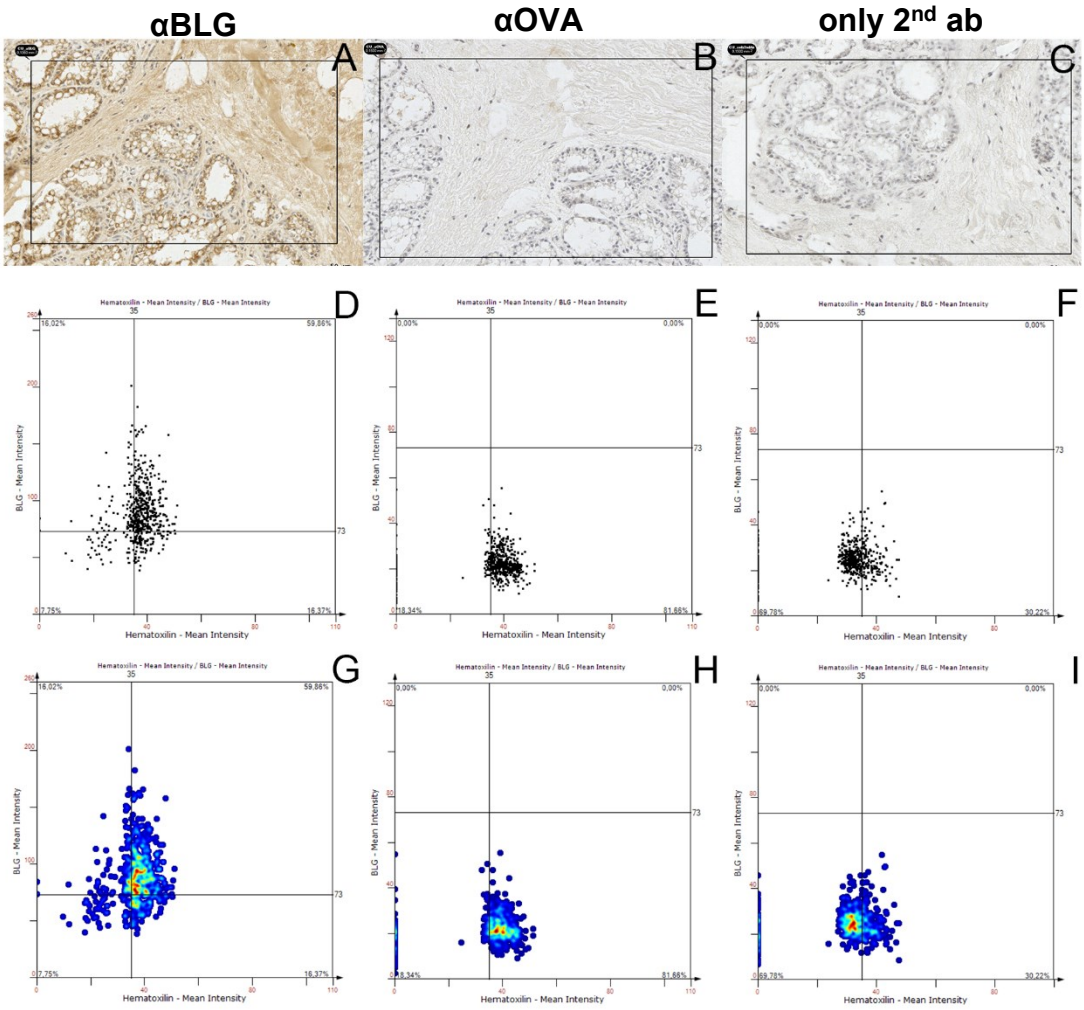

Figure 18 HistoQuest Analysis for BLG and Haematoxylin mean intensity. A – C: marked 0,15mm² big region of interest (ROI) which was analysed by HistoQuest. D – F: Scattergram showing bins according to measured Haematoxylin and BLG mean intensity. G – I: Heatmap of scattergram showing bins according to measured Haematoxylin and BLG mean intensity. D – I: all thresholds were set on the BLG stained scattergram (D, G) in the 25% percentile and applied to both controls (E, F, H, I).

In figure 18, scattergrams of the investigated ROI generated by HistoQuest are presented. The program could identify no positive signals in the negative controls which to the human eye also remain completely negative and the scattergrams show similar populations. The overlay of all three scattergrams (figure 20 -A) shows the obtained bins for all three samples. The overlapping bins for the negative samples are clearly distinguishable from the BLG stained sample indicating a successful staining. Looking at the histograms (figure 20 -B/-C), haematoxylin produced relatively equal signal in all samples. More intense signal in the αBLG stained sample might be a result of the BLG staining interfering with the haematoxylin detection. In coherence with literature BLG is clearly present in the bovine udder.

A **Color Separation Parameters**

Parameter	Value
Color	
Color RGB	[143, 140, 183]
Is Mixture	No
Intensity	200

B **Color Separation Parameters**

Parameter	Value
Color	
Color RGB	[231, 202, 123]
Is Mixture	No
Intensity	200

Figure 20 Colour separation parameters for cow udder. In A the colour setting for haematoxylin detection in cow udder is shown. In B the colour for DAB – BLG detection in cow udder is shown.

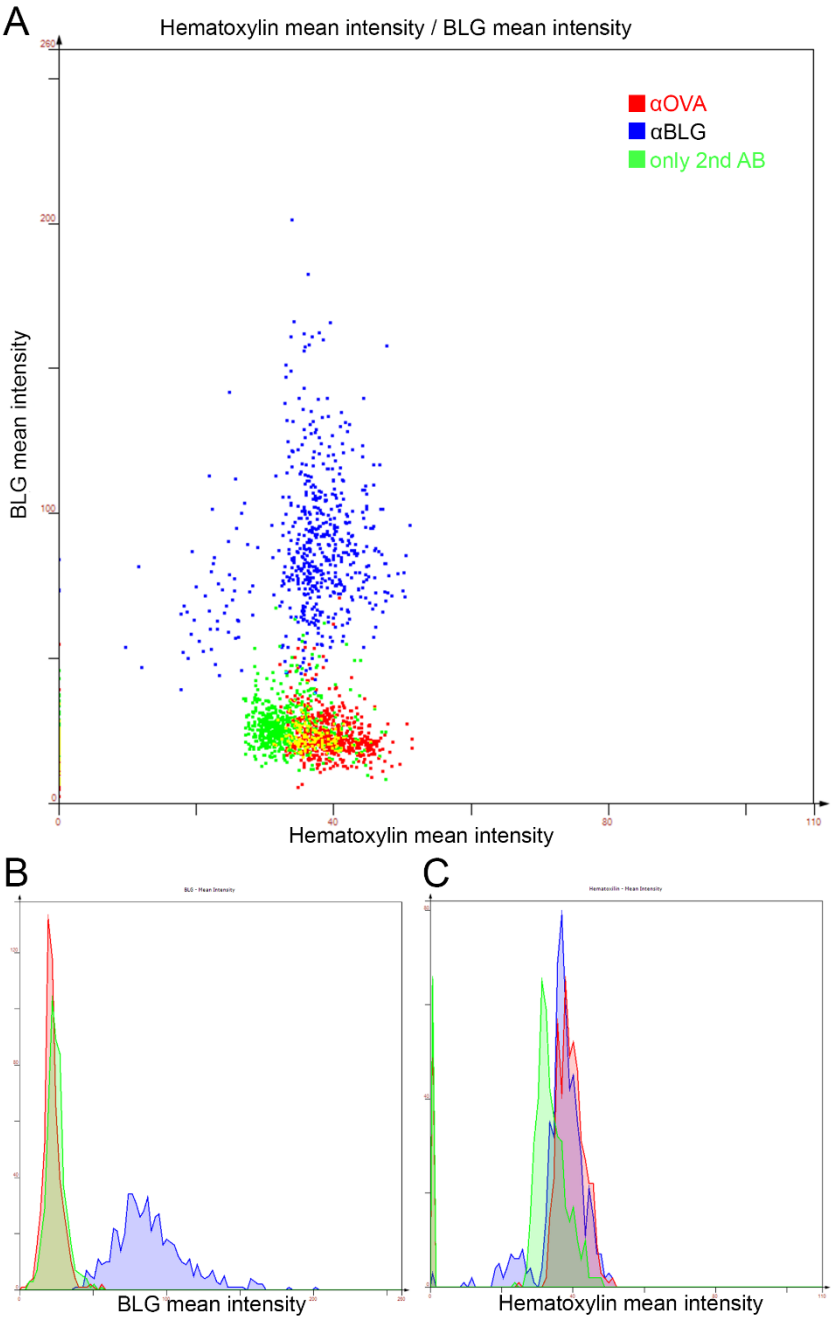

Figure 19 Overlay of measured BLG/Haematoxylin mean intensity in cow udder. In A overlay of all detected bins according to measured BLG and haematoxylin mean intensity is shown for cow udder αBLG, αOVA and only 2nd Ab stained samples. Additionally, in B and C histogram overlays for cow udder αBLG, αOVA and only 2nd Ab stained samples are shown for BLG (B) and haematoxylin (C).

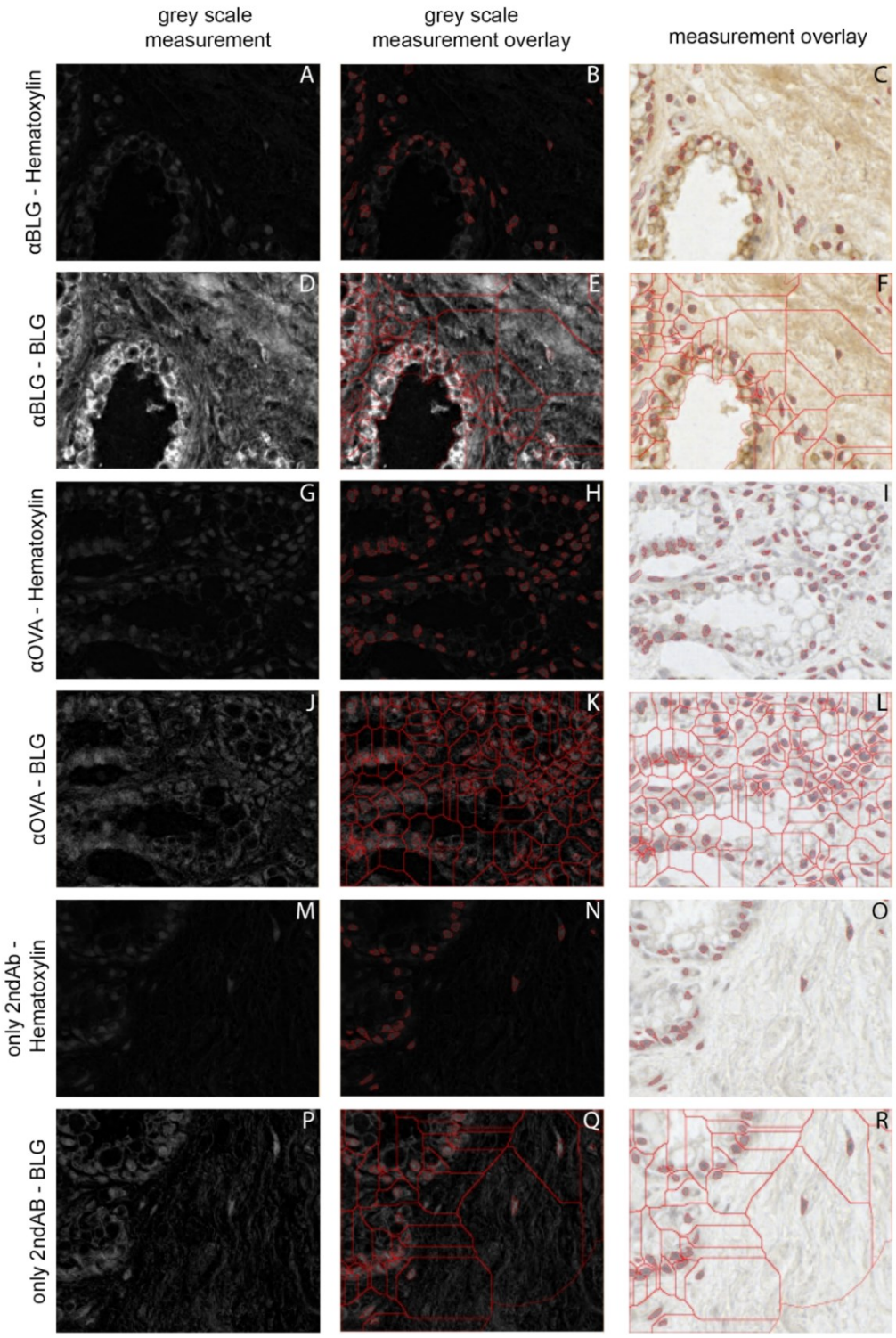

Figure 21 HistoQuest analysis marker detection and colour separation. In A, D, G, J, M, P grey scale image is shown and bright pixels show positive signal. B, C, E, F, H, I, K, L, N, O, Q, R show calculated borders (in red) of either cell or nucleus in grey scale or in colour. A – F: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow udder tissue. G – L: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow udder tissue. M – R: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow udder tissue. A – C, G – I and M – O shows detection of haematoxylin and D – F, J – L and P – Q shows BLG detection.

Histological analysis of cow udder tissue

In αBLG stained cow udder section, BLG seems to be present throughout the whole specimen. Yet, some parts are stained stronger than others, such as the epithelial lining of the alveolar glands. As shown in figure 22- B by the black arrows these epithelial cells are stained the strongest. Moreover, BLG is clearly present in the extracellular matrix surrounding the alveolar milk glands, while the cell lining of bigger milk duct has less BLG (figure 22 - C). The red arrows in figure 22 - A show highly dividing tissue parts which are mostly free of BLG. Therefore, BLG seems not to be specific to the alveolar gland epithelial cells, but present throughout the udder tissue.

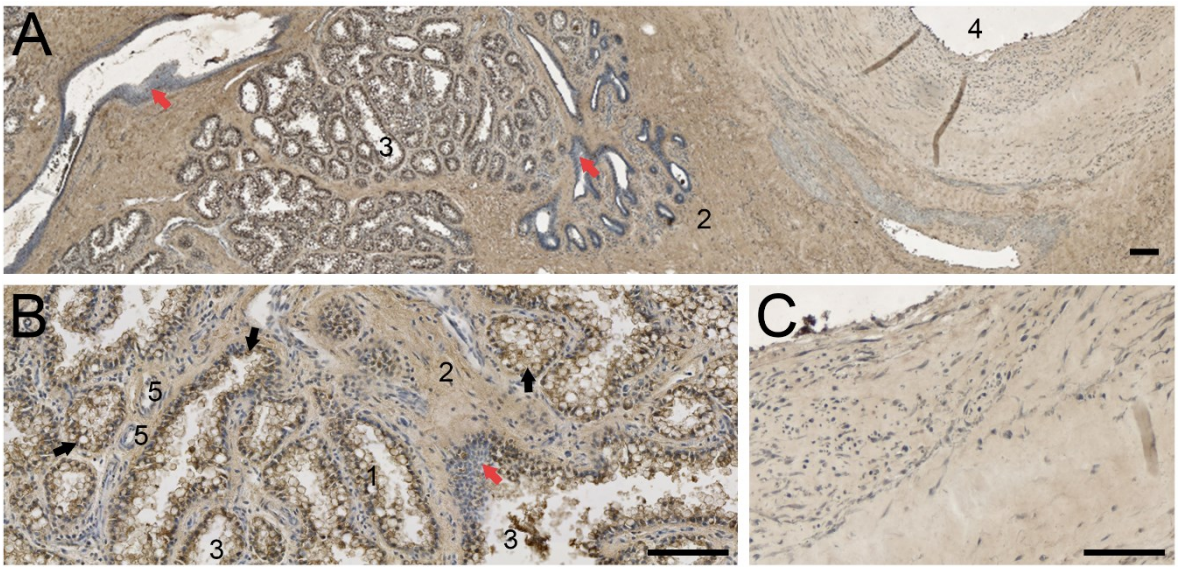

Figure 22 BLG in cow udder. In A several typical structures for the bovine udder are shown including alveolar gland tissue (1), extracellular matrix (2), milk ducts of the alveolar gland (3) as well as bigger milk ducts (4). Additionally, blood vessels can be found (5). Scalebars = 100 µm

4.1.2 Immunohistochemical staining of BLG in cow kidney tissue

Cow kidney tissue was stained in the same manner as the cow udder and is described in detail in 3.2.1.

HistoQuest analysis

Immunohistochemical staining of cow kidney for BLG showed that the protein is indeed present. After setting of the regions (0.15 mm², 63x acquisition) in all three samples, the marker colours were chosen (figure 25) with the autodetect tool. Thereafter, HistoQuest analysis of chosen regions was performed. In cow kidney, in αBLG 935 cells, in αOVA 611 cells and in only the 2nd antibody 474 cells (haematoxylin positive) were counted. In the αBLG, 702 were BLG-positive, which translates to a total of 75.08%. According to HistoQuest, in the αOVA sample 3 cells (0.49%) and with only 2ndantibody 1 cell (0.21%) were BLG positive. These findings are presented graphically in figure 23.

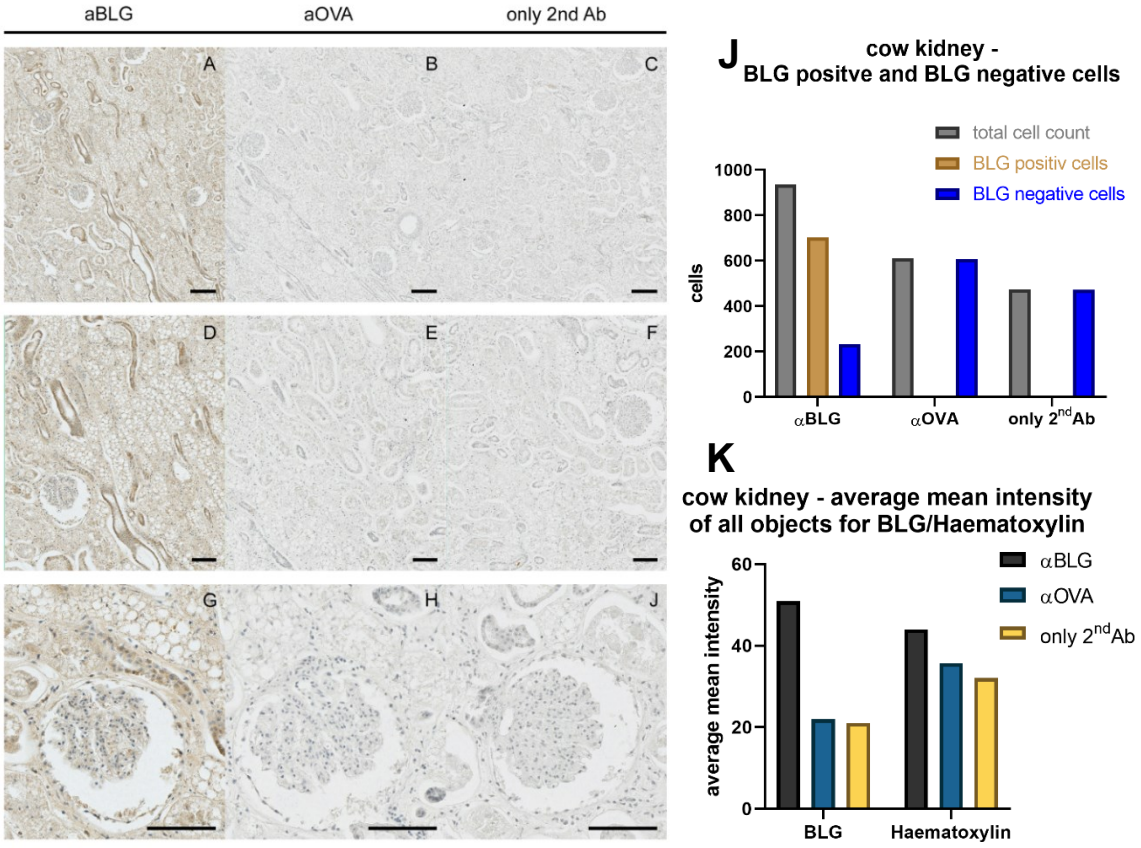

Figure 23 Immunohistochemical analysis of cow kidney for BLG. A, D, G: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and Haematoxylin stained cow kidney tissues. B, E, H: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and Haematoxylin stained cow kidney tissues. C, F, J: only 2nd Ab (1:25000) and Haematoxylin stained cow kidney tissues. A-C: scale bar = 200 µm, D-I: scale bar = 100 µm. J: HistoQuest analysis of 0.15 mm² big ROI (region of interest) for BLG positive cells shows that no BLG is present in negative controls (αOVA, only 2nd Ab). K: Average mean intensity measured by HistoQuest for BLG and Haematoxylin staining in per selected ROI.

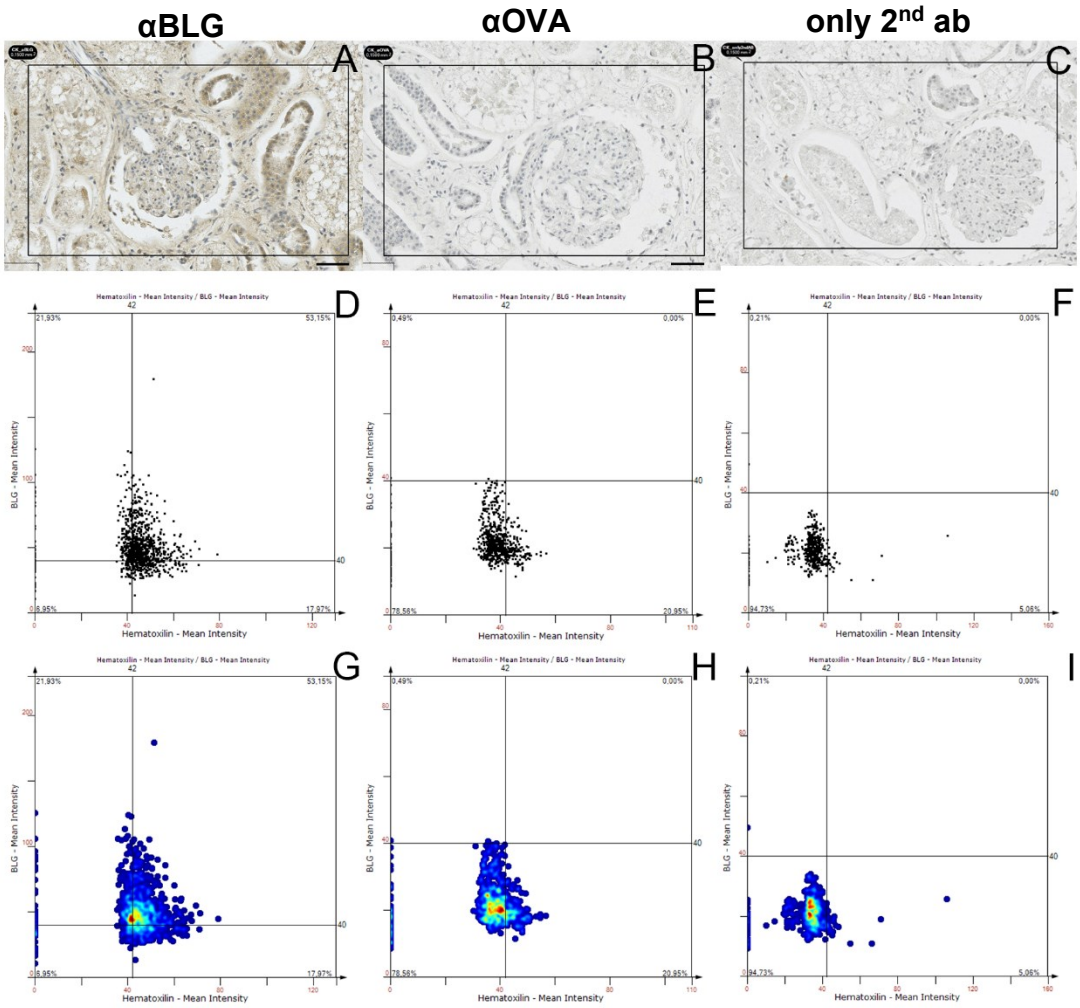

Figure 24 HistoQuest Analysis for BLG and Haematoxylin mean intensity. A – C: marked 0.15mm² big region of interest (ROI) which was analysed by HistoQuest. D – F: Scattergram showing bins according to measured Haematoxylin and BLG mean intensity. G – I: Heatmap of scattergram showing bins according to measured Haematoxylin and BLG mean intensity. D – I: all thresholds were set on the BLG stained scattergram (D, G) in the 25% percentile and applied to both controls (E, F, H, I).

Moreover, in figure 23 a histochemical staining of the cow kidney tissue is shown representatively. To the human eye there is no positive BLG staining in both controls. The ROIs which were used for the HistoQuest analysis is shown in figure 24 as well as resulting scattergrams. These scattergrams show the distribution of measured events according to the measured mean intensity of detected markers with applied cut-of-values. These diagrams show that most cells are both BLG and haematoxylin positive in the αBLG stained sample. In the negative controls, only in the αOVA sample some events are close to the border, there are no events above the BLG threshold. Noteworthy, haematoxylin had lower mean intensities in the negative controls. An overlay of all three obtained scattergrams is shown in figure 26. Here we could observe that the αBLG stained sample showed overall higher mean intensity values. The two negative controls nicely showed considerable overlay which is also illustrated in the histograms of both markers in figure 26.

A

Color Separation Parameters

Parameter	Value
Color	
Color RGB	[152, 170, 210]
Is Mixture	No
Intensity	200

B

Color Separation Parameters

Parameter	Value
Color	
Color RGB	[226, 191, 120]
Is Mixture	No
Intensity	200

Figure 26 Colour Separation Parameters for cow kidney. In A the colour for haematoxylin detection in cow kidney is shown. In B the colour for DAB – BLG detection in cow kidney is shown.

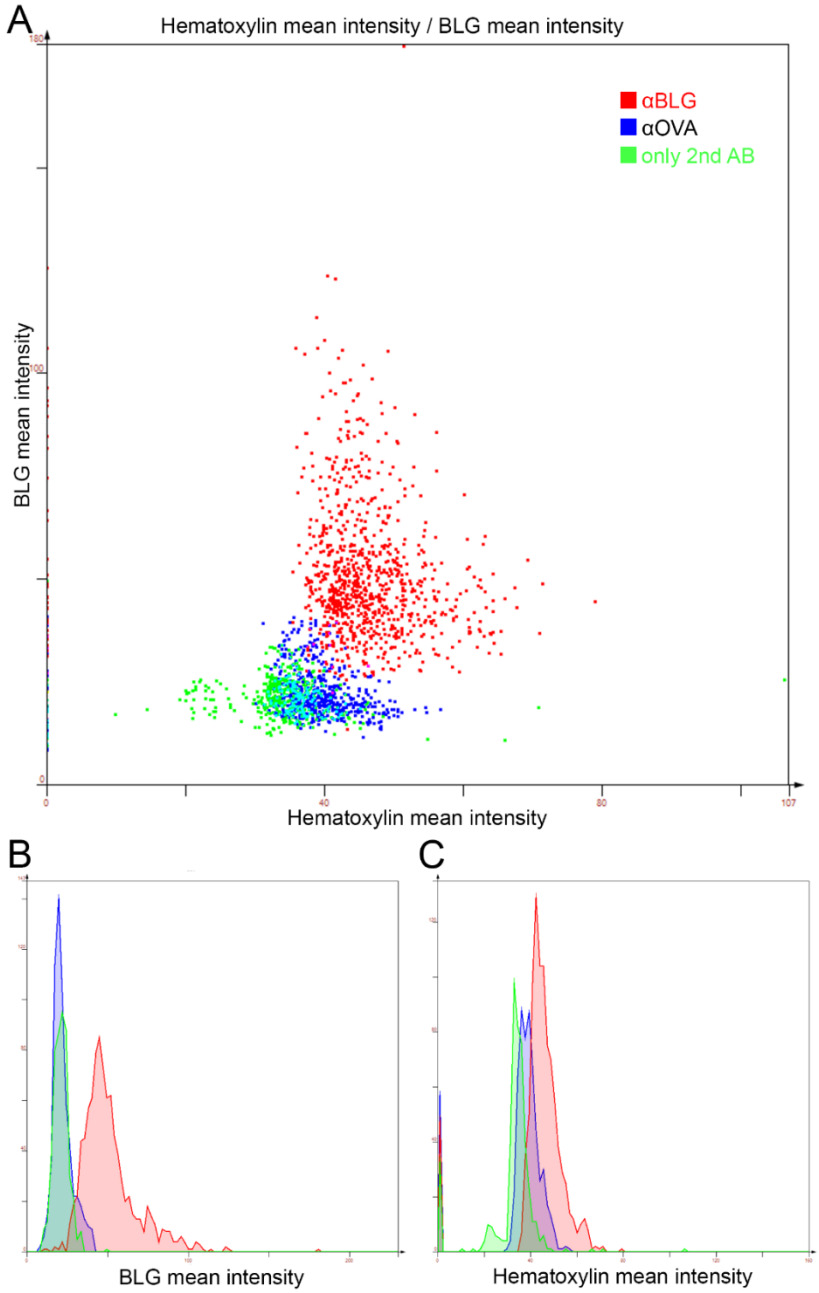

Figure 25 Overlay of measured BLG/Haematoxylin mean intensity in cow kidney. In A overlay of all detected bins according to measured BLG and haematoxylin mean intensity is shown for cow kidney αBLG, αOVA and only 2nd Ab stained samples. Additionally, in B and C histogram overlays for cow kidney αBLG, αOVA and only 2nd Ab stained samples are shown for BLG (B) and haematoxylin (C).

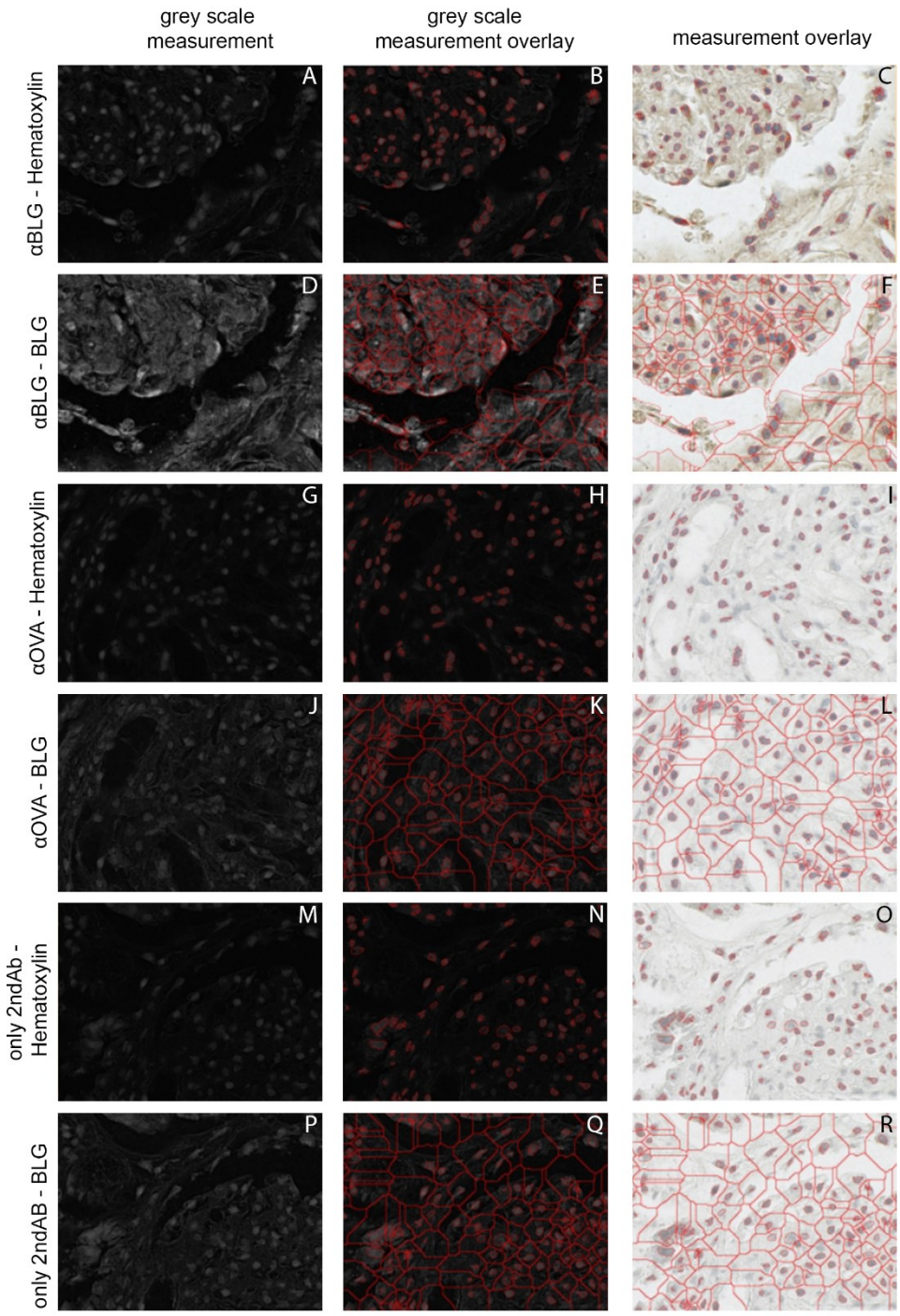

Figure 27 HistoQuest Analysis marker detection and colour separation. In A, D, G, J, M, P grey scale image is shown and light pixels show positive detection. B, C, E, F, H, I, K, L, N, O, Q, R show calculated borders (in red) of either cell or nucleus in grey scale or in colour. A – F: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow kidney tissue. G – L: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow kidney tissue. M – R: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and Haematoxylin stained cow kidney tissue.

Histological analysis of cow kidney tissue

Looking on the obtained acquisition of the cow kidney although BLG seems to be present in small amounts throughout the whole kidney section, but also more positive structures in certain places can be observed. Exemplary acquisitions are presented in figure 28, demonstrating BLG staining throughout the sections. In cow kidney highly BLG positive structures are the tubular parts of the nephron. Within the glomeruli, small aggregations of strongly BLG positive sites can be found, indicating filtration of BLG from the blood, therefore making BLG presence in the bovine blood highly likely.

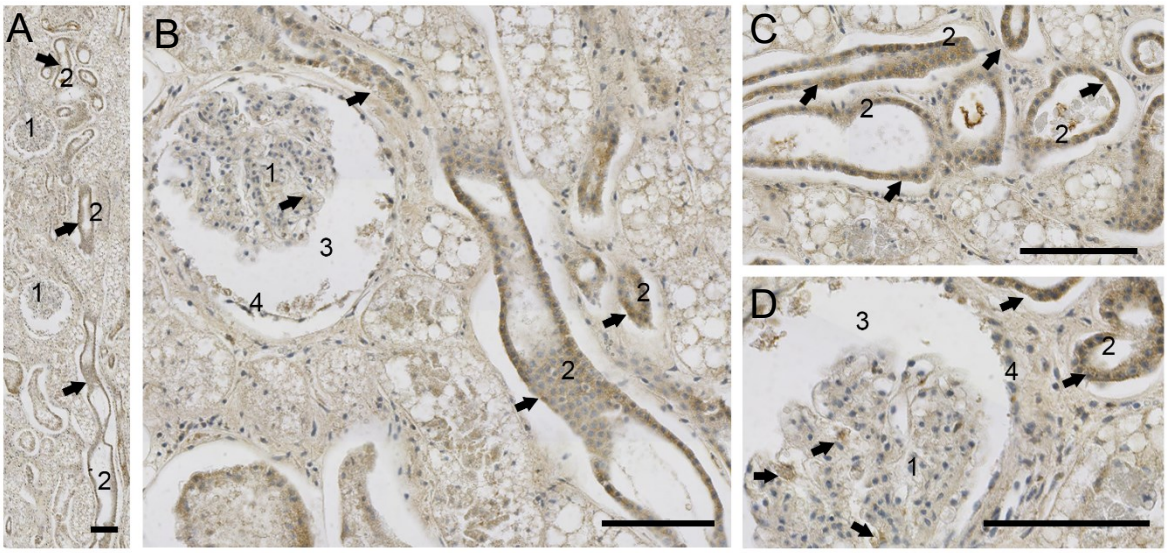

Figure 28 BLG in cow kidney. BLG seems to be present throughout the bovine kidney. In A several typical structures for the kidney cortex are shown such as the glomerulus (1) and the tubular parts of the nephron (2). Additionally, in A nearly the full distal tube is shown. In B a closer view of A shows that the tubular structures of the nephron are stained the strongest (black arrows). C and D show respective structures in different regions of the acquisition. 3 – capsule space, 4 – inner layer of the Bowman´s capsule. Scalebars = 100 µm

4.1.3 Immunohistochemical analysis of BLG in cow adrenal gland tissue

Cow adrenal gland tissue was stained in the same manner described in the previous two cow organs and the detailed protocol enclosed in 3.2.1.

HistoQuest analysis

Immunohistochemical staining of cow adrenal gland for BLG surprisingly revealed BLG presence. Following the setting of the ROI (0.15 mm², 63x acquisition) in all three samples, the marker colours were chosen (figure 31) again with the autodetect tool. Performed HistoQuest analysis revealed in the αBLG stained sample 520 cells, in αOVA 838 cells and in only the 2nd antibody 566 cells (haematoxylin positive). In the αBLG out of 520 cell 400 were BLG positive equalling 76.92% of all counted cells. In the αOVA sample 1 out of 837 cells (0.12%) and 0 out of 566 cells (0%) were BLG positive. These findings are shown in figure 29.

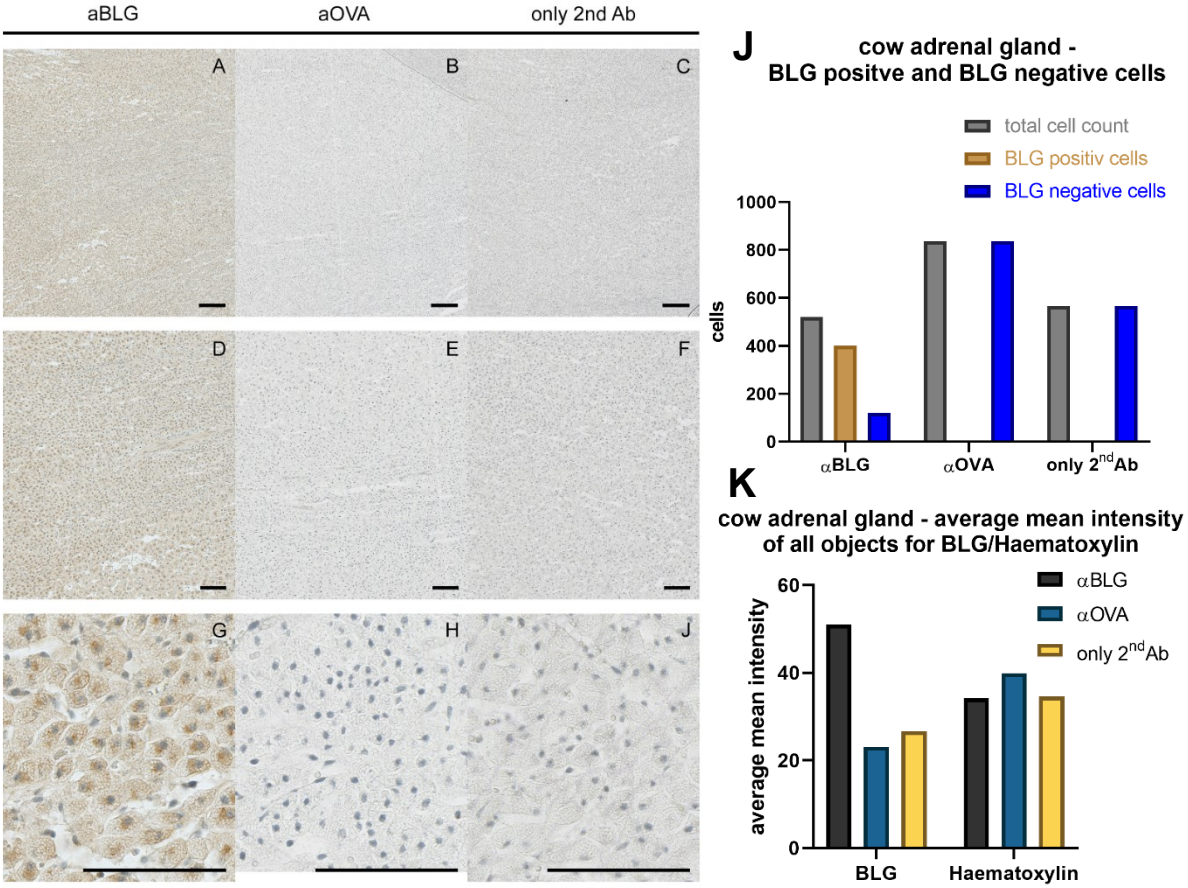

Figure 29 Immunohistochemical analysis of cow adrenal gland for BLG. A, D, G: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and Haematoxylin stained cow adrenal gland tissues. B, E, H: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and Haematoxylin stained cow adrenal gland tissues. C, F, J: only 2nd Ab (1:25000) and Haematoxylin stained cow kidney tissues. A-C: scale bar = 200 µm, D-I: scale bar = 100 µm. J: HistoQuest analysis of 0.15 mm² big ROI (region of interest) for BLG positive cells shows that no BLG is present in negative controls (αOVA, only 2nd Ab). K: Average mean intensity measured by HistoQuest for BLG and Haematoxylin staining per selected ROI

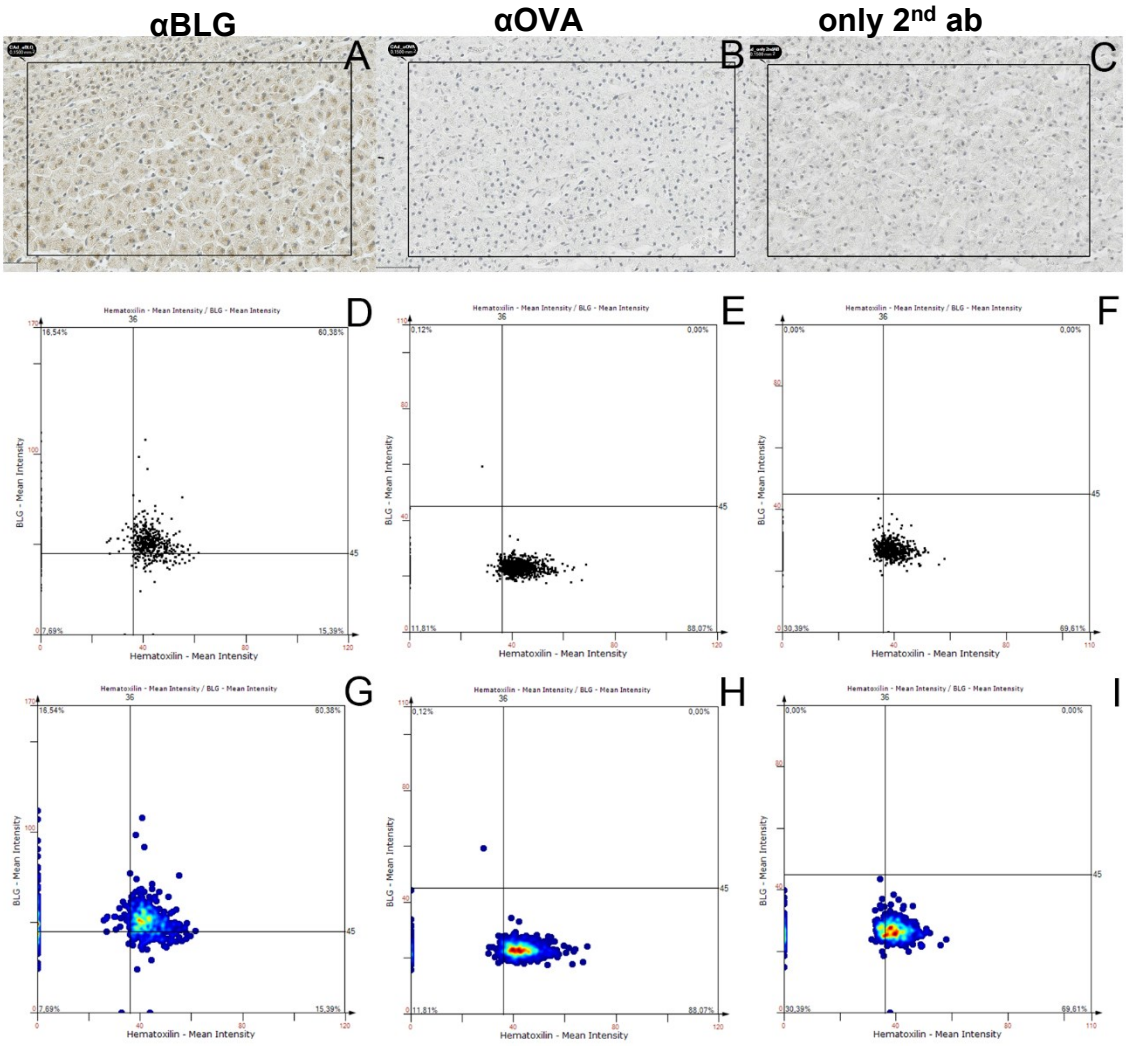

Figure 30 HistoQuest Analysis for BLG and Haematoxylin mean intensity. A – C: marked 0,15mm² big region of interest (ROI) which was analysed by HistoQuest. D – F: Scattergram showing bins according to measured Haematoxylin and BLG mean intensity. G – I: Heatmap of scattergram showing bins according to measured Haematoxylin and BLG mean intensity. D – I: all thresholds were set on the BLG stained scattergram (D, G) in the 25% percentile and applied to both controls (E, F, H, I).

In figure 30 the histochemical staining of the cow adrenal gland tissue as scattergrams of the ROIs analysed by HistoQuest are shown. By the human eye no positive BLG staining is detectable in both negative controls. The scattergrams similarly to the previous investigated organs, show that most cells of αBLG stained sample are BLG positive. Haematoxylin had comparable intensities across all samples. An overlay of all three obtained scattergrams (figure 32) shows that obtained bins for the negative samples are mostly overlapping and the BLG stained ROI produced clearly distinguishable bins.

A Color Separation Parameters

Parameter	Value
Color	
Color RGB	[148, 164, 212]
Is Mixture	No
Intensity	200

B Color Separation Parameters

Parameter	Value
Color	
Color RGB	[226, 196, 134]
Is Mixture	No
Intensity	200

Figure 31 Colour Separation Parameters for cow adrenal gland. In A the colour for haematoxylin detection in cow adrenal gland is shown. In B the colour for DAB – BLG detection in cow adrenal gland is shown.

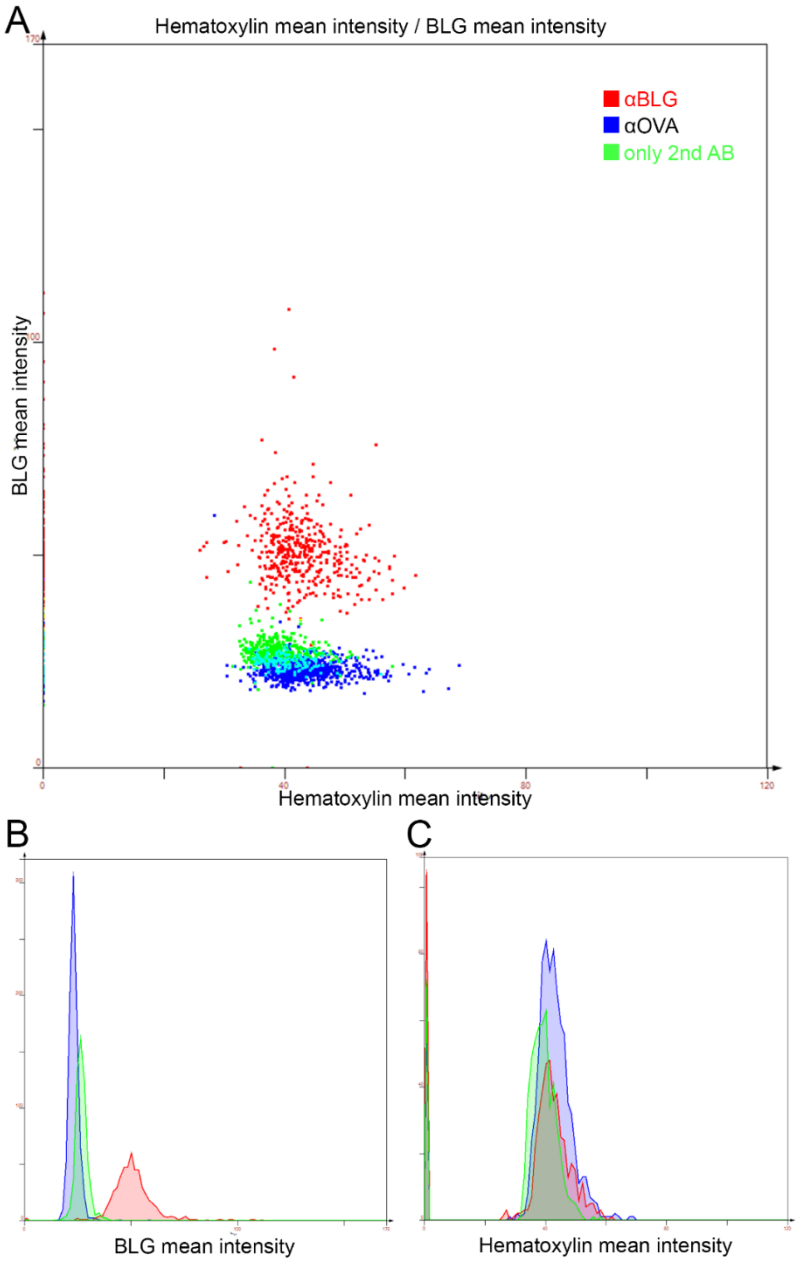

Figure 32 Overlay of measured BLG/Haematoxylin mean intensity in cow adrenal gland. In A overlay of all detected bins according to measured BLG and haematoxylin mean intensity is shown for cow adrenal gland αBLG, αOVA and only 2nd Ab stained samples. Additionally, in B and C histogram overlays for cow adrenal gland αBLG, αOVA and only 2nd Ab stained samples are shown for BLG (B) and haematoxylin (C).

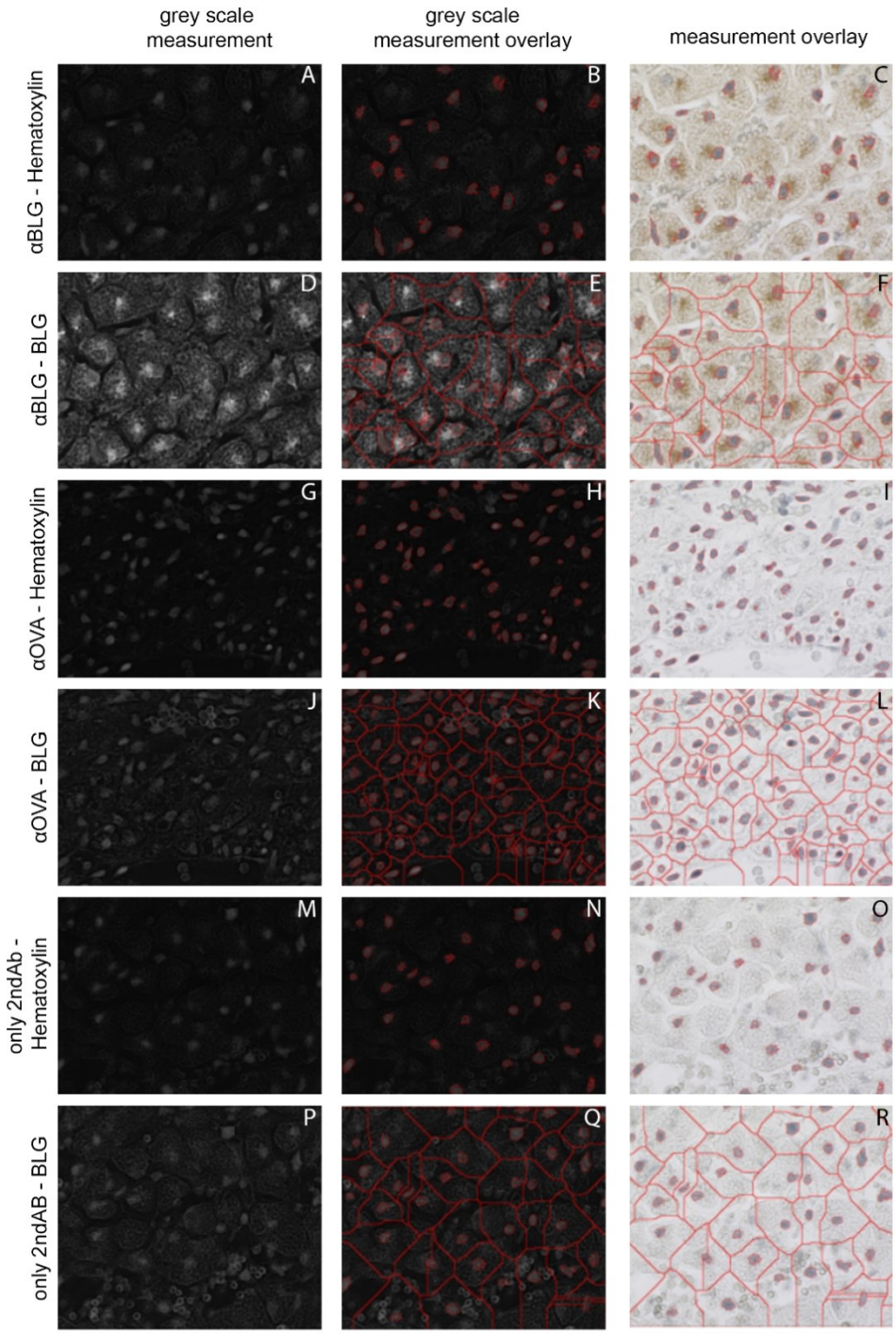

Figure 33 HistoQuest Analysis marker detection and colour separation. In A, D, G, J, M, P grey scale image is shown and bright pixels show positive detection. B, C, E, F, H, I, K, L, N, O, Q, R show calculated borders (in red) of either cell or nucleus in grey scale or in colour. A – F: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow adrenal gland tissue. G – L: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow adrenal gland tissue. M – R: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow adrenal gland tissue. A – C, G – I and M – O shows detection of haematoxylin and D – F, J – L and P – Q shows BLG detection.

Histological analysis of cow adrenal gland tissue

Reviewing the obtained acquisitions, BLG seems to be mostly present in the cortex (figure 34 -1) of the adrenal gland, although the medulla (figure 34 -2) is not entirely negative itself. Moreover, within the medulla stronger stained cell patches can be found. The capsule (figure 34 -7) of the adrenal gland is only slightly positive, the strongest stained cells are found in the zona glomerulosa (figure 34 -8). While the cells of the third cortex layer, the zona reticularis (figure 34 -5) are homogeneously stained, within the cells of the middle cortex layer (figure 34 - 4) BLG aggregations can be found as indicated by the black arrows in figure 34 -C.

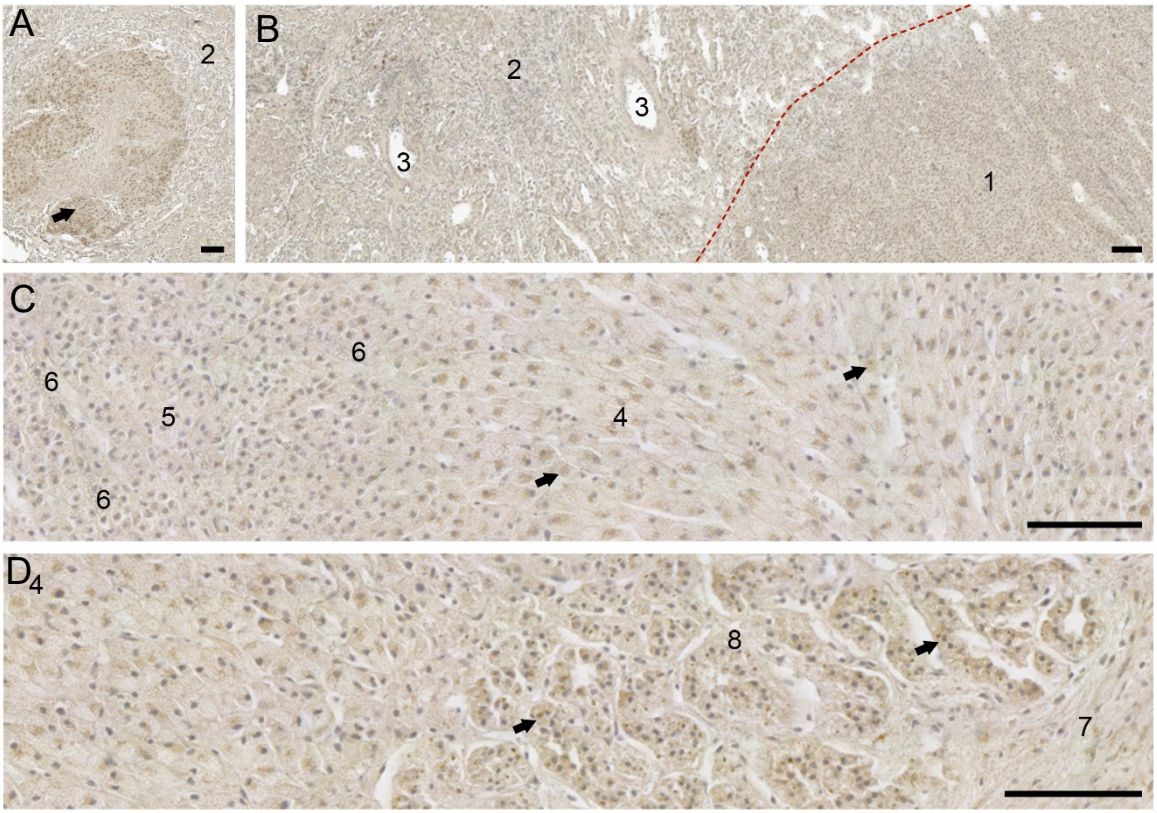

Figure 34 BLG in cow adrenal gland. Although present throughout the adrenal gland tissue, the cortex (1) is stronger stained by αBLG than the medulla (2). Here the zona glomerulosa (8) and the zona fasciculata (4) have the strongest BLG staining in comparison to the third layer – zona reticularis (5). Also, clearly visible in the cortex are the foamy vesicles (6). 3 – blood vessel, 7 – capsula. Red dotted line: border between medulla and cortex; scalebars = 100 µm

4.1.4 Immunohistochemical analysis of BLG in bull testis tissue

Bull testis tissue was stained in the same manner as investigated cow tissues and is described in detail in 3.2.1.

HistoQuest analysis

Originally, the testis was included into the experiments as a male exclusive control organ. Therefore, the detection of BLG in the sections was an intriguing surprise. ROIs and marker colours for HistoQuest analysis were set in the same manner as for the cow tissues (figure 37). HistoQuest analysis of chosen ROIs identified in αBLG stained sample 721 cells, in αOVA 507 cells and in only 2nd antibody 664 cells (haematoxylin positive). In the αBLG stained sample 650 cells were BLG positive (90.15%). The αOVA sample contained 7 cells (1.38%) and only 2nd antibody control 6 out of cells (0.90%) were BLG positive (figure 35).

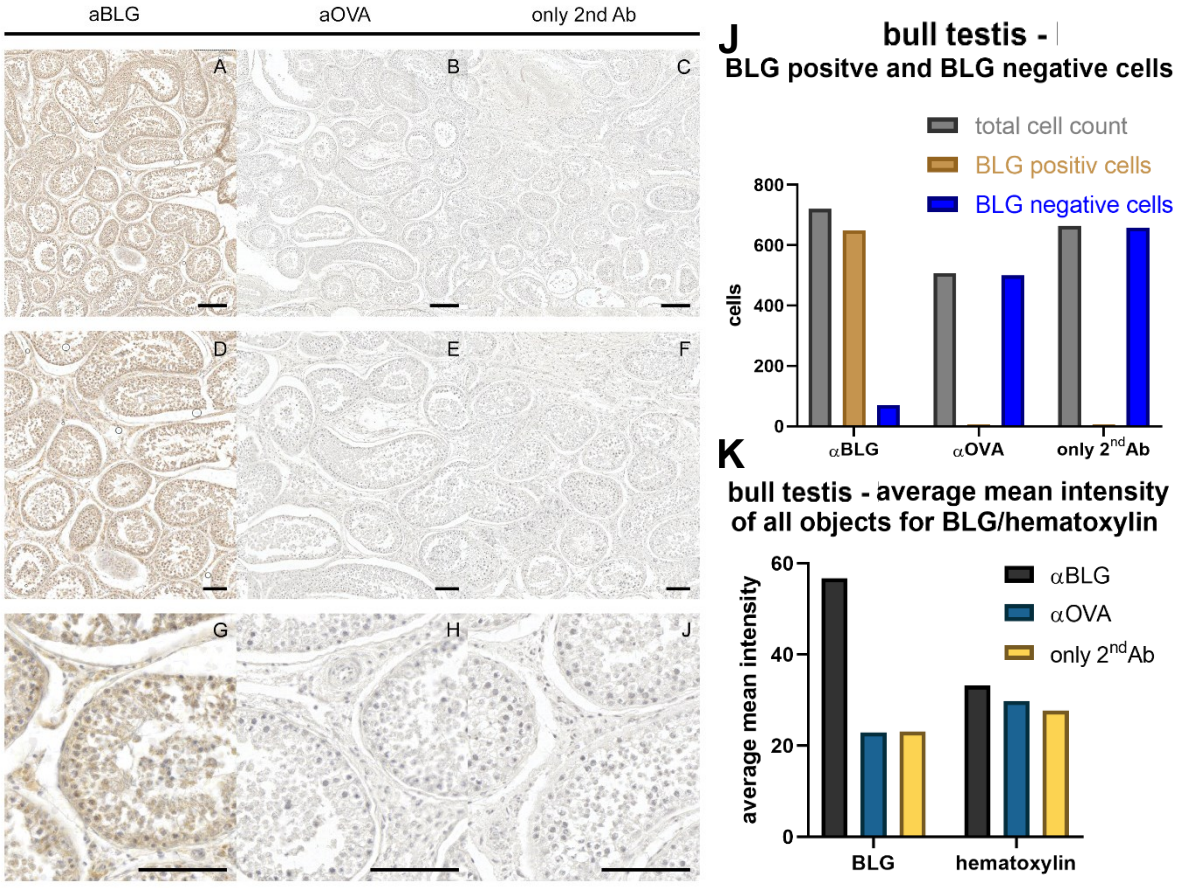

Figure 35 Immunohistochemical analysis of bull testis for BLG. A, D, G: αBLG - DAB (1:1500, 2nd Ab: 1:25000) and Haematoxylin stained cow kidney tissues. B, E, H: αOVA - DAB (1:1500, 2nd Ab: 1:25000) and Haematoxylin stained cow kidney tissues. C, F, J: only 2nd Ab (1:25000) and Haematoxylin stained cow kidney tissues. A-C: scale bar = 200 µm, D-I: scale bar = 100 µm. J: HistoQuest analysis of 0.15 mm² big ROI (region of interest) for BLG positive cells shows that no BLG is present in negative controls (αOVA, only 2nd Ab). K: Average mean intensity measured by HistoQuest for BLG and Haematoxylin staining in per selected ROI. ROI for J, K derives from 1:2000 primary Ab and 1:25000 secondary Ab stained samples.

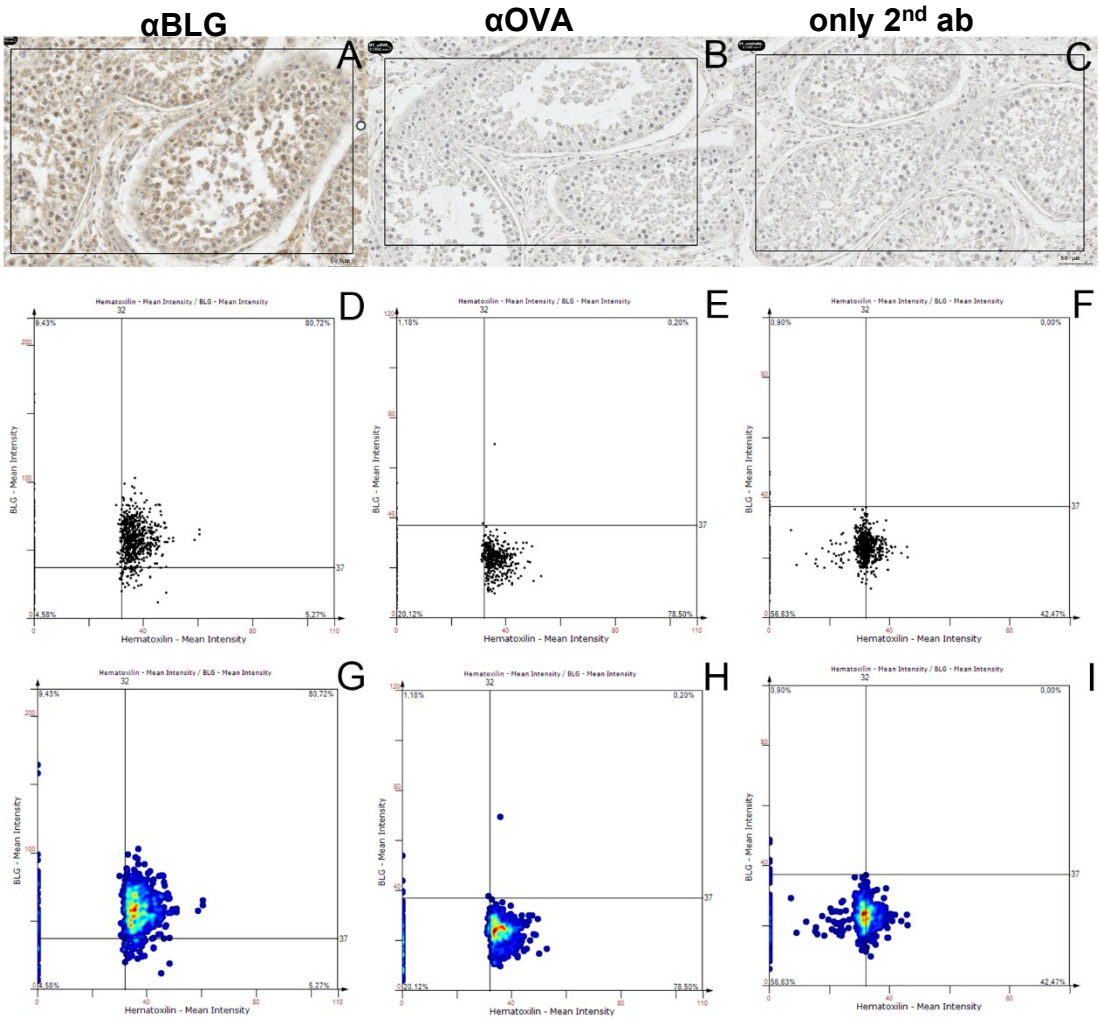

Figure 36 HistoQuest Analysis for BLG and Haematoxylin mean intensity. A – C: marked 0,15mm² big region of interest (ROI) which was analysed by HistoQuest. D – F: Scattergram showing bins according to measured Haematoxylin and BLG mean intensity. G – I: Heatmap of scattergram showing bins according to measured Haematoxylin and BLG mean intensity. D – I: all thresholds were set on the BLG stained scattergram (D, G) in the 25% percentile and applied to both controls (E, F, H, I).

In figure 36 the scattergrams ROIs of the bull testis analysed and generated by HistoQuest are shown. Although HistoQuest identified a small number of positive cells in the negative controls, to the human eye no positive signals can be identified. On the scattergram overlay (figure 38) the negative controls are fairly similar and the αBLG stained sample can be clearly distinguished. The bigger peaks for the only 2nd antibody stained sample are due to more identified cells compared to the αOVA sample.

A Color Separation Parameters

Parameter	Value
Color	
Color RGB	[148, 147, 196]
Is Mixture	No
Intensity	200

B Color Separation Parameters

Parameter	Value
Color	
Color RGB	[193, 154, 66]
Is Mixture	No
Intensity	200

Figure 37 Colour Separation Parameters for bull testis. In A the colour for haematoxylin detection in bull testis is shown. In B the colour for DAB – BLG detection in bull testis is shown.

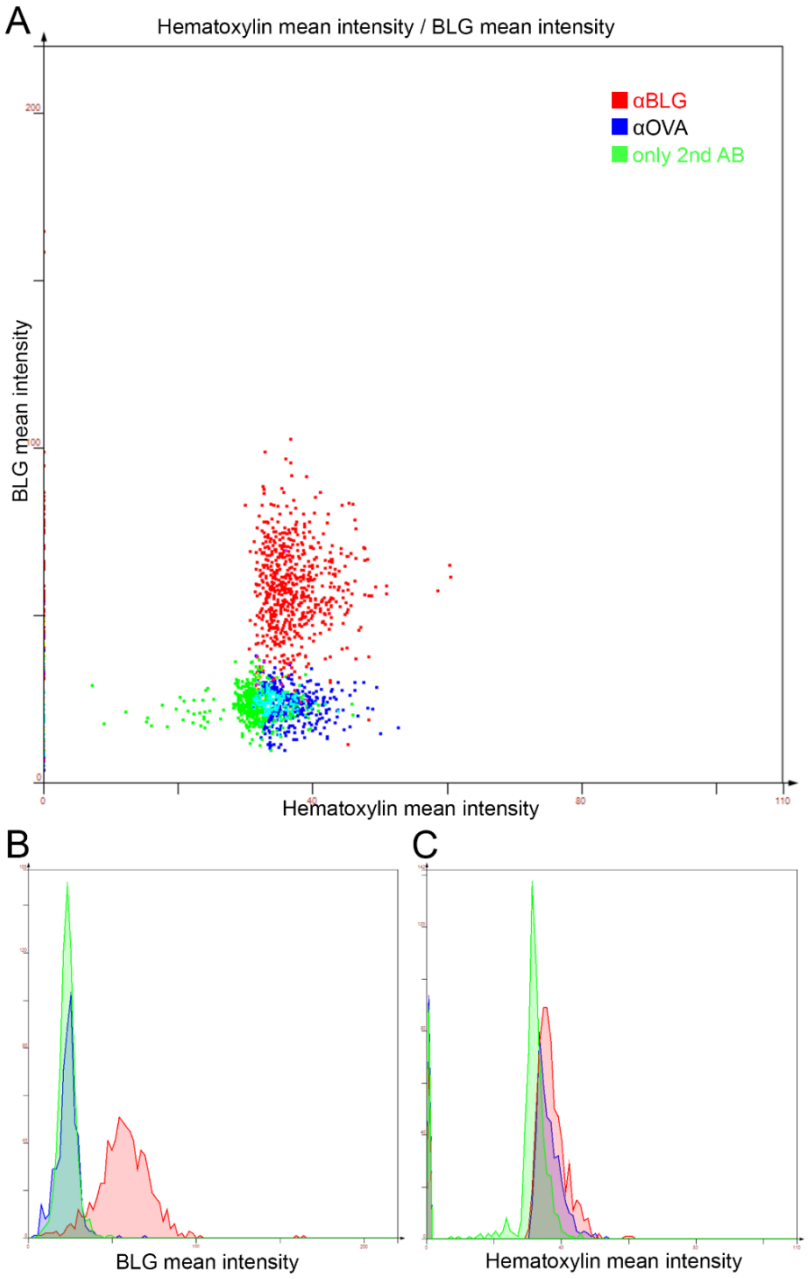

Figure 38 Overlay of measured BLG/Haematoxylin mean intensity in bull testis. In A overlay of all detected bins according to measured BLG and haematoxylin mean intensity is shown for bull testis αBLG, αOVA and only 2nd Ab stained samples. Additionally, in B and C histogram overlays for bull testis αBLG, αOVA and only 2nd Ab stained samples are shown for BLG (B) and haematoxylin (C).

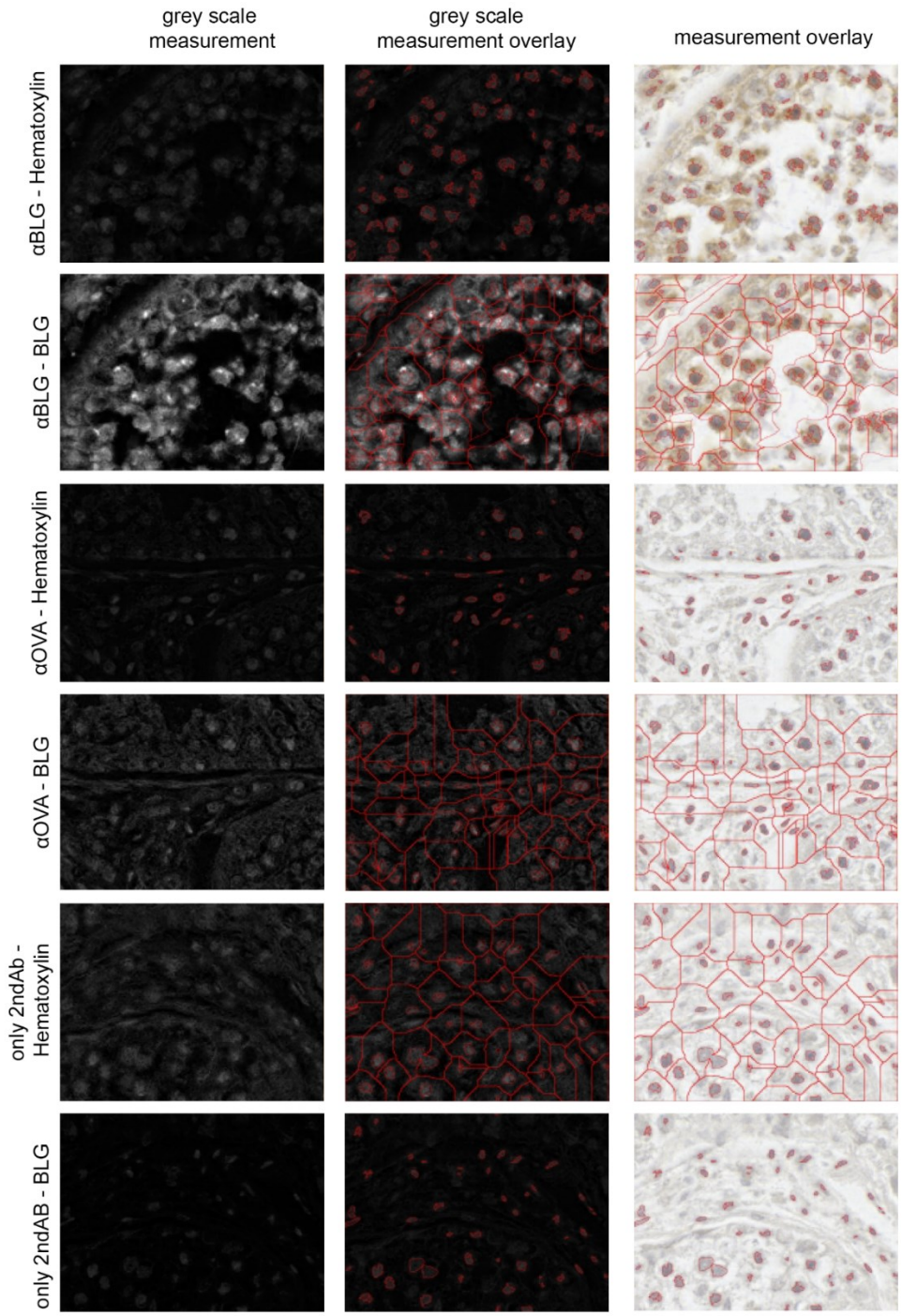

Figure 39 HistoQuest Analysis marker detection and colour separation. In A, D, G, J, M, P grey scale image is shown and light pixels show positive detection. B, C, E, F, H, I, K, L, N, O, Q, R show calculated borders (in red) of either cell or nucleus in grey scale or in colour. A – F: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull testis tissue. G – L: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull testis tissue. M – R: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull testis tissue. A – C, G – I and M – O shows detection of haematoxylin and D – F, J – L and P – Q shows BLG detection

Histological analysis of bull testis tissue

In αBLG stained bovine testis samples, BLG surprisingly is highly present and further confirming that BLG is not unique to the female. In lower magnifications of the testis, BLG seems to be rather equally distributed. Investigating the samples with higher magnifications show that although BLG is slightly present throughout the tissue, there are also stronger signals. This highly positive cells/regions can be found both in the tubuli seminiferi contorti (figure 40 -1). While the lamina propria (figure 40 -3) is only slightly stained, the lumen of primary spermatocytes (figure 40 -2) has higher levels of BLG. Moreover, also cells with higher BLG levels can be found in the interstitial tissue (figure 40 -5) surrounding the tubuli seminiferi contorti (figure 40 -1). This is of high interest as the interstitial tissue, as already described in the introduction, contains the Leydig cells, which are responsible for the production of several androgen hormones.

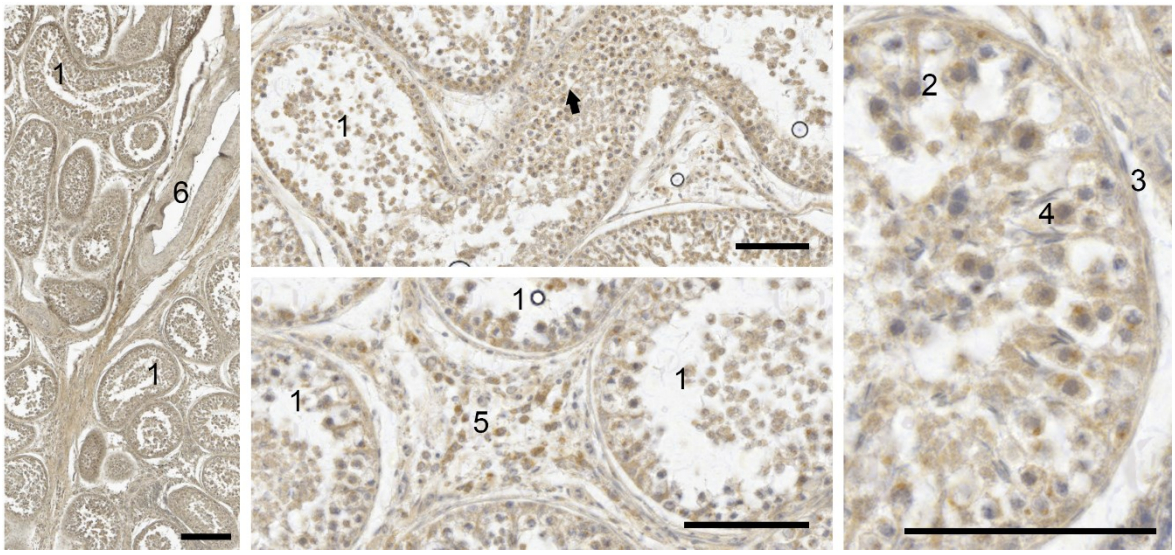

Figure 40 BLG in bovine testis. BLG is present in the bovine testis. It can be found in the cells within the tubuli seminiferi contorti (1) containing the primary spermatogonia (2) and spermatocytes (4) and within certain cells within the interstitial tissue (5). 3 – lamina propria, 6 – blood vessel.

4.1.5 Immunohistochemical analysis of BLG in bull kidney tissue

Immunohistochemical staining of bull kidney tissue was conducted in the same manner as in previous shown tissue sections and is described in detail in 3.2.1.

HistoQuest analysis

Since our working group already found BLG in the urine of bulls, the detection of BLG by immunohistochemistry in the bull kidney was not surprising. Again, for HistoQuest analysis used ROIs and marker colours were set in the same manner as for the cow tissues. The analysis of chosen ROIs identified 739 cells in αBLG stained sample, 550 cells in αOVA and 740 cells in only 2nd antibody (haematoxylin positive). In the αBLG stained sample 550 cells were BLG positive (74.42%). The αOVA sample contained 1 (0.18%) and only 2nd antibody control 0 cells were BLG positive (figure 41). The percentage of BLG positive cells in the bull kidney is therefore comparable to that of cow kidney (~75%).

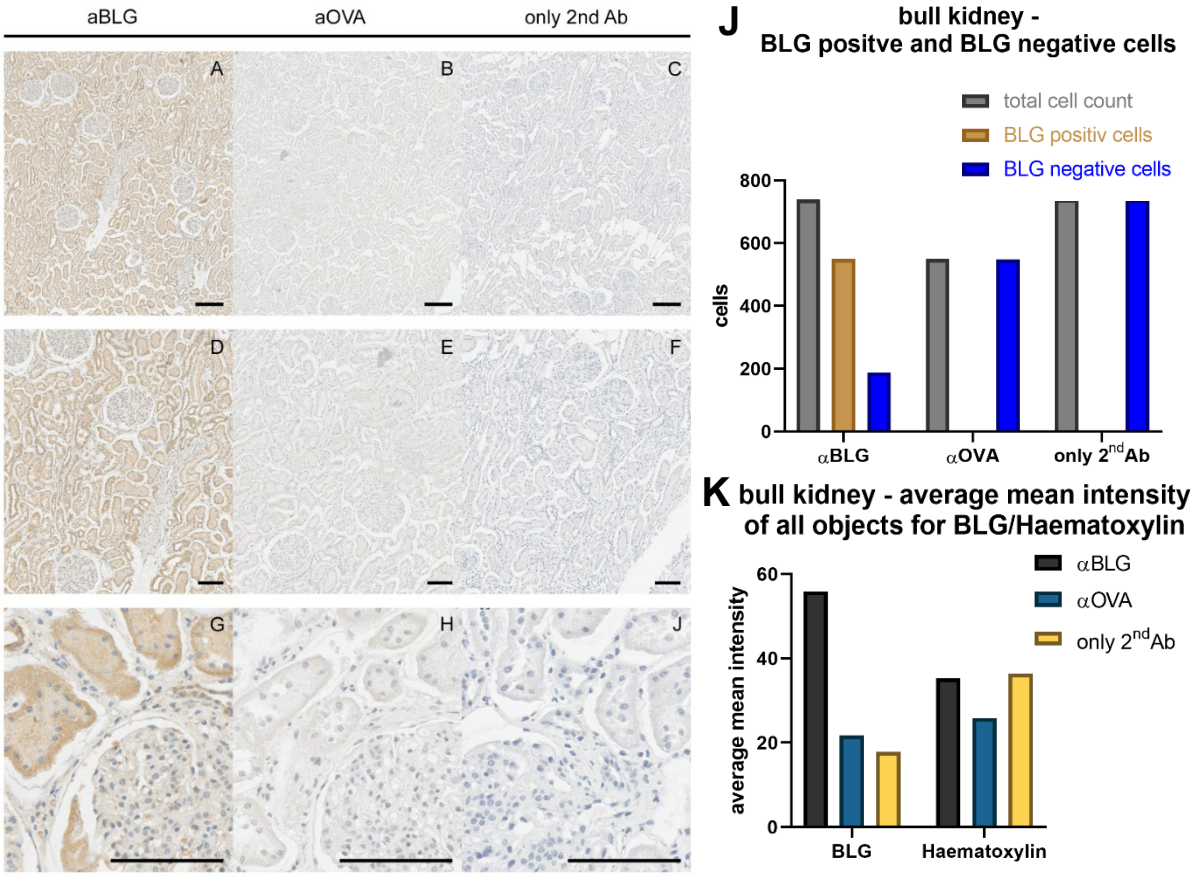

Figure 41 Immunohistochemical analysis of bull kidney for BLG. A, D, G: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained cow kidney tissues. B, E, H: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and Haematoxylin stained bull kidney tissues. C, F, J: only 2nd Ab (1:25000) and Haematoxylin stained bull kidney tissues. A-C: scale bar = 200 µm, D-I: scale bar = 100 µm. J: HistoQuest analysis of 0.15 mm² big ROI (region of interest) for BLG positive cells shows that no BLG is present in negative controls (αOVA, only 2nd Ab). K: Average mean intensity measured by HistoQuest for BLG and haematoxylin staining in per selected ROI.

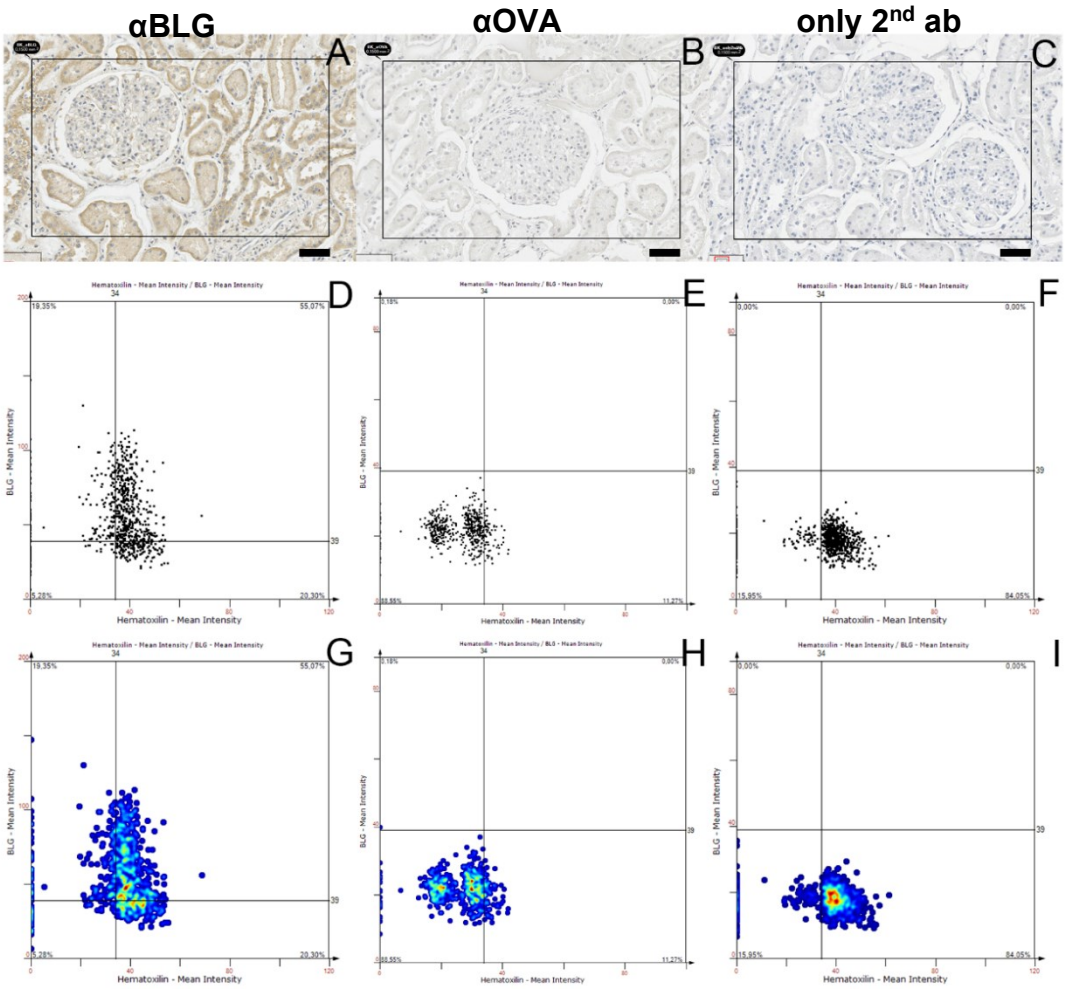

Figure 42 HistoQuest Analysis for BLG and Haematoxylin mean intensity. A – C: marked 0,15mm² big region of interest (ROI) which was analysed by HistoQuest. D – F: Scattergram showing bins according to measured haematoxylin and BLG mean intensity. G – I: Heatmap of scattergram showing bins according to measured haematoxylin and BLG mean intensity. D – I: all thresholds were set on the BLG stained scattergram (D, G) in the 25% percentile and applied to both controls (E, F, H, I).

In figure 42 besides the chosen ROIs, the obtained scattergrams are shown. No BLG positive signal detectable in the negative controls. These scattergrams show the distribution of measured events according to the measured mean intensity of detected markers with applied cut of values. In the αBLG stained sample most cells are both BLG and haematoxylin positive. In the negative controls only in the αOVA sample one event is above the BLG cut-off and no event exceeds the BLG threshold in the only 2nd antibody control. Although treated equally, haematoxylin staining of αOVA turned out weaker as in the other tissue samples. In combination with the manual correction this led to two peaks in haematoxylin mean intensity and consequently two populations as seen in figure 42-H. In the overlay of all three scattergrams (figure 44) the negative controls had comparable low BLG mean intensities but different haematoxylin values. Bins derived from the αBLG stained sample are clearly distinguishable from the populations of the negative controls.

A Color Separation Parameters

Parameter	Value
Color	
Color RGB	[148, 164, 212]
Is Mixture	No
Intensity	200

B Color Separation Parameters

Parameter	Value
Color	
Color RGB	[218, 196, 115]
Is Mixture	No
Intensity	200

Figure 44 Colour Separation Parameters for bull kidney. In A the colour for haematoxylin detection in bull kidney is shown. In B the colour for DAB – BLG detection in bull kidney is shown.

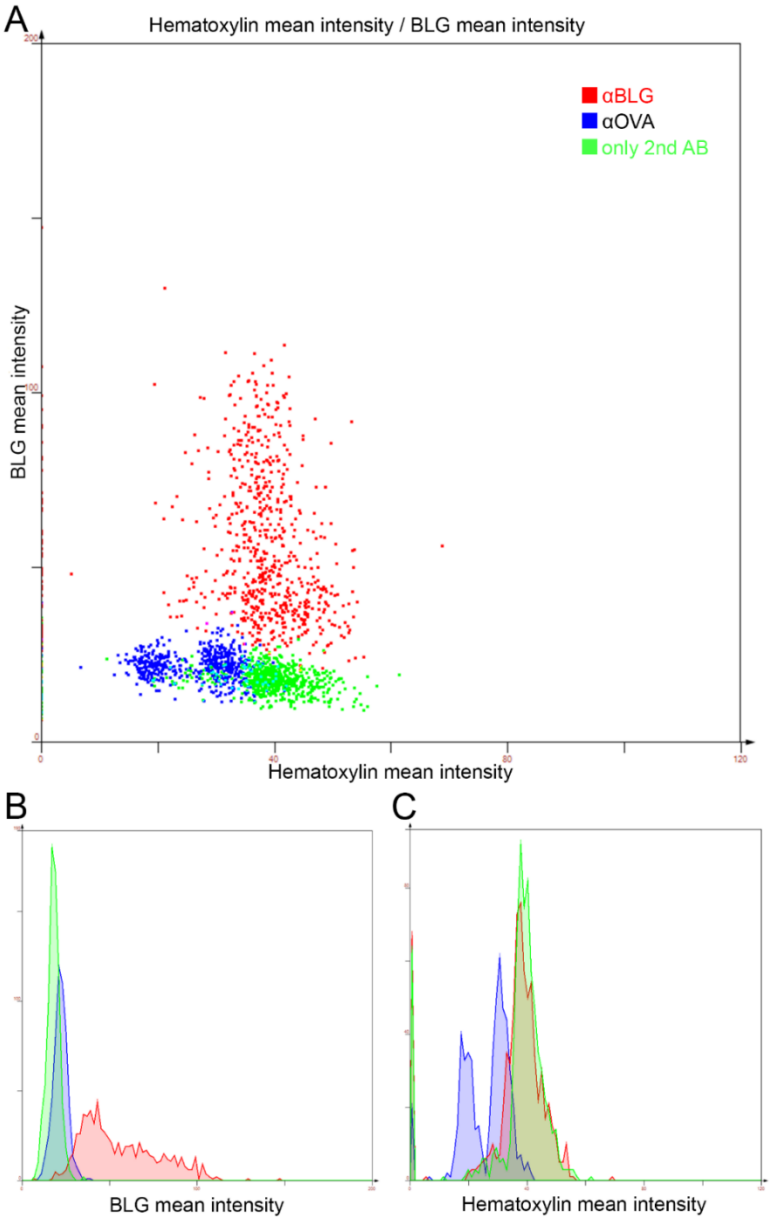

Figure 43 Overlay of measured BLG/Haematoxylin mean intensity in bull kidney. In A overlay of all detected bins according to measured BLG and haematoxylin mean intensity is shown for bull kidney αBLG, αOVA and only 2nd Ab stained samples. Additionally, in B and C histogram overlays for bull kidney αBLG, αOVA and only 2nd Ab stained samples are shown for BLG (B) and haematoxylin (C).

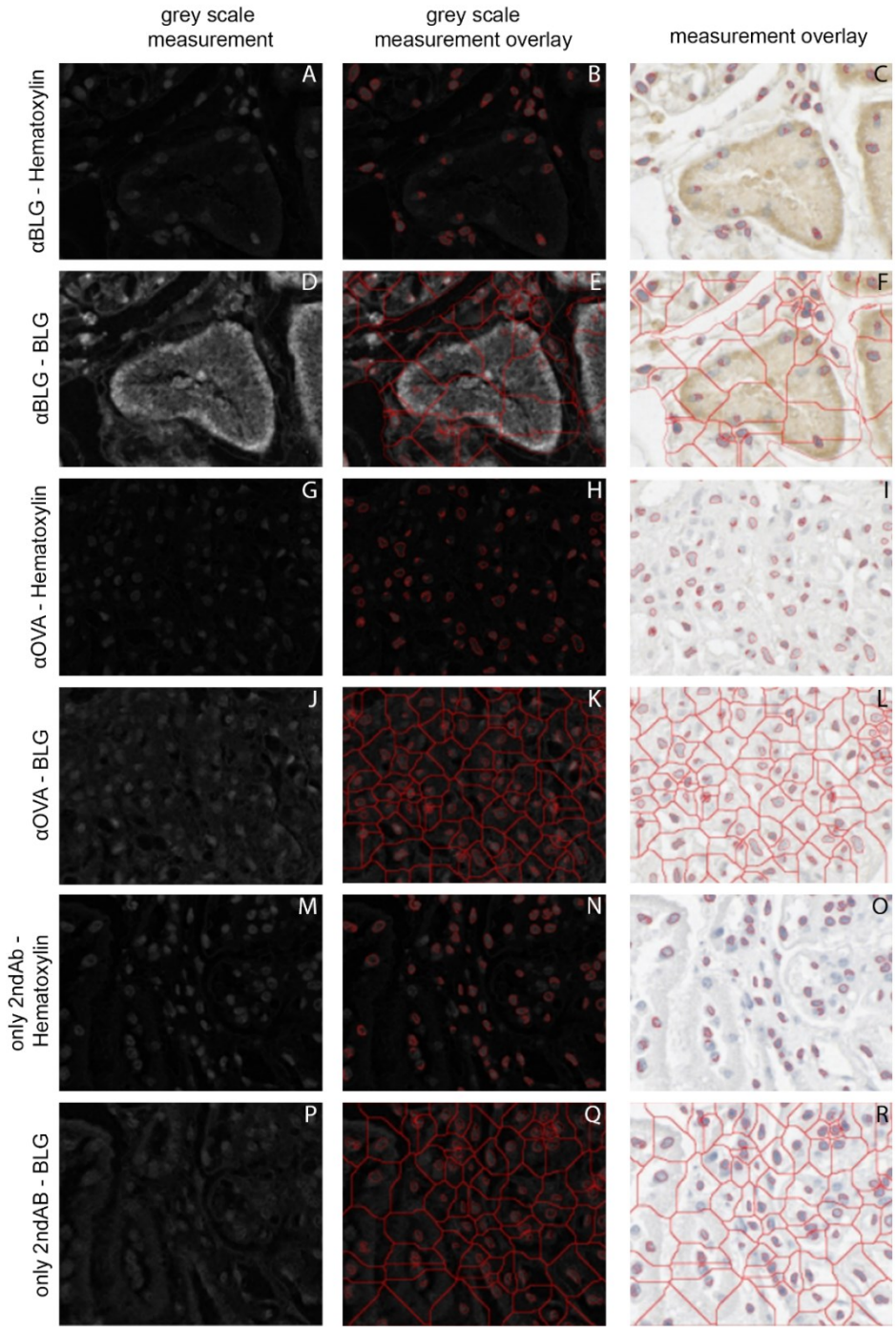

Figure 45 HistoQuest Analysis marker detection and colour separation. In A, D, G, J, M, P grey scale image is shown and light pixels show positive detection. B, C, E, F, H, I, K, L, N, O, Q, R show calculated borders (in red) of either cell or nucleus in grey scale or in colour. A – F: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull kidney tissue. G – L: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull kidney tissue. M – R: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull kidney tissue. A – C, G – I and M – O shows detection of haematoxylin and D – F, J – L and P – Q shows BLG detection.

Histological analysis of bull kidney tissue

BLG seems to be most present in the tubular parts of the nephrons. Since the cortex, which contains the glomeruli, is stained more intensively than the hardly stained collecting ducts (figure 46 - C), it indicates that BLG is at least partially reabsorbed. Also, the blood vessel found in the presented section are hardly stained in comparison to the tubuli of the nephrons. Comparable to the cow kidney, again small strongly stained BLG aggregates are present in the glomerulus.

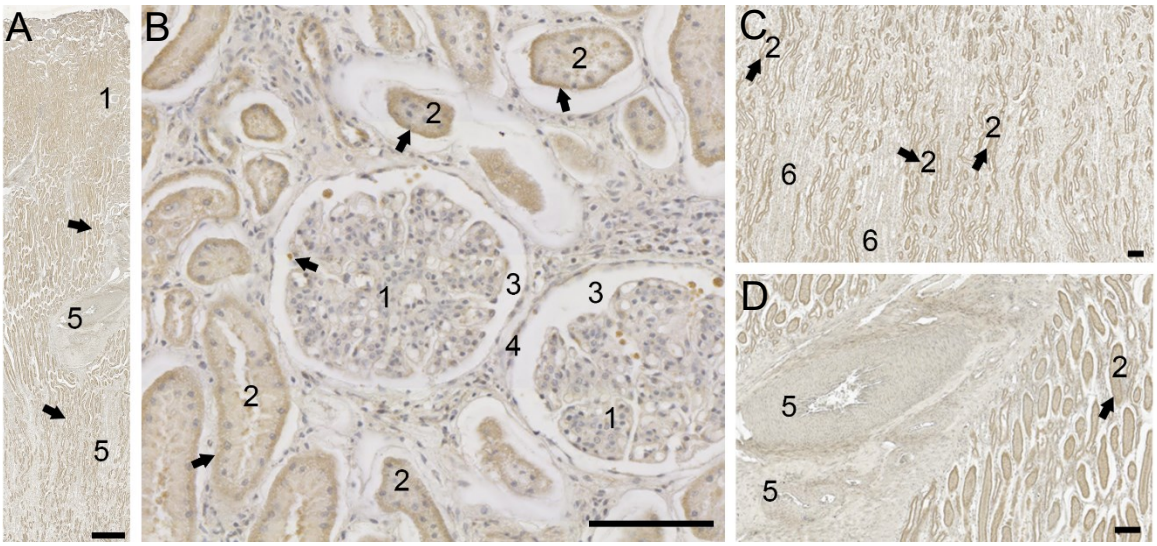

Figure 46 BLG in bull kidney. BLG seems to be present throughout the male kidney. In A a picture of the cortex leading down into the medulla is shown. In B several typical structures for the kidney cortex are shown such as the glomerulus (1) and the tubular parts of the nephron (2). Picture C shows the medulla right below the kidney cortex. No glomeruli can be found there, but the tubular parts of the nephrons (2) leads deep into the medulla together with collective tubes (6) which gather the urine. As already seen in the female kidney sample, the tubular structures of the nephron are stained the strongest (black arrows). C and D show respective structures in different regions of the acquisition. 3 – capsule space, 4 – inner layer of the Bowman´s capsule, 5 – blood vessel. Scalebars = 100 µm

4.1.6 Immunohistochemical analysis of BLG in bull adrenal gland tissue

Bull adrenal gland tissue was stained as all other here presented organs according to 3.2.1.

HistoQuest analysis

Comparably to the cow adrenal gland, BLG could be detected via immunohistochemistry also in the bull adrenal gland. For the HistoQuest analysis again ROI (0.15 mm², 63x acquisition) for all three samples as well as the marker colours (figure 49) were set as in all other investigated organs. The HistoQuest analysis revealed in the αBLG stained sample 562 cells, in αOVA 532 cells and in only the 2nd antibody 563 cells (haematoxylin positive). In the αBLG 422 cells BLG positive equalling 75.09% of all counted cells. No BLG positive events were detected by HistoQuest for the negative controls. These findings are shown in figure 47.

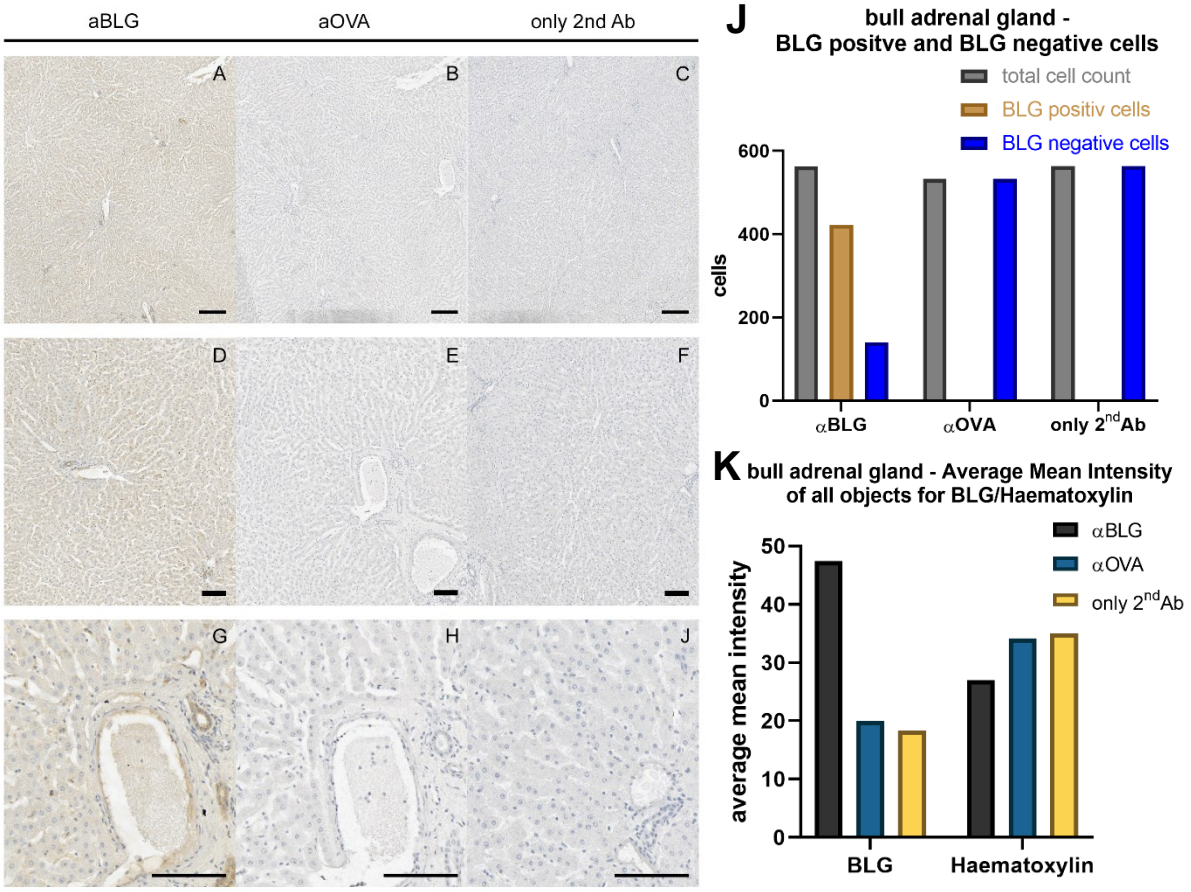

Figure 47 Immunohistochemical analysis of bull adrenal gland for BLG. A, D, G: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and Haematoxylin stained bull adrenal gland tissues. B, E, H: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and Haematoxylin stained bull adrenal gland tissues. C, F, J: only 2nd Ab (1:25000) and Haematoxylin stained bull adrenal gland tissues. A-C: scale bar = 200 µm, D-I: scale bar = 100 µm. J: HistoQuest analysis of 0.15 mm² big ROI (region of interest) for BLG positive cells shows that no BLG is present in negative controls (αOVA, only 2nd Ab). K: Average mean intensity measured by HistoQuest for BLG and Haematoxylin staining in per selected ROI.

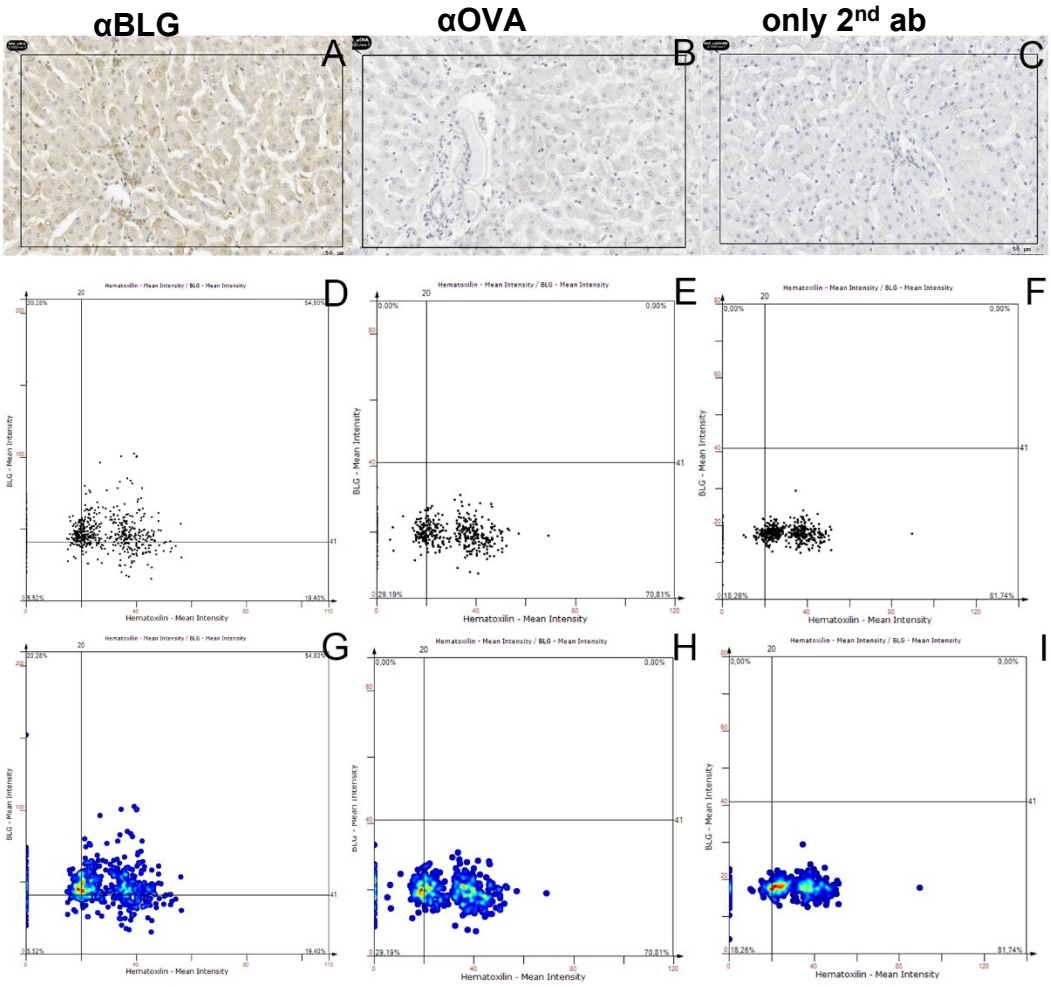

Figure 48 HistoQuest Analysis for BLG and Haematoxylin mean intensity. A – C: marked 0.15mm² big region of interest (ROI) which was analysed by HistoQuest. D – F: Scattergram showing bins according to measured Haematoxylin and BLG mean intensity. G – I: Heatmap of scattergram showing bins according to measured Haematoxylin and BLG mean intensity. D – I: all thresholds were set on the BLG stained scattergram (D, G) in the 25% percentile and applied to both controls (E, F, H, I).

In figure 48 the chosen ROI with the corresponding scattergrams are shown. In all scattergrams two populations are present which are a result of a less intense haematoxylin staining and applied manual correction. The less intense haematoxylin as well as inhomogeneous staining of the nuclei are a result of less dense chromatin of the non-dividing cells. Nevertheless, as shown in figure 50 -A the αBLG stained sample is clearly distinguishable from both negative controls which are overlapping. Moreover, the overall mean intensities of haematoxylin are comparable between the sample and its controls. In the here presented data, clearly a BLG positive population could be measured by the HistoQuest program for bull adrenal gland tissue. Also looking at the obtained acquisitions no positive staining was present in the negative controls, while BLG positive staining was clearly visible in the αBLG stained sample of the bull adrenal gland.

A Color Separation Parameters

Parameter	Value
Color	
Color RGB	[146, 158, 202]
Is Mixture	No
Intensity	200

B Color Separation Parameters

Parameter	Value
Color	
Color RGB	[220, 201, 133]
Is Mixture	No
Intensity	200

Figure 49 Colour Separation Parameters for bull adrenal gland. In A the colour for haematoxylin detection in bull adrenal gland is shown. In B the colour for DAB – BLG detection in bull adrenal gland is shown.

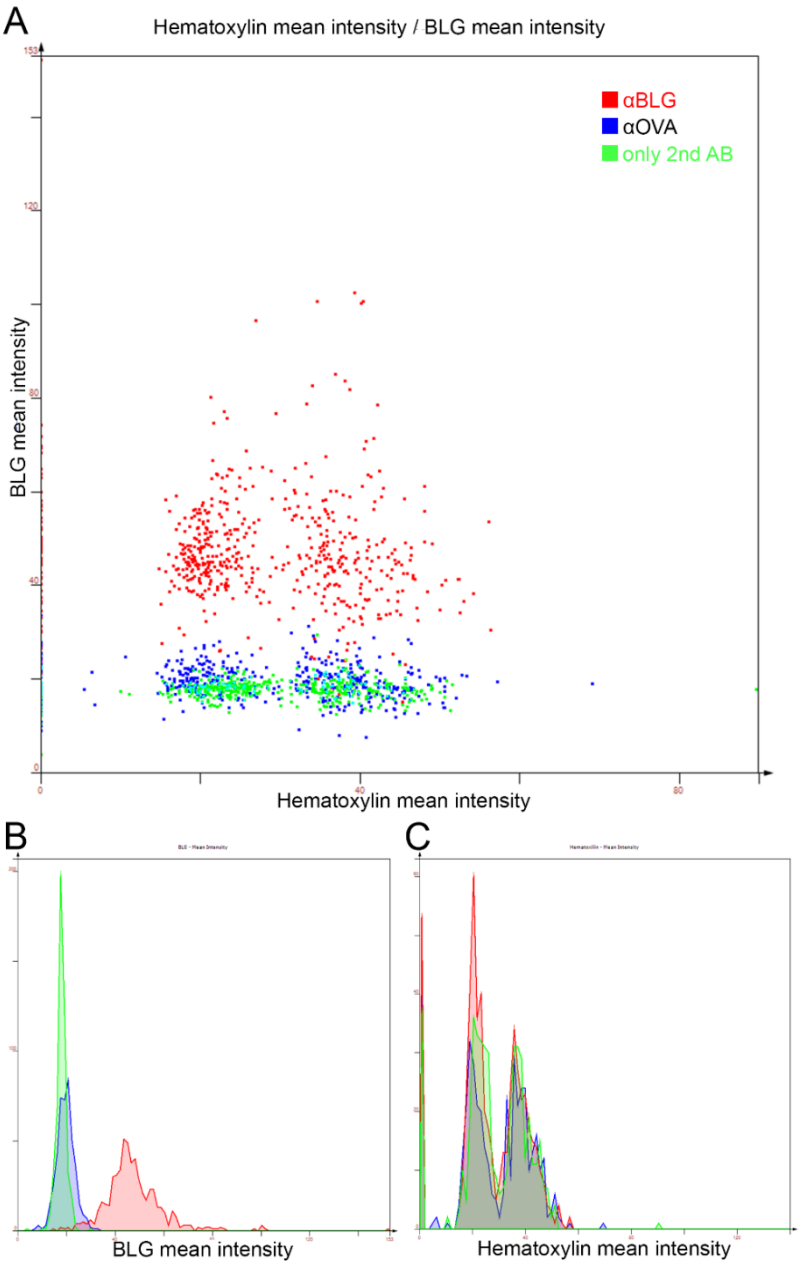

Figure 50 Overlay of measured BLG/Haematoxylin mean intensity in bull adrenal gland. In A overlay of all detected bins according to measured BLG and haematoxylin mean intensity is shown for bull adrenal gland αBLG, αOVA and only 2nd Ab stained samples. Additionally, in B and C histogram overlays for bull adrenal gland αBLG, αOVA and only 2nd Ab stained samples are shown for BLG (B) and haematoxylin (C).

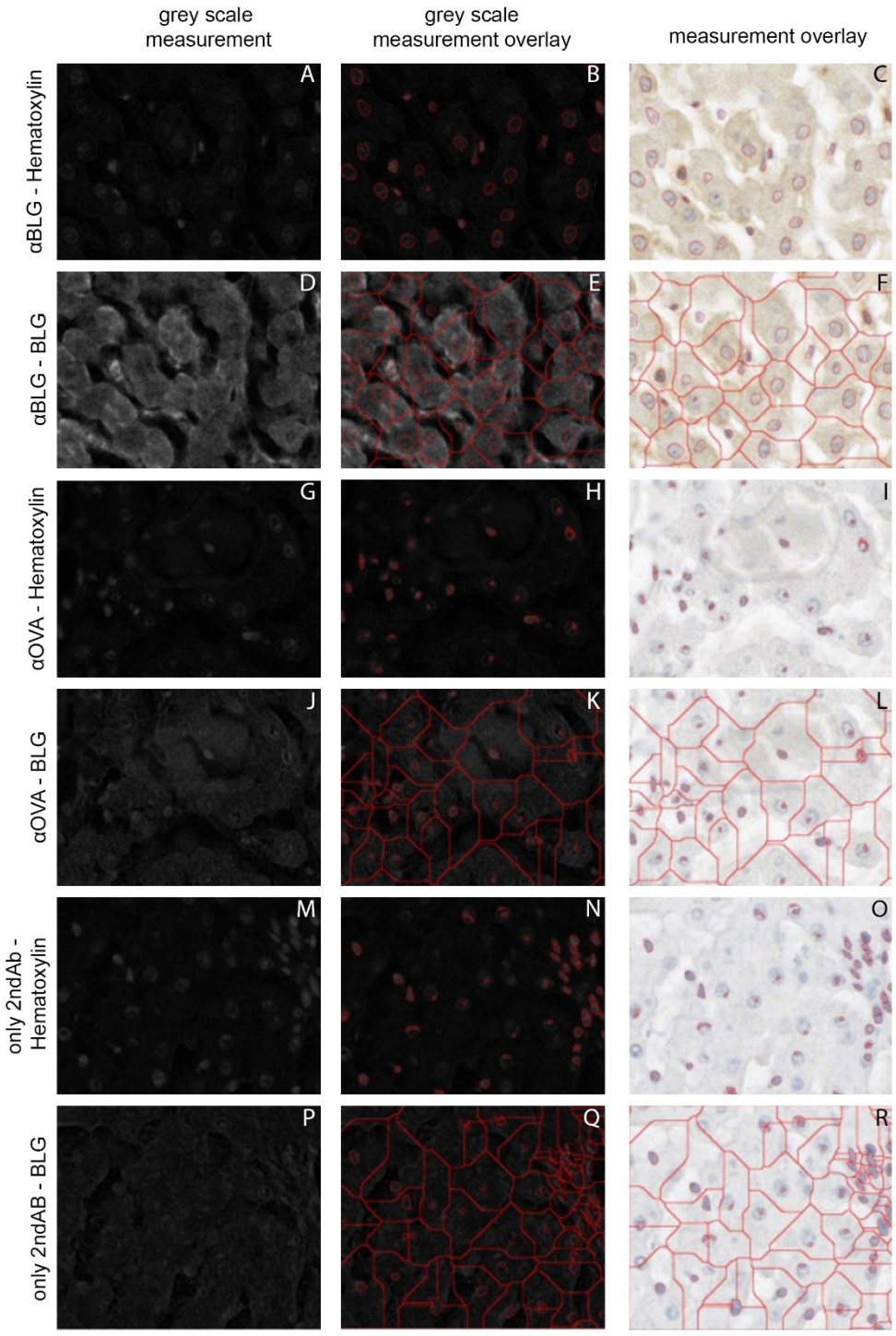

Figure 51 HistoQuest Analysis marker detection and colour separation. In A, D, G, J, M, P grey scale image is shown and light pixels show positive detection. B, C, E, F, H, I, K, L, N, O, Q, R show calculated borders (in red) of either cell or nucleus in grey scale or in colour. A – F: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull adrenal gland tissue. G – L: αOVA - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull adrenal gland tissue. M – R: αBLG - DAB (1:2000, 2nd Ab: 1:25000) and haematoxylin stained bull adrenal gland tissue. A – C, G – I and M – O shows detection of haematoxylin and D – F, J – L and P – Q shows BLG detection.

Histological analysis of bull adrenal gland tissue

The αBLG stained bull adrenal gland sample contains mostly medulla tissue (figure 52 -1) which stained homogenously. There are no special aggregations of BLG within the cells, in contrast to the cortex tissue of the cow adrenal gland. The connective tissue (figure 52 -2) engulfing a duct of the adrenal gland is less stained than the medulla tissue (figure 52 -1). Interestingly the epithelial cells covering certain vessels (figure 52 -3) have a higher BLG content (black arrows).

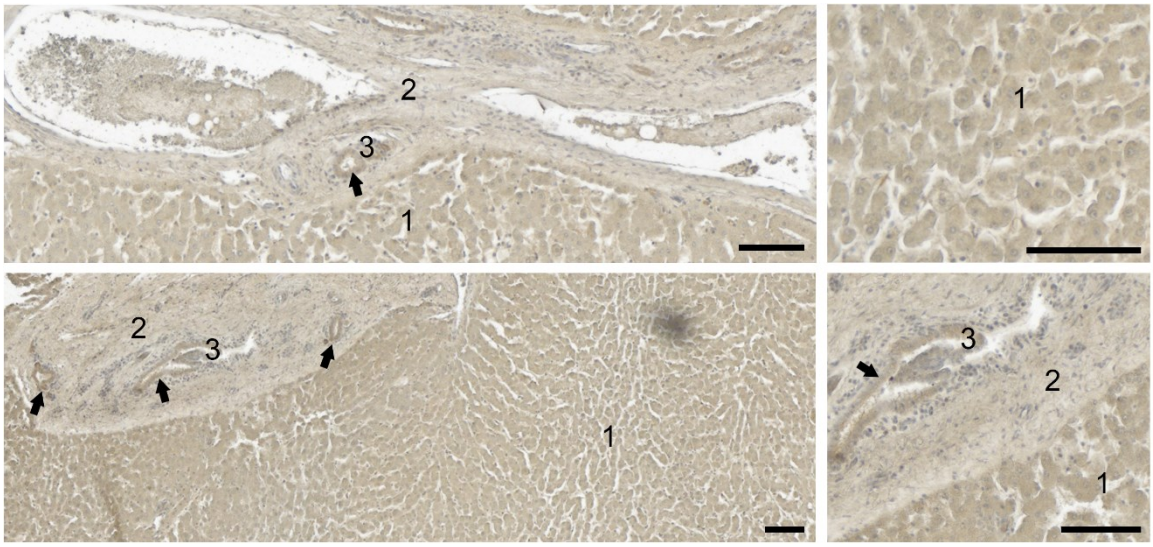

Figure 52 BLG in bull adrenal gland. BLG is present throughout the adrenal gland tissue of the medulla (1), yet the connective tissue surrounding a bigger duct (2) is less positive. Epithelial cells (black arrows) of some vessels (3) also have a higher BLG content. Scalebars = 100 µm

4.2. Assessment of BLG content in cattle tissue via protein extracts

Although the histochemical analysis has shown that BLG is present in all tested organs, we decided to additionally verify our findings by application of other methods. To do so on protein level, protein extracts were produced (described in 3.2.2). The total protein content of these extracts was determined via NanoDrop (table 9) and extracts were tested by western blot and ELISA. Former method was conducted to verify correct BLG detection by the IHC-antibody and latter was conducted to quantify present BLG.

Table 8 Mean protein concentration of protein extracts. Before application to ELISA or western blot, protein extracts were measured for their total protein content in mg/ml by applying 1 µl to NanoDrop machine.

Total protein content of produced protein extract in mg/ml					
CU	CK	BT	BK	BAd	CiL
71.2	116	81.7	81.3	29.6	116

4.2.1 Western blot

Western blot was performed with protein extracts from cow udder, cow kidney, bull testis, bull kidney and bull adrenal gland. Unfortunately, no additional cow adrenal gland could be obtained. Figure 53 shows that the western blots results are in accordance with our IHC findings. BLG could be primarily detected in its monomeric state at the same height as control BLG (10 ng / 20 µl) which produced a lane at the height of approximately 18 kDA as described in literature.

Unsurprisingly strongest signals were obtained from the udder sample as well as generally in the female tissue. While the protein extracts derived from the female individual delivered stronger signals, the protein was also clearly present in all male tested tissues. Also, the signal strength decreased with higher dilutions ([1:2], [1:5], [1:10], [1:25], [1:50], [1:75]). Additionally, no signal was emitted by both negative controls, ovalbumin (10 ng / 20 µl) and protein extract derived from chicken liver (1:25 from original sample). Less diluted samples did not completely enter the SDS-Page during gel electrophoresis. This resulted in higher and/or smeared bands.

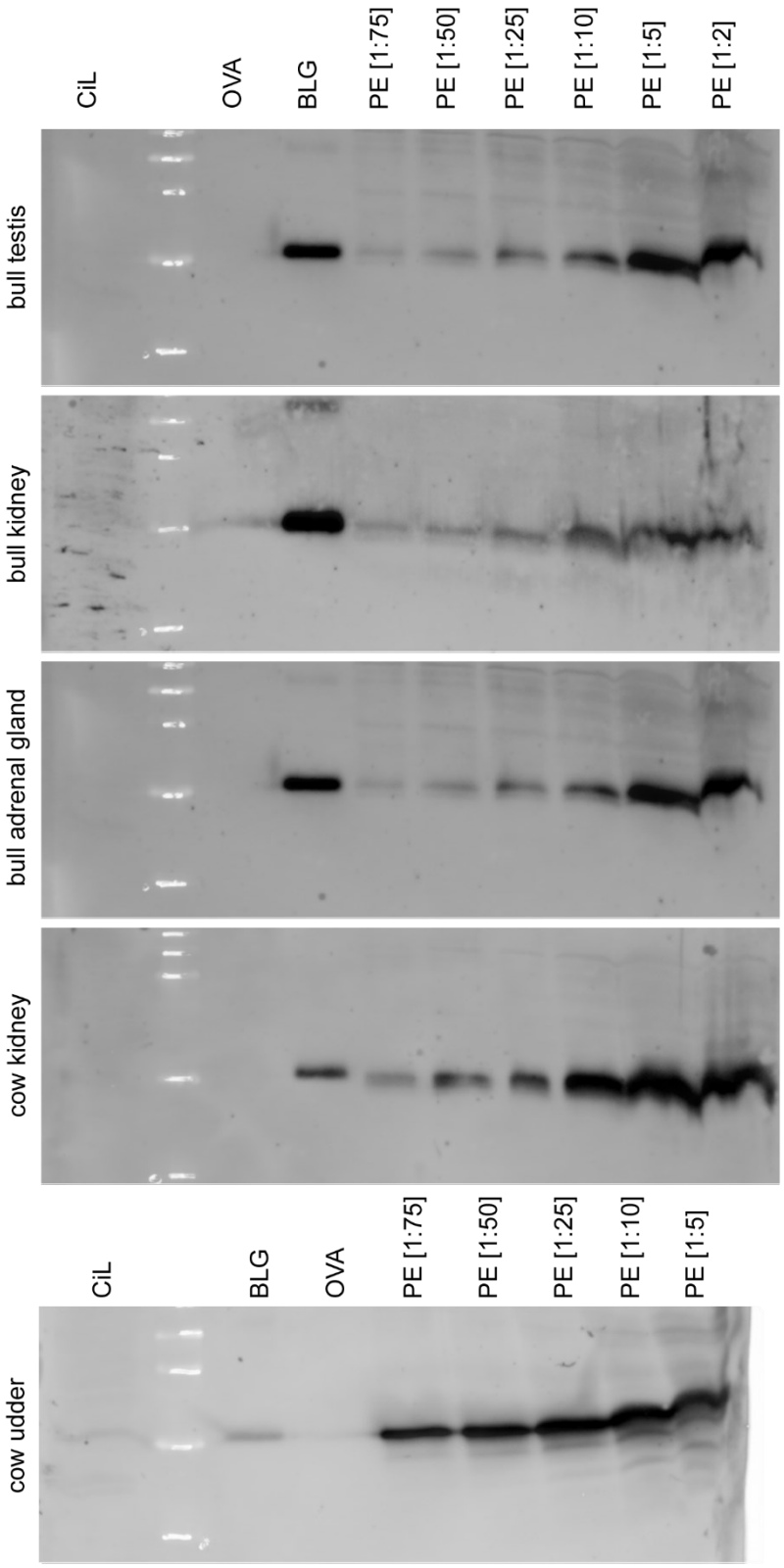

Figure 53 Western blot of protein extracts. Different dilution of produced protein extracts (PE) analysed via western blot reveal BLG presence in all tested bovine samples but not in the ovine controls (OVA – ovalbumin, CiL – chicken liver). Higher protein concentration travelled slower through the SDS-Page resulting in slightly higher bands and smears. BLG and OVA were applied with 10 ng/20 µl. CiL was diluted 1:25 from original protein extract.

4.2.2 ELISA

Since in all bovine protein extracts BLG could be detected, quantitative BLG amount was assessed with a BLG-specific ELISA kit (Bethyl Laboratories Inc., Cat. No. E10-125). The obtained BLG amount was then divided by the total protein content of the respective protein extract. This delivered the amount of BLG in ng per mg total protein content. As no BLG could be detected by western blot in chicken liver protein extracts, obtained values in ELISA from this sample were subtracted from all other tested samples as background. The test was repeated 4 times and the average of the results is presented in figure 54.

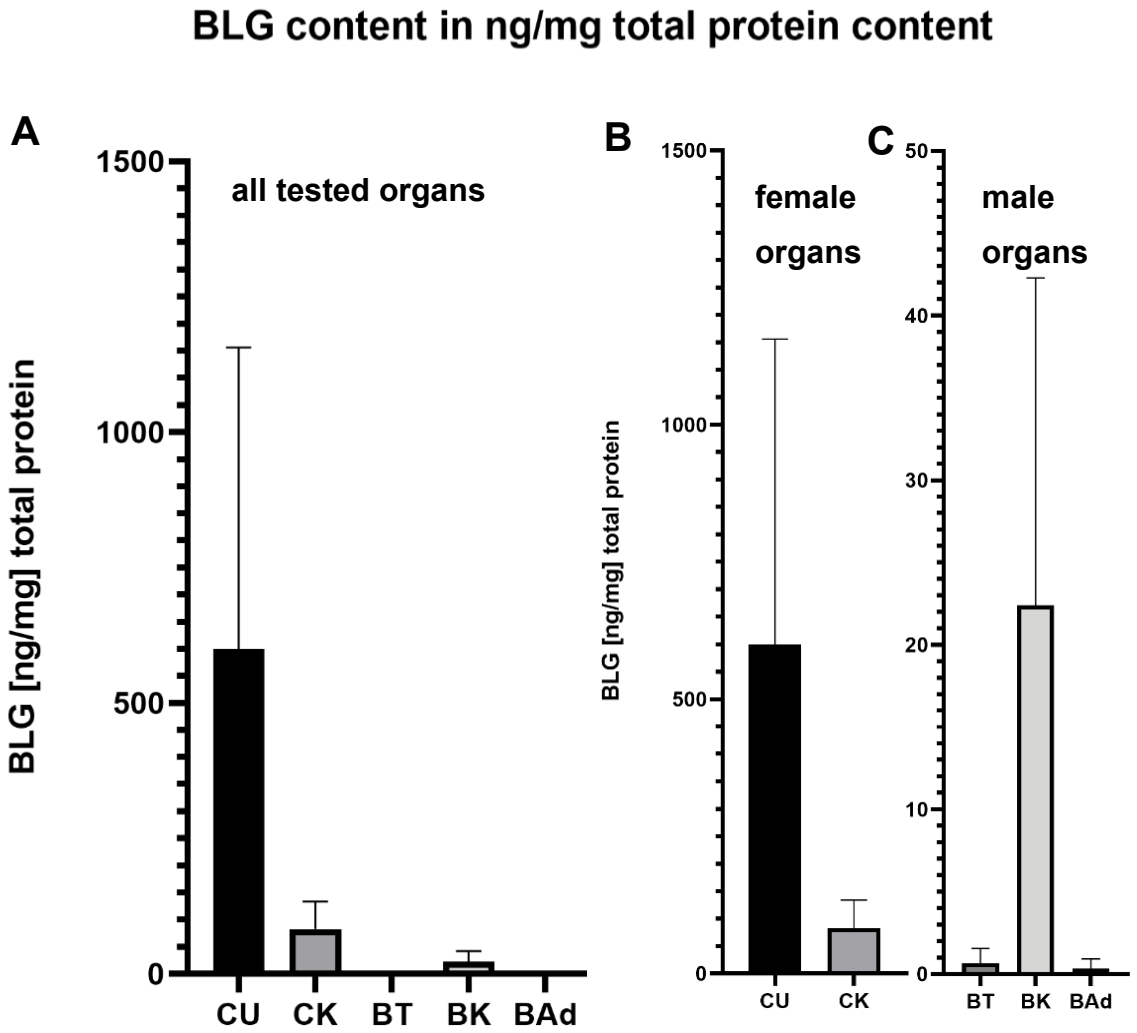

Figure 54 BLG content found in protein extracts by ELISA. A – comparison of all tested protein extracts in one graph shows that BLG is most present in protein extracts derived from female organs, especially cow udder. B – comparison of female organs BLG content. C - comparison of male organs BLG content shows that the protein is most abundant in the kidney and present in small amounts in both adrenal gland and testis. For all presented results chicken liver results were subtracted as background. CU – cow udder, CK – cow kidney, BT- bull testis, BK – bull kidney, BAd – bull adrenal gland.

According to obtained ELISA results (table 10), again BLG is most present in the cow udder and kidney. In the bull samples, BLG is most abundant in the kidney followed by testis and adrenal gland. In the two tested individuals the bull had about one third of BLG compared to the female individual´s kidney. Interestingly bull adrenal gland and bull testis had similar amount of BLG, although low compared to BLG present in kidneys of both individuals.

Table 9 Exact detected BLG values in tested protein extracts. This tables shows the mean of all BLG concentration values for tested bovine organs both pre- and post-subtraction of chicken liver background.

Ø total protein content of protein extracts [mg/ml]					
cow udder	cow kidney	bull testis	bull kidney	bull adrenal gland	chicken liver
49,7	73,1	46,65	52,05	27,9	196,3
Ø BLG content per total protein content of protein extracts [ng/mg] without background					
cow udder	cow kidney	bull testis	bull kidney	bull adrenal gland	chicken liver
599,61	82,83	0,84	22,42	0,54	0
Ø BLG content per total protein content of protein extracts [ng/mg] with background					
cow udder	cow kidney	bull testis	bull kidney	bull adrenal gland	chicken liver
599,85	83,07	1,09	22,66	0,78	0,24

4.2.3 Analysis of cow blood

As ELISA as well as IHC and western blot results show BLG is highly present in the bovine kidney we wondered whether these elevated concentrations are a result of elevated expression or due to the filtration of BLG in the blood. Therefore, BLG could be transported to the kidney via blood and the kidney might not fully reabsorb the protein resulting in BLG presence in the urine.

Although testing blood for BLG presence does not exclude possible BLG expression in kidney tissue, investigation of BLG in bovine blood is an interesting research question. We could obtain blood from one euthanized cow. Both serum and plasma were collected and a 1:20 dilution of the sample was tested by the already established ELISA. Again, the total protein concentration of blood serum and plasma were determined via the NanoDrop. Obtained results are presented in figure 55 and show that BLG is present in the blood. Moreover, there is no significant difference between BLG content of blood serum or blood plasma, however blood plasma yielded more constant values with used ELISA-kit.

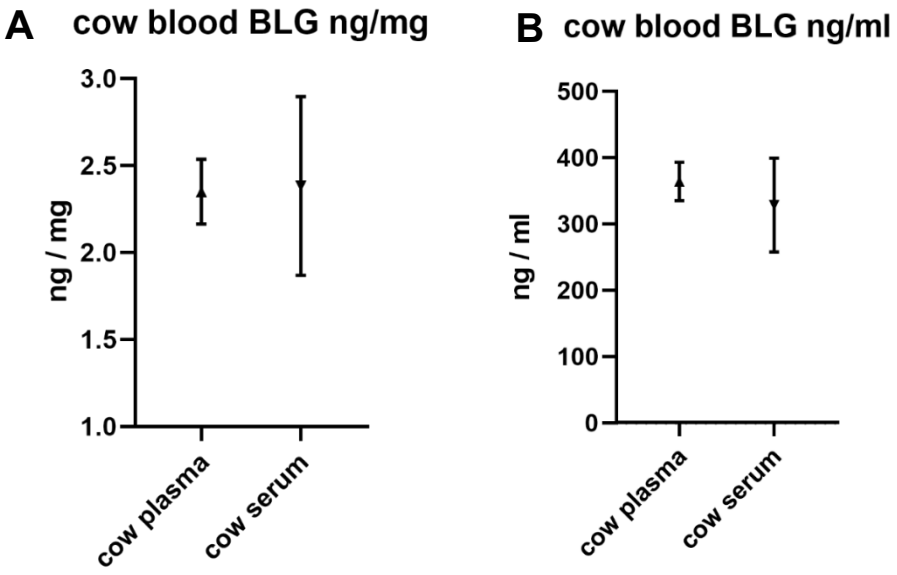

Figure 55 BLG content of cow blood serum and plasma. A – BLG content of cow blood serum and plasma in ng per total protein content in mg. B – BLG content of cow blood serum and plasma per ml of blood. Both A and B show that BLG is present and detectable via ELISA in the obtained cow blood sample. Moreover, no significant difference between blood serum and plasma is visible.

4.2.4 Analysis of cow saliva

As ELISA results for cow blood revealed positive results for BLG presence we investigated if BLG might also be excreted by mucosal glands and therefore might additionally be present in bovine saliva. Therefore, saliva of cows, ox, bull and horse was collected. The sample was frozen for storage and before use centrifuged for 15 minutes at 2500g to remove food debris. Supernatant was used for further analysis. Cow saliva was tested again by BLG-specific ELISA kit according to manufactures manual except all volumes were cut by half due to limited sample amount and tested twice.

In figure 57 the BLG concentration per total protein content is presented. Values for cow saliva sample 3 (CS3) and horse saliva sample (HoS1) are not included as there was no sample left for Nanodrop measurement for these sample. Results for the total ng of BLG per ml is shown in figure 56 and includes CS3 and HoS1.

Since this initial testing of 5 bovine samples showed promising result a second sample collection round was conducted. In total 10 additional cow saliva samples and 5 bull saliva samples could be collected, 2 female calf saliva samples 1 male calf saliva sample. Moreover, a human saliva sample was donated. If sample amount enabled it, all samples were tested trice via ELISA and the mean results are presented in figure 58 and figure 59. From CS10 only 1 meaningful value

could be obtained as the other two measurements were out of range due to being too high. There was not enough sample for a third measurement for BS4, BS6 and CS1.

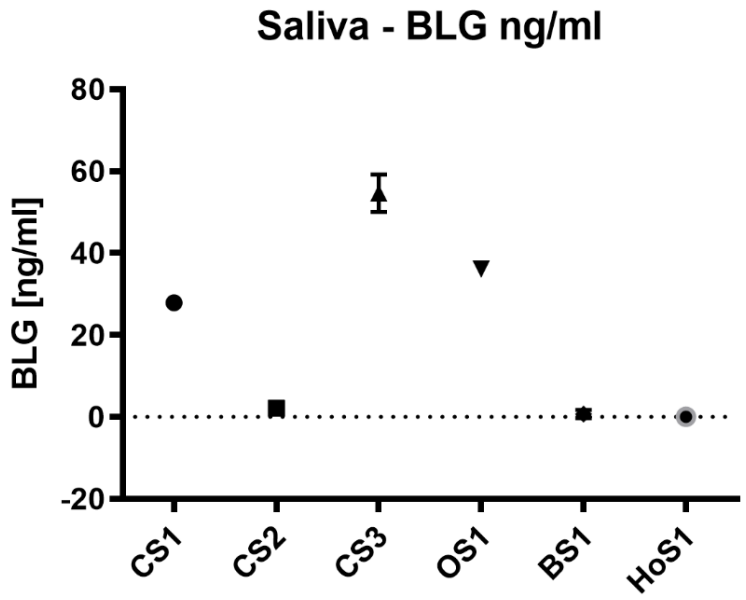

Figure 57 BLG in bovine saliva in ng per ml. CS1 (cow saliva 1 – A9), CS3 (cow saliva 3 – A11) and OS1 (Ox saliva 1 – A12) showed clearly BLG presence. In CS2 (cow saliva 2 – A10) as well as BS1 (bull saliva 1 – A13) only small amounts of BLG (close to background) could be detected per volume. Saliva collected from horse remained negative (HoS1- horse saliva 1 – A14)

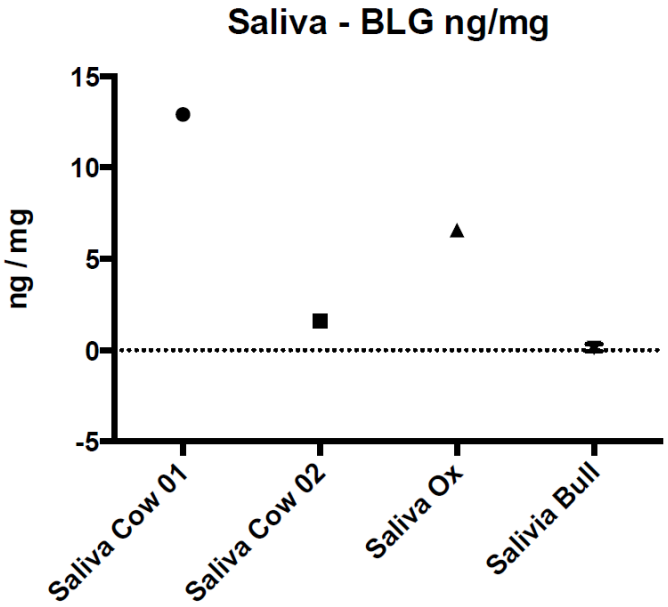

Figure 56 BLG in bovine saliva in ng per mg total protein. When adjusting determined BLG values according to total protein concentration of the sample BLG is still clearly present in CS1 (cow saliva 1 – A9) and OS1 (Ox saliva 1 – A12). Although lower than CS1 and OS1 the CS2 (cow saliva 2 – A10) is now also clearly BLG positive. BLG presence for bull saliva (BS1 – A13) remains very low to negative for the tested individual.

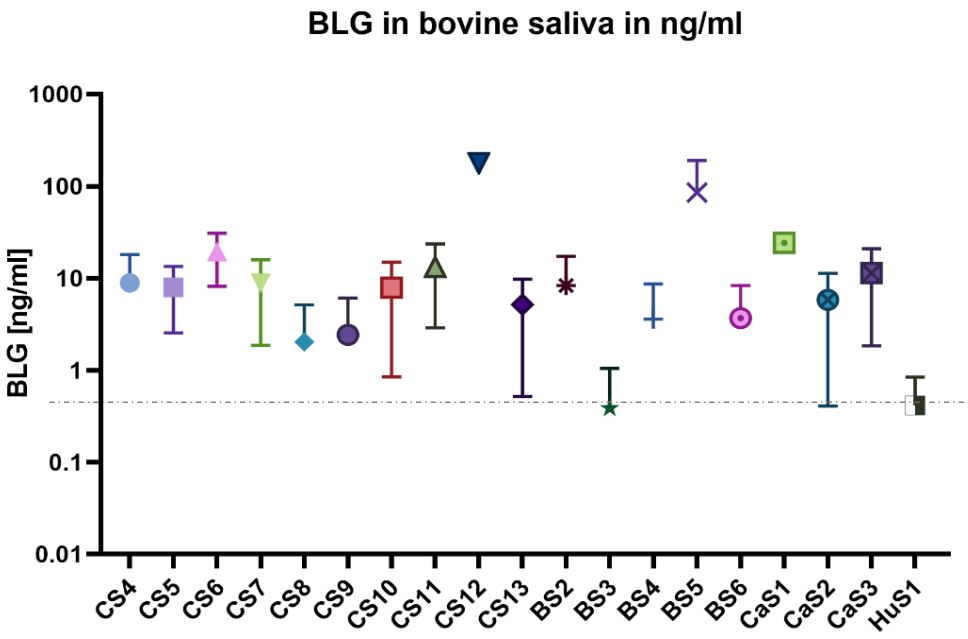

Figure 59 BLG in bovine saliva in ng/ml. BLG content in saliva seems to be rather individual and BLG is present in both sexes as well as the young even though they were not fed with milk. CaS1- CaS2 are female. CaS3 sample belongs to a bull calf. CS – cow saliva, BS – bull saliva, CaS – calf saliva, HuS1 – human saliva.

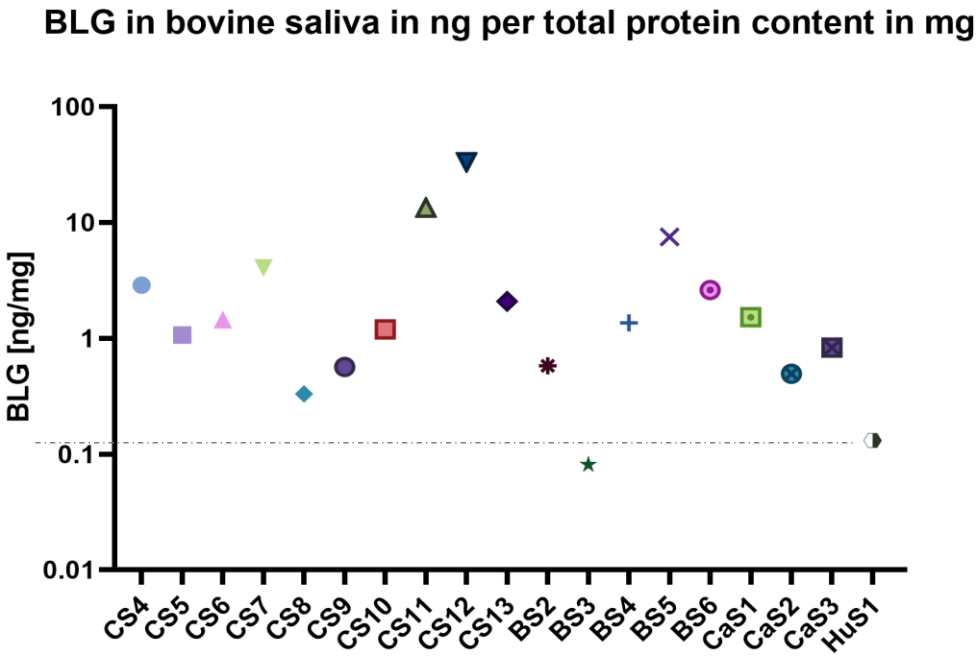

Figure 58 BLG in bovine saliva in ng/mg total protein content. Protein concentration of saliva sample was tested via NanoDrop and obtained results were set in relation with the ELISA results for those samples. CS4-CS7, CS10-CS13, BS4-BS6 and CaS1 samples contained more than 1 ng/mg BLG. CS – cow saliva, BS – bull saliva, CaS – calf saliva, HuS1 – human saliva.

Nevertheless, out of 13 tested cow samples (figure 58), 8 samples (CS4 -CS7, CS10-CS-13) contained more than 1 ng/mg BLG in their saliva. In the other tested samples, 3 out of 6 bull saliva samples, 1 out of 3 tested calf saliva samples and the single ox saliva sample contained more than 1 ng/mg BLG. Totally, 23 bovine saliva samples were tested and 12 samples contained more than 1 ng/mg BLG (table 11).

Table 10 Obtained values for BLG in bovine saliva samples. BLG content of saliva was determined via ELISA and in the table obtained quantifications are presented in ng BLG per ml saliva and ng BLG per mg total saliva proteins. All green boxes indicate samples containing more than 1ng BLG per mg total protein content.

Sample	BLG [ng/ml]	BLG [ng/mg]	Sample	BLG [ng/ml]	BLG [ng/mg]	Sample	BLG [ng/ml]	BLG [ng/mg]
Cow saliva samples			Bull saliva samples			non bovine saliva samples		
CS1			BS1			HoS1		-
CS2			BS2	8,33	0,58	HuS1	0,41	0,13
CS3		-	BS3	0,39	0,08			
CS4	8,89	2,87	BS4	3,59	1,36			
CS5	7,97	1,07	BS5	85,01	7,52			
CS6	19,49	1,44	BS6	3,68	1,74			
CS7	8,86	4,06	OxS1					
CS8	2,03	0,33	calf saliva samples					
CS9	2,43	0,57	CaS1	24,2	1,01			
CS10	7,89	1,19	CaS2	5,83	0,49			
CS11	13,21	13,4	CaS3	11,4	0,83			
CS12	177,4	11,04						
CS13	5,15	2,09						

4.3 Analysing BLG expression in cattle tissue via RT-PCR

Since BLG on protein level was present in multiple organs apart from bovine udder and is apparently also present in the male sex, the expression of the protein in different organs needed to be investigated. Additionally, to BLG gene expression, expression of two house-keeping genes was analysed (ACTB and GAPDH).

For the expression analysis, a reverse transcription PCR was conducted. Although RT-PCR in contrast to RT-qPCR does not allow quantification, it allows qualitative analysis confirming either BLG production in the specific organ or not. RNA was extracted from the frozen tissue as described in 3.2.3 and stored at -80°C until reverse transcription. Prior to reverse transcription, extracted RNA was tested first by NanoDrop and samples exhibiting inferior purity were discarded. Thereafter, 2 µl samples were run on agarose gel for further evaluation of the sample (figure 60 - B). The agarose gel electrophoresis revealed that mostly shorter RNA pieces have been preserved. Although obtained RNA exhibited not ideal preservation and integrity, according to NanoDrop measurements the sample were free from DNA contamination.

Finally, RNA was reverse transcribed as described in 3.2.3 and obtained cDNA was used. The results of the conducted RT-PCR (figure 60 -A) indicate that BLG is produced in the udder as already described in literature. Additionally, PCR amplification could be observed in the testis sample. Both kidney samples appear to be negative indicating no active BLG transcription in the bovine kidney, yet this might also be a result of the rather low RNA integrity of the tested samples. Nevertheless, if BLG transcription in the bovine kidney is present it most likely occurs on a lower level than that of the bovine udder and testis.

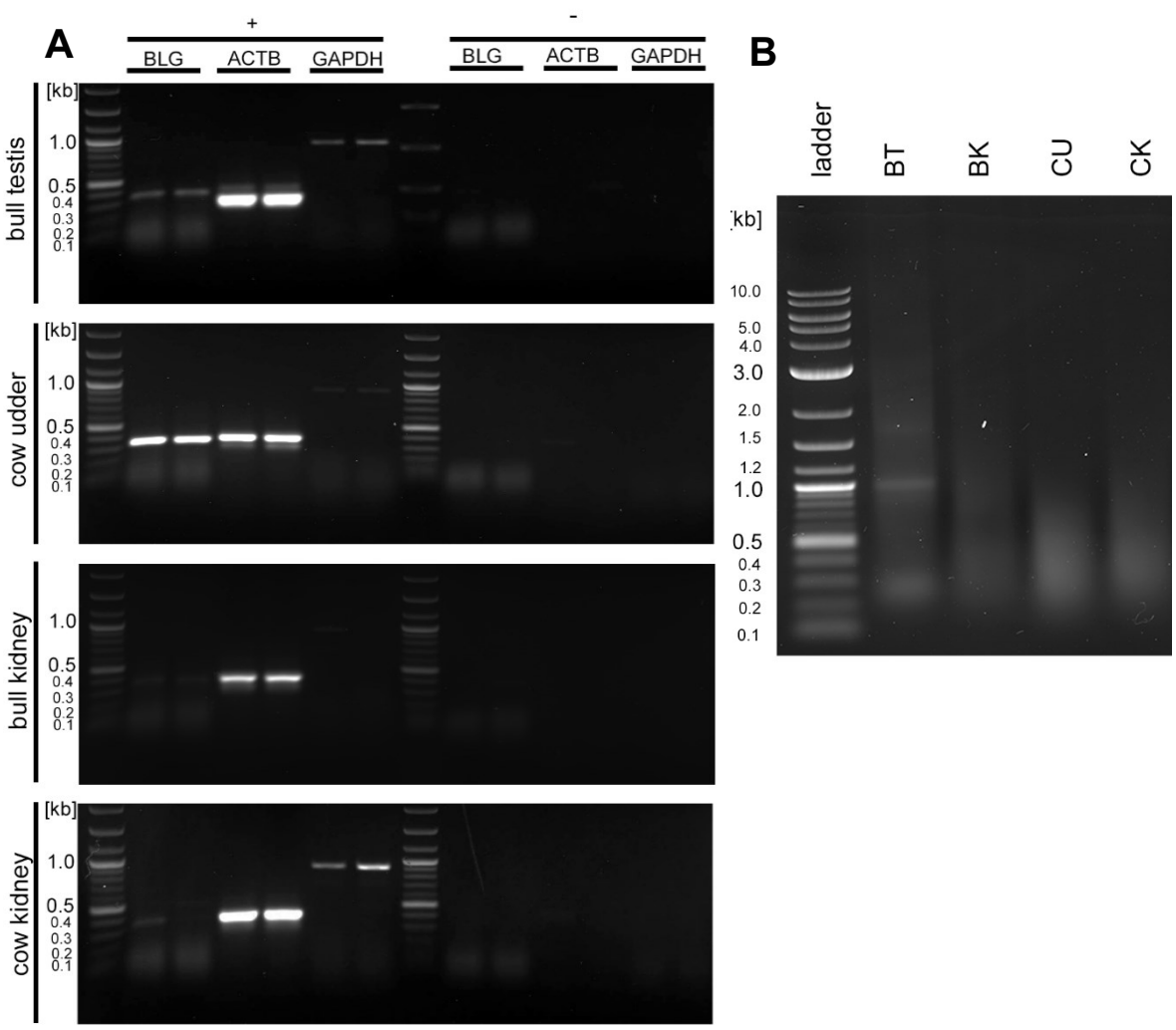

Figure 60 Agarose gel electrophoresis with PCR amplicons and RNA sample obtained from frozen tissue. A – gel electrophoresis with obtained PCR amplicons form bull testis/kidney and cow udder/kidney show that the LGB gene is transcribed in cow udder and bull testis. B – Agarose gel was run with 2 µl RNA samples showing that mostly small RNA pieces have been preserved. BT – bull testis RNA, BK – bull kidney RNA, CU – cow udder RNA, CK – cow kidney RNA, + = with DNA, - = negative control (DNA free water)

4.4 Comparison of BLG in different organs by sex

Immunohistochemically BLG could be detected in all investigated samples independently of sex. Moreover, BLG presence could be detected in the majority of analysed cells. In table 12 all obtained HistoQuest analysations are summarized. Interestingly the bull testis contained the most positive cells, although the bull kidney contained the highest BLG levels measured by the ELISA. In the here presented investigations the bovine kidney, was the best comparable organ between both sexes. Although BLG levels determined by ELISA for both sexes showed lower levels of BLG in the bull kidney, both kidney samples exhibit similar patterns for BLG in the histochemical analysis which are presented in figure 61.

Concerning the IHC results for the adrenal gland, cow adrenal gland sections contained mostly cortex and a small amount of medulla while the sections of the bull showed mainly medulla and no cortex. In the cow the cells of the cortex contained more BLG than cells of the medulla. Here especially cells which belong to the zona glomerulosa had the strongest BLG staining followed by cells of the zona fasciculata which contained aggregations of BLG within their cell lumen. These aggregations are not present in the zona reticularis which is the least positive part of the cortex. Yet this last layer is still more positive than the medulla where BLG is solely present in small clusters. The vesicles, which can be found throughout the tissue, do not contain BLG.

Table 11 TissueQuest results for all tested bovine organs. The obtained acquisitions were analysed by TissueQuest and revealed similar amount of positive and negative cells for both sexes.

αBLG stained sample	total cells	BLG positive cells	BLG negative cells
sex specific organs			
cow udder	568	431 (~75%)	137 (~25%)
bull testis	721	650 (~90%)	71 (~10%)
kidney			
cow kidney	935	702 (~75%)	233 (~25%)
bull kidney	739	550 (~74%)	189 (~26%)
adrenal gland			
cow (cortex)	520	400 (~77%)	80 (~23%)
bull (medulla)	562	422 (~75%)	140 (~25%)

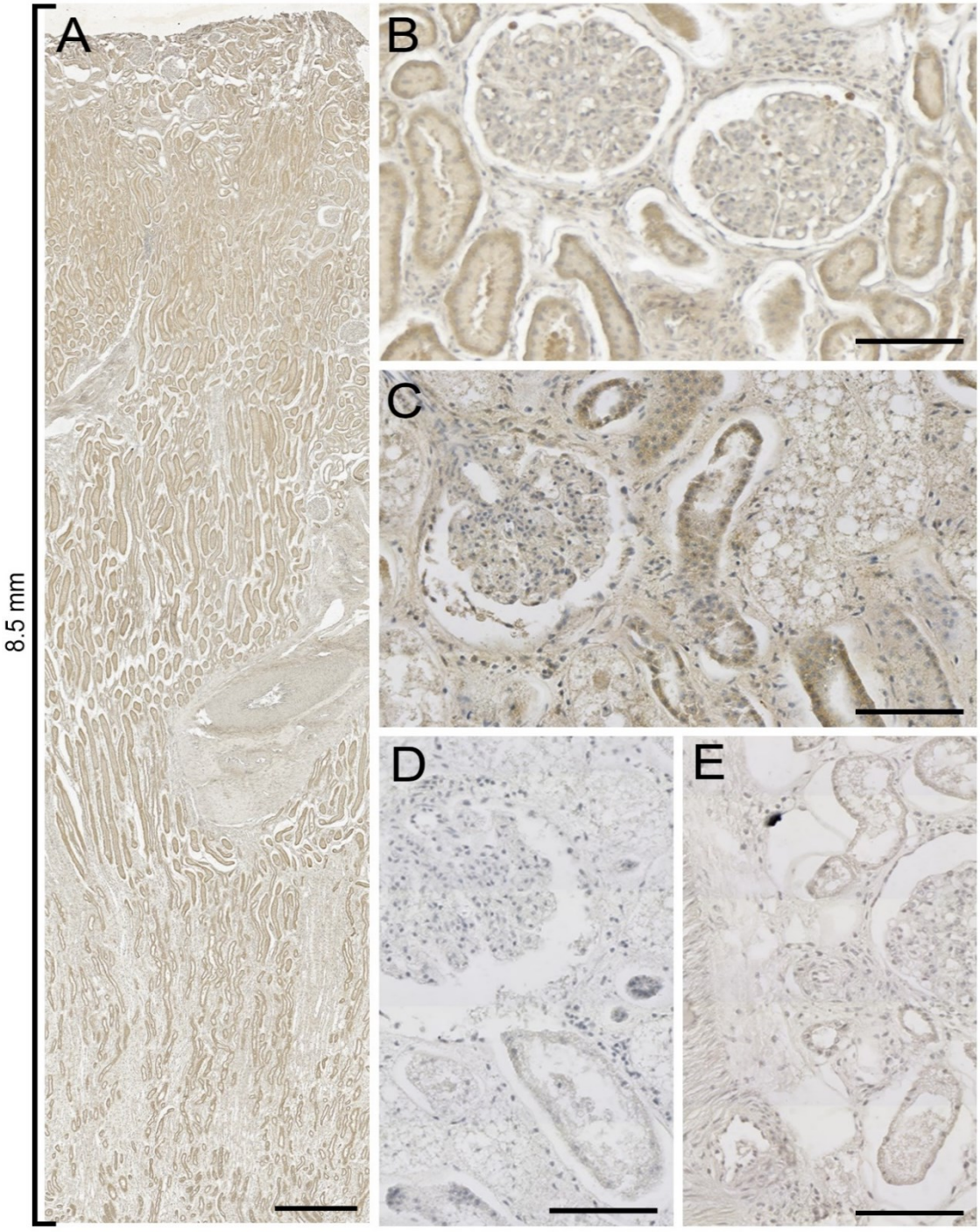

Figure 61 IHC of bovine kidney. Both bull and cow have BLG in their kidney´s. A-B – bull kidney αBLG stained, C – cow kidney αBLG, D – cow kidney negative control, E – bull kidney negative control, A: scale bar –1 mm, B-E: scale bar – 100 µm.

5. Discussion

Recent studies have shown that growing up on a farm protects children from developing asthma and allergies in their adult live. Interestingly, not all farms seem to be equally protective and among all farms, dairy farms seem to provide the strongest protection against developing a hyperreactive immune system. This raises the question what makes this kind of stables so unique? Following Strachan´s hypothesis a possible solution could be the presence of specific beneficial microbiota, however, another solution may be found in the dust of cow sheds or even the animals themselves. Our previous analysis has shown that the bovine protein BLG can be found in high amounts in cow stable dust. Furthermore, BLG has been detected in the urine of both cows and bulls.

Due to the presence of BLG in urine of both genders and its high concentrations in stable dust, it is unlikely that milk and female mammary gland are the proteins only origin points. In the here conducted experiments, BLG could be successfully detected in an array of bovine organs independently of sex. BLG could be detected immunohistochemically in the udder, kidney, and adrenal gland of a cow and in the testis, kidney as well as adrenal gland of a bull. Although we only had access to a low number of individuals (1 female, 1 male) for the immunohistochemical investigation, and the protein could also be detected by ELISA as well as western blot. Overall, the here presented findings contradict the general assumption that BLG is a unique milk and mammalian gland cell protein.

Immunohistochemical investigations of the bovine udder have first been conducted in the 1970ties. These investigations were done with a single fluorophore-linked αBLG monoclonal antibody and revealed BLG presence in the epithelial cells of the alveolar tissue but not in the surrounding tissue. In our experiments with the bovine udder, we revealed BLG presence in surrounding tissue as well. The discrepancy of these findings might be due our far gentler fixation method, the use of a dual antibody system as well as the use of a polyclonal antibody, which allows the binding of several epitopes resulting in an amplification of the signal. Since polyclonal antibodies come with the danger of cross-reactivity and a cross-reactivity between BLG and bovine α-lactalbumin has been described in literature, the used antibody was tested via ELISA (Appendix) for such a cross-reactivity. The used antibody showed no signs of binding α-lactalbumin. In concordance with previous findings, BLG presence was highest in the epithelial cells, but the protein could also be found in the surrounding connective tissue. BLG was not present in proliferating parts of the tissue and reduced in the tissue of the big milk ducts (figure 21) In the cow, the udder was the organ with the highest BLG content. The presence of BLG in the bovine udder was also shown by ELISA and western blot, confirming the IHC findings. Moreover, BLG expression could be shown by RT-PCR despite low RNA integrity.

Although the bovine testis is not equivalent to the udder (which would be the bovine ovaries) it was chosen due to belonging to the reproductive system and its secretory as well as hormonal function, its accessibility and for being exclusive for the male sex. As already explained in the introduction of this work, BLG production in literature has solely been ascribed to the female, and until now to the best of our knowledge has not been associated or described to the male sex. Therefore, our detection of BLG in male reproductive tissue is a highly interesting finding. Moreover, since the bull does not produce milk to feed a calf this finding further strengthens the believe that BLG does not solely serve as a nutrient and amino acid source. Summarizing the findings of this part, BLG could be found in the testis via IHC, ELISA, western blot and RT-PCR. The presence of BLG in the bovine testis has been shown by three different methods and two different antibodies systems on protein level. Aggregations of the protein are present in the primary spermatocytes as well as in cells of the interstitial tissue. Additionally, also RT-PCR revealed that BLG is produced in the testis. This indicates that the protein might be of reproductive importance.

Since BLG is present in high amounts in stable dust both in dairy stables as well as in cattle farms only inhabited by bulls, BLG had to leave the bovine body in an alternative way to milk which we found to be the bovine urine. Accordingly, immunohistochemistry as well as ELISA and western blot showed that BLG is present in the kidney. It was present in both sexes and in the here investigated samples, according to the immunoassays, the cow kidney contained more BLG than the bull kidney. Among all bull organs, however, the kidney was the organ with the highest BLG presence.

Contrasting this, the immunohistochemical analysis by TissueQuest revealed no relevant difference between the sexes (table 12). Immunohistochemistry showed that the nephrons exhibit the highest BLG presence, while other parts such as glomeruli and surrounding tissue were less positive. Moreover, the medulla which harbours less nephrons was also less positive. Although the low RIN values of the extracted RNA might also be responsible for a negative result of the RT-PCR for LGB mRNA, it is assumable that BLG enters the kidney via the blood and is filtered back into the blood stream through the nephron. BLG presence in bovine urine can thereby be explained by either an incomplete reabsorption or a special loading-status of the BLG-protein which is not reabsorbed.

The bovine adrenal gland was included in the organs of interest due to its hormonal function and proximity to the kidney. Unexpectedly the organ tested positive in the IHC for both genders as well as in the ELISA and western blot for the bull. Due to the lack of sample material no ELISA or western blot could be performed for cow adrenal gland and no RT-PCR for both sexes. Therefore, for bovine adrenal gland, BLG presence could only be confirmed on protein level.

Since BLG is present in the urine, could be identified in the kidney and our RT-PCR indicate that the protein is not produced within the kidney, the protein needed to be transported there. Therefore, a cow blood sample was tested for BLG presence. And indeed, the protein was present in the blood. This indicates a base level of expression of the protein in both genders and possible transport through the blood. To confirm this assumption, however, a bigger sample size and investigations on both sexes are needed.

As in some publications it was theorized that the protein might support the digestive system and help the calf to digest milk, we also collected saliva samples from a dairy farm and a cattle farm (inhabited only by bulls) and tested these for eventual BLG content. In the collected samples, BLG could be found in all individuals to various degrees. However, in some animals these levels were below 1 ng/mg of total protein while in other animals 10 ng/mg were exceeded. Cows in general had higher levels in their saliva than bulls. The inconsistent protein levels however could be more likely explained by contamination by the surrounding and environment, as BLG can be found plentily in stable dust. In order to investigate possible BLG production and release in the saliva of cattle, an immunohistochemical analysis of the salivary gland would yield more reliable results.

As BLG seems to possess more than a nutritive function, it would be interesting to compare, whether similar proteins also exist in human. According to literature, the most similar protein to bovine BLG in humans is the human placental protein-14 (PP14) which is also known as progesterone-associated endometrial protein (PAEP) or glycodelin. In humans this protein is produced in the late luteal phase of the menstrual cycle as well as the first trimester of pregnancy. This protein has been first isolated from the placenta, but its primary point of synthesis is the endometrium. Apart from this, PP14 can be found in the blood of pregnant women and is present in high quantities in the amniotic fluids, uterine luminal fluid. Additionally, PP14 has been found to have immunosuppressive properties by influencing activation of natural killer and T-cells. Today 4 different glycosylation forms (A-, F-, S-, C-) are known and have also been found in seminal plasma and the oocyte. There the protein plays a role in the fertilization of the egg. Unlike BLG, which is secreted into bovine milk, PP14 is not present in human breast milk.[24,46] Our finding of BLG in the bovine testis indicates that BLG might have a similar function for the reproductive and the immune system in cattle and probably also other mammals producing BLG in their milk.

Additionally, scientists have produced BLG-knockout cattle, as the production of BLG-free milk by breeding animals lacking the protein would be a rather lucrative business for two reasons: First, BLG-free milk is hypoallergenic, and additionally milking already BLG-free milk is less expensive than extracting it from normal cow´s milk. Second, BLG-free milk is far better digestible than BLG containing milk. Until today, scientists were able to produce a cow and bull without BLG

expression, but no reports of successful breeding of such cattle can be found both on PubMed and the internet. Considering our findings of BLG expression and presence in bovine testis as well as likely functional similarities with human PP14, these genetically modified animals might be viable but infertile. To prove this theory, however, further investigations are necessary.

5.1 Summary and conclusion

In summary, we have conducted several experiments to investigate the presence of the lipocalin BLG in different bovine organs of both cattle sexes. By immunohistochemistry, BLG could be found in bovine udder, testis, kidney and adrenal gland of a cow and a bull (table 13).

Table 12 Summery of all experimental results. Both cow (f) and bull (m) organs harboured detectable amounts of BLG in their organs, / = not conducted.

sex	organ	IHC	ELISA	WB	RT-PCR
f	udder	pos.	pos.	pos.	pos.
m	testis	pos.	pos.	pos.	pos.
f	kidney	pos.	pos.	pos.	neg.
m	kidney	pos.	pos.	pos.	neg.
f	adrenal gland	pos.	/	/	/
m	adrenal gland	pos.	pos.	pos.	/

Determination of BLG via protein extracts of aforementioned organs revealed that although the cow organs harboured more BLG, also the tested bull organs contained detectable amounts of the protein. Moreover, reverse transcription PCR revealed that BLG is transcribed in the testis of the bull showing that the proteins presence is not the product of environmental exposure, uptake or contamination. The here presented findings show that BLG is not a unique mammary tissue protein and is not solely present in the female. BLG presence in females as well as male cattle tissues and organs indicates that this protein of the lipocalin-family may harbour other functions in addition to being a nutrient for the calf. Previous results of our working group showed that depending on the proteins loading state, different immune responses have been observed. Therefore, BLG might have an immune-modulatory effect within cattle as well as influence the immune system of a suckling calf. This influence on the immune response might also occur in humans via cow´s milk consumption as well as environmental exposure during living near cow sheds. To prove these assumptions, further investigations in vitro as well as in vivo with BLG and its ligands are being conducted.

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6.1 List of abbreviations

ab	antibody
ACTB	beta actin gene
AGP	acid glycoprotein
APC	antigen presenting cells
BLG	beta lactoglobulin
Bos d 5	beta lactoglobulin (allergen)
$CD4^+$	Cluster of Differentiation 4 positive
Cys	cysteine
DAB	3,3′-Diaminobenzidine
ELISA	enzyme-linked immunosorbent assay
Fc-region	fragment crystallization-region
FSH	follicle stimulation hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HFPE	Hope-fixed paraffine embedded
HOPE	Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect
IFNγ	interferon-γ
Ig	immunoglobulin
IHC	immunohistochemistry
Il	interleukin
LCN2	lipocalin 2
LGB	beta-lactoglobulin gene
mRNA	messenger RNA
NGAL	neutrophil gelatinase-associated lipocalin
OVA	ovalbumin
RBP	retinol binding protein
ROI	region of interest
RT-PCR	reverse transcription polymerase chain reaction
Th2	T-helper cells type 2
PE	protein extract
PP14	placental protein 14
WB	western blot

6.2 List of tables

6.3 List of figures

Except for figure 1 (PDB ID: 3NPO, Loch, J. et al. Two modes of fatty acid binding to bovine β-lactoglobulin--crystallographic and spectroscopic studies. J. Mol. Recognit. JMR 24, 341–349 (2011).)[17], all here presented pictures and figures have been produced by the author of this work (Simona Winkler) via Adobe Illustrator CS6, Adobe Photoshop CS6, GraphPad Prism 8 or HistoQuest.

7. Appendix

7.1 Sequences of possible PCR-Products

Expected primer Products according to the online tool Sequence Manipulation Suite: PCR Products (https://www.bioinformatics.org/sms2/pcr_products.html)

ACTB:

>125 bp product from linear template ACTB_mRNA:NM_173979.3 , base 438 to base 562 (ACTBfor5 - ACTBrev5).
CGTGAGAAGATGACCCAGATCATGTTCGAGACCTTCAACACCCCTGCCATGTACGTGGCC
ATCCAGGCTGTGCTGTCCCTGTATGCCTCTGGCCGCACCACCGGCATCGTGATGGACTCC
GGTGA

GAPDH:

>3834 bp product from linear template GAPDH_gene:NC_037332.1, base 11 to base 3844 (For - Rev).
CCTGCCCGTTCGACAGATAGCCGTAACTTCTGTGCTGTGCCAGGTAAATGCCTGGCCGAG
AGGAGCCACAAAGGGCTGGGGATAGGCCCACCCAAAAGGGACGAGGGGGACGCGCGCCGG
GTGTGCGGCTGCGGGGGCTGGGGTCGACAGATCGCGCCCAGCTCCCCGCATTGCAGGGGC
GGGAGGAAGGACGTGCGGAAGCGCAGGTCGGGGATGGAGGCTTGGGGGGAGGGGTGGGGA
GGGGCTGGTGGGGAGAGGTGGTCAGCGCGGGATTCCATCCTCTTAGCCCCGCGCTCTAAT
GTTCACCTTCCTCTGCCCATAGCCGCATCCCTGAGACAAGATGGTGAAGGTCGGAGTGAA
CGGGTGAGTTACGGGCAGGGGTGGAGTGGAGTGCAGTGGGGTGGGGCAGGGTAGTCTTGC
GGTCCTCACCGCATTGCGCTAGCGGGTCTCTGCAAGCGCGAGGAGAACCTGAATAGGGTG
GTCGTACCCACCGCCCCCCGCAAGGAGAACTCAAGGTCAGCGCTCAGACCTGGAGGAGGC
CCTCGAAGGCCTCGTGTGCCCCCCACCTCCGGCTCTTGCGCCTCCTGTAACTGCGCCCCT
GCGGAACCTTCCACCACCTTGCGGCAGATACACAGGAGGTTCCAATTTTTGCTTGGGCCG
CATCCCCTAATCCCGCACCTGGGGCTCTTTTCTTTCCTTTCGCGCTCTGCGGGATCACGT
GCCTGGGAGGAGCCCGTCCCCCACCACCCCCCTAGCCCGTGCCCCCAGCAGGCGCGGCCC
CGAGGGTGCAGGAGGCACAGCTGCGCACAGAACCAGCTGGGAGGGGTCATCTGCGGTAAC
CCAGCCGCCATGTTGCAACTTGCTAGGAAGGAAATGAATGAACTGCCCGTTAGGAATCCT
TCCTTAGCCTTGCCGTCTTTTCGCCTTCCTTGCCCATGACTAACGCTTCTACCCCTGCCT
CGTGGCATGGAGTCACGTTTAGCCCATAGGCTAGGTGAGGCCAGGCTTTCCCGTTCTTGC
AGCTAGACCTCAACTCTGACCCTAGTAACCCCAGACCACCACTTAAACCGCCAGCCAAAT
TCCGCGGCCCCTCCTCCACTCCCAGGAATGAGAGGATGCTTTTCCTAGATGGTTCTCTGA
GGAATTCAAAACGTGGGCTTATGGGAGTGGTCTTGTAGTTTGGGGGTGTTTCTCCTTTCC
TTTTCCGGAGAGGTGTGGTGGCCCTGGCTGGGTGCCATGCTTGGGGGAGCTGAGTCATGG
ATGGGTACTTAGAAAATTTCCACCACAAAATGGCTCCCAGAGCTGCAGCCCCTGTGGTCT
TGCCGGCTCTCCTCGAAGCTCCTTTCCCCCATCTTCGCCTGGAGCTGACCCCACCCTAAA
GGCCAGGCTTTAAAGGTCACCAGGAGGATTAAGTGTCTGTGAGCCCCGGGAATCCTGCCC
TTCTCCCCATCCCATCCTTCAGAAACCAGATCCCCGTTCCACCTAAGCTGGGGTTAAATA
TAGCTGCCTGACCTTTCTGCAGCTGGGGGCCTGGGCTTTTGCTCTCCGCCCCCCACCTTT
CCCCCCCCTACTCCCTAATACCTGTTGCTCCCAATCCTGGTTTTTGGAAAAGAGCTTGGA
AGGACAGGTGCTGAGCAAGTCAGAAAGAACCCCAGGATGAGGGGATTGAGAAACCAGTTT
TCCCTCTCTCCTCTTGTGGGGGAGAGGGTAGGTTTTCCCGTCCAGTTCATGCTGACCTTT
GCTCTTGCCCTTTGAGCTTGGTGATGCTGAGTGGACTAGTGTTTGCAGTTCAGAGGGTGT
AGCCCGAAGTTGGGCCAAACAGGAGCAAGCGTTTGCAGAGAGTAGGTGTGATGAATGCCA
TGTGCCTGCCACGTGCCCAGCCTGTAGGTGGCAGTAGCTGCGCACCATCCCATCAACTCA
ACAGATTGCCAATTACTAGAGGGTGGGCAAGCCTTTGTGCCTGATTACCTATGAGCCTTT
CACTCTGTGGGCTGACAGGGTTGCTTAAGAAAAATAAAGTGAGTTCTCGCGAGACTTCGT
GGGGGTAACAGAGTGGCCTCTTTGAAAGAGGGTGAATGGGCCGGGGGCTGCTTCCATGCC
TAGGAGGAATCCCCTCCCCCTCACCCCTTCTCGTCTCTTGTCCCCCTCAGATTCGGCCGC

ATCGGGCGCCTGGTCACCAGGGCTGCTTTTAATTCTGGCAAAGTGGACATCGTCGCCATC
AATGACCCCTTCATTGACCTTCACTACATGGTGAGTGCTACCCCACAGCAGGTGGGGTCA
GAGCCTGGCTGAGGTGTGGCCCCTTGACGCCCCGCTCCTTTGTGCCCCCACCCCCCCCCA
GGTCTACATGTTCCAGTATGATTCCACCCACGGCAAGTTCAACGGCACAGTCAAGGCAGA
GAACGGGAAGCTCGTCATCAATGGAAAGGCCATCACCATCTTCCAGGAGTAAGTGTGAGA
GACGGAACAGGAAGAACTTGGCCTTGGAGGAGGCACTGGGACGGGGTGATCACCTGGGGT
CTGTGGTACCCTGTGTCCCTGAGCTTTGTCCTGTCTTCCCTCATAGGCGAGATCCTGCCA
ACATCAAGTGGGGTGATGCTGGTGCTGAGTATGTGGTGGAGTCCACTGGGGTCTTCACTA
CCATGGAGAAGGCTGGGGTGAGCAGCGAGGGGCTGGAGAGGAGGGTGTAACAGGACGATG
GGGGGGAAAGCGGACTTAGGAATGTGAGAGCCCGGGTGCAATTAACTTGCTCCTTGCTGC
AGGCTCACTTGAAGGGTGGCGCCAAGAGGGTCATCATCTCTGCACCTTCTGCCGATGCCC
CCATGTTTGTGATGGGCGTGAACCACGAGAAGTATAACAACACCCTCAAGATTGTCAGGT
GAGCGCAGCAGAGAGGAGGTGGGGAAGCGGGCTGATAGTGCCCCCTGGTGCCCAGTGGAC
GTGCCCCTGACTTGCCTCCCTCTTTTCCAGCAATGCCTCCTGCACCACCAACTGCTTGGC
CCCCCTGGCCAAGGTCATCCATGACCACTTTGGCATCGTGGAGGGACTTATGGTAGGAGT
GGTGGGAAACTTGAACACACTCAGTTCTCAGCTAGTATGGGACCCTTCTGCCTCCTGCAG
AGCAGAGCCCTTCAGAGCGATCTGTGTCCCTTAATTCTTCCCCAGGGGAGGGGGAGGTGA
AGGAGGGCATGGCCTGGTGAGCACCCAGTGGGTGGTCTCCTCCTCCCGCAGACCACCGTC
CACGCCATCACTGCCACCCAGAAGACTGTGGATGGCCCCTCCGGGAAGCTGTGGCGTGAC
GGCCGAGGGGCTGCCCAGAATATCATCCCTGCTTCTACTGGCGCTGCCAAGGCCGTGGGC
AAGGTCATCCCTGAGCTCAACGGGTAAGACCCTTGACTGTTTTCTGCCATGTGGACCCAG
GGAGCTGGGAGGAGCAGTGCAGACTGACCCTGCTTCCTTGTGTCCACAGGAAGCTCACTG
GCATGGCCTTCCGCGTCCCCACTCCCAACGTGTCTGTTGTGGATCTGACCTGCCGCCTGG
AGAAACCTGTGAGTTTGGGTTGGAGCAGCTTCGATGGCCTGCGGGGCCAGCGCGGGGGAG
TTGCAAGACTGGAACTCCTGTGACCATCTGTTTTTTACCTTGTCAGGCCAAGTATGATGA
GATCAAGAAGGTGGTGAAGCAGGCGTCAGAGGGCCCTCTCAAGGGCATTCTAGGCTACAC
TGAGGACCAGGTACAGTCAGTGGGTCAGGGGACTGGTGTCTGCATGGCTCCCGGGAGCCA
GGCGGGATTCCATATGAGGCCCTCTCTTCCCCCAGGTTGTCTCCTGCGACTTCA

>914 bp product from linear template GAPDH_mRNA:NM_001034034.2, base 11 to base 924 (For - Rev).
CCTGCCCGTTCGACAGATAGCCGTAACTTCTGTGCTGTGCCAGCCGCATCCCTGAGACAA
GATGGTGAAGGTCGGAGTGAACGGATTCGGCCGCATCGGGCGCCTGGTCACCAGGGCTGC
TTTTAATTCTGGCAAAGTGGACATCGTCGCCATCAATGACCCCTTCATTGACCTTCACTA
CATGGTCTACATGTTCCAGTATGATTCCACCCACGGCAAGTTCAACGGCACAGTCAAGGC
AGAGAACGGGAAGCTCGTCATCAATGGAAAGGCCATCACCATCTTCCAGGAGCGAGATCC
TGCCAACATCAAGTGGGGTGATGCTGGTGCTGAGTATGTGGTGGAGTCCACTGGGGTCTT
CACTACCATGGAGAAGGCTGGGGCTCACTTGAAGGGTGGCGCCAAGAGGGTCATCATCTC
TGCACCTTCTGCCGATGCCCCCATGTTTGTGATGGGCGTGAACCACGAGAAGTATAACAA
CACCCTCAAGATTGTCAGCAATGCCTCCTGCACCACCAACTGCTTGGCCCCCCTGGCCAA
GGTCATCCATGACCACTTTGGCATCGTGGAGGGACTTATGACCACTGTCCACGCCATCAC
TGCCACCCAGAAGACTGTGGATGGCCCCTCCGGGAAGCTGTGGCGTGACGGCCGAGGGGC
TGCCCAGAATATCATCCCTGCTTCTACTGGCGCTGCCAAGGCCGTGGGCAAGGTCATCCC
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