

RESEARCH ARTICLE

# Tissue Specificity of Human Angiotensin I-Converting Enzyme

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## Abstract

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## Background

Angiotensin-converting enzyme (ACE), which metabolizes many peptides and plays a key role in blood pressure regulation and vascular remodeling, as well as in reproductive functions, is expressed as a type-1 membrane glycoprotein on the surface of endothelial and epithelial cells. ACE also presents as a soluble form in biological fluids, among which seminal fluid being the richest in ACE content - 50-fold more than that in blood.

## Methods/Principal Findings

We performed conformational fingerprinting of lung and seminal fluid ACEs using a set of monoclonal antibodies (mAbs) to 17 epitopes of human ACE and determined the effects of potential ACE-binding partners on mAbs binding to these two different ACEs. Patterns of mAbs binding to ACEs from lung and from seminal fluid dramatically differed, which reflects difference in the local conformations of these ACEs, likely due to different patterns of ACE glycosylation in the lung endothelial cells and epithelial cells of epididymis/prostate (source of seminal fluid ACE), confirmed by mass-spectrometry of ACEs tryptic digests.

## Conclusions

Dramatic differences in the local conformations of seminal fluid and lung ACEs, as well as the effects of ACE-binding partners on mAbs binding to these ACEs, suggest different regulation of ACE functions and shedding from epithelial cells in epididymis and prostate and endothelial cells of lung capillaries. The differences in local conformation of ACE could be the base for the generation of mAbs distinguishing tissue-specific ACEs.

**Abbreviations:** ACE, angiotensin-converting enzyme; ZPHL, carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine; HHL, hippuryl-L-histidyl-L-leucine; LMW, low molecular weight.

## Introduction

Angiotensin I-converting enzyme (ACE, CD143) is a  $Zn^{2+}$  peptidyl dipeptidase which plays key roles in the regulation of blood pressure and in the development of vascular pathology and remodeling. ACE is constitutively expressed on the surface of endothelial cells, epithelial and neuroepithelial cells and cells of the immune system (macrophages, dendritic cells, reviewed in [1–3]).

In addition to membrane-bound ACE, blood, seminal fluid and other biological fluids contain a variable amount of soluble ACE. Blood ACE likely originates from the vascular endothelium [4], mostly lung endothelial cells, because lung capillaries exhibit nearly 100% ACE expression compared to only 10–15% ACE-positive capillaries in the systemic circulation [5]. ACE enters the circulating pool via a proteolytic cleavage from the cell surface [6–7] by still unidentified membrane-bound ACE secretase [8].

Human seminal fluid ACE likely originates from glandular epithelial cells of epididymis and prostate, that express significant amount of somatic ACE [9–14]. Human seminal fluid contains 50-fold more ACE than blood [15–17]. However, the level of somatic ACE expression in male reproductive tract is comparable to somatic ACE expression in endothelial cells of capillaries [14, 18]. Therefore extremely high level of ACE in seminal fluid could be due to the higher ratio of the surface of epithelial cells producing ACE in the reproductive tract to the volume of seminal fluid than the ratio of the surface of ACE-producing endothelial cells of lung capillaries to the blood volume. Alternatively, it could be due to increased shedding of ACE from the surface of epithelial cells of epididymis and prostate in comparison to ACE shedding from endothelial cells.

There are at least two possible reasons of increased shedding: 1) Increased expression of ACE secretase in glandular epithelial cells of epididymis and prostate (but unfortunately the nature of ACE secretase is still unknown); 2) Different conformations of ACE on the surface of endothelial and epithelial cells, which, in turn, may lead to either different exposure of stalk region, where ACE secretase cleaves ACE from cell surface, or different regulation of ACE shedding from different cells by the presence of putative ACE-binding proteins/ACE effectors.

Species specificity of ACE is apparent; however, more subtle tissue specificity of the enzyme can influence ACE functions both *in vitro* and *in vivo*. We demonstrated recently that the pattern of mAbs binding to the conformational epitopes on the surface of ACE, “**conformational fingerprint of ACE**”, is an extremely sensitive marker of the local conformational changes in ACE molecule due to mutations, denaturation, inhibitor binding, presence of membrane anchor, etc. We proved the concept that the conformation of ACE is cell- and tissue-specific and stems likely from different post-translational modifications, including different glycosylation, on the example of ACE from endothelial cells and ACE from macrophages and dendritic cell of sarcoid granuloma. The ACE fingerprint, therefore, has a potential for the disclosure of the cells from which ACE originates [19].

We applied this approach here to detect structural (conformational) differences of ACEs originated from endothelial cells (lung ACE) and epithelial cells (male reproductive tract). We believe that structural differences in ACEs from different organs, which we found, could be a base for the development of antibodies, which will distinguish ACEs shed to the blood circulation from different organs by their origin.

## Experimental Section

### ACEs from different sources

The work was carried out in accordance with The Code of Ethics of World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Boards of the Bakulev

Center of Cardiovascular Surgery, Moscow University and the University of Illinois at Chicago. Written informed consent from the donor or the next of kin was obtained for the use of this sample in research. Human citrated plasma, seminal fluid, lung, prostate and epididymis tissue homogenates were used as sources of somatic two-domain ACE. Lung and seminal fluid ACEs were purified using lisinopril affinity chromatography exactly as in [20]. Estimation of the percentage of ACE molecules carrying hydrophobic anchor (membrane form) in lung ACE preparation was performed by phase separation in Triton X-114 solution [21].

### ACE activity assay

ACE activity in blood plasma, seminal fluid, homogenates of human organs was measured using a fluorimetric assay with two ACE substrates, 2 mM Z-Phe-His-Leu (ZPHL) and 5 mM Hip-His-Leu (HHL), at pH 8.3 [22]. Briefly, 20  $\mu$ l aliquots of samples were added to 200  $\mu$ l of ACE substrate and incubated for the appropriate time at 37°C. The His-Leu product was quantified fluorimetrically via complexing with *o*-phthalaldehyde. Inhibition of ACE activity by ACE inhibitors was performed by incubation of ACE (2 nM) with up to 0.1  $\mu$ M enalaprilat and up to 1  $\mu$ M teprotide, at pH 7.5 for one hour and then measurement of residual ACE activity with 0.2 mM ZPHL as a substrate at the same pH value. Inhibition of ACE activity with anti-catalytic anti-ACE mAbs was performed by 10  $\mu$ g/ $\mu$ l mAbs as described in [23].

### Immunological characterization of ACEs

Ninety six-well plates (Corning, Corning, NY) were coated with anti-ACE mAbs via goat anti-mouse IgG (Pierce, Rockford, IL) bridge [24] and incubated with different sources of ACE, which were equilibrated for ACE activity with ZPHL as a substrate. After washing off unbound ACE, plate-bound ACE activity was measured by adding a substrate for ACE (ZPHL) directly into the wells [24]. Sixteen mAbs to human ACE were generated in our lab [19], while mAb BB9 [25] was kindly provided by Paul J Simmons (then Brown Foundation of Molecular Medicine, University of Texas Health Science Center, Houston, TX, USA).

Immunochemical characterization of ACE in the presence of different effectors was performed as described above, ACE solution being mixed with a solution of effectors just before application on the plate wells. The effectors used were heat-inactivated citrated plasma, heat-inactivated seminal fluid, 3 kDa filtrate of human citrated plasma, albumin, and bilirubin. Heat-inactivated plasma and seminal fluid were prepared by heating at 65°C for 45 min and checked for the absence of ACE activity; 3 kDa filtrate was prepared by filtration of human citrated plasma on Vivaspin 500 MWCO 3000 concentrator (GE Health Care, Sartorius Corp., Bohemia, NY) at 4°C and 12,000 g for 2 h.

### Immune response to ACE from seminal fluid

Mice were immunized five times with pure seminal fluid ACE at 10  $\mu$ g per i.p. injection. The sera were tested against this immunogen in ELISA and against two ACEs, pure seminal fluid ACE and pure lung ACE, in plate precipitation assay. Upon reaching antibody titers  $5 \times 10^5$ – $10^6$ , as measured by ELISA, mice were boosted and isolated splenocytes were fused to mouse myeloma cells 653-Ag8. The resulting populations grown in 96-well plates were subjected to primary screening with two pure ACEs, seminal fluid ACE and lung ACE, in plate precipitation assay in simultaneous experiments.

## Estimation of the size of the surface influenced by oligosaccharide

Sialylated biantennary complex oligosaccharide was modeled with the program Insight II (Accelrys Inc., San Diego, CA) and attached to the each potential site of glycosylation on the surface of the C domain (PDB 108A) of ACE. Molecular dynamics during 500 psec was applied for the estimation of the movement of the glycan and the neighboring amino acid residues within 15 Å from definite Asn residue.

## Desialylation of ACE

Desialylation of ACE was performed by neuraminidase from *Vibrio Cholerae* (Sigma-Aldrich, MO) in 0.1 M MES buffer, pH 6.0, containing 0.15 M KCl and 0.4 mM CaCl<sub>2</sub>, at 25°C. The process was controlled by ion-exchanged chromatography of 1 μM solutions of native and desialylated ACEs on Agilent1100 chromatograph with electrochemical cell. Chromatograms of native and desialylated ACEs were treated by the program Agilent Chemstation A.09.01 (Santa Clara, CA). For the calibration curve, solutions of neuraminic acids of 1, 10, and 20 μM were used.

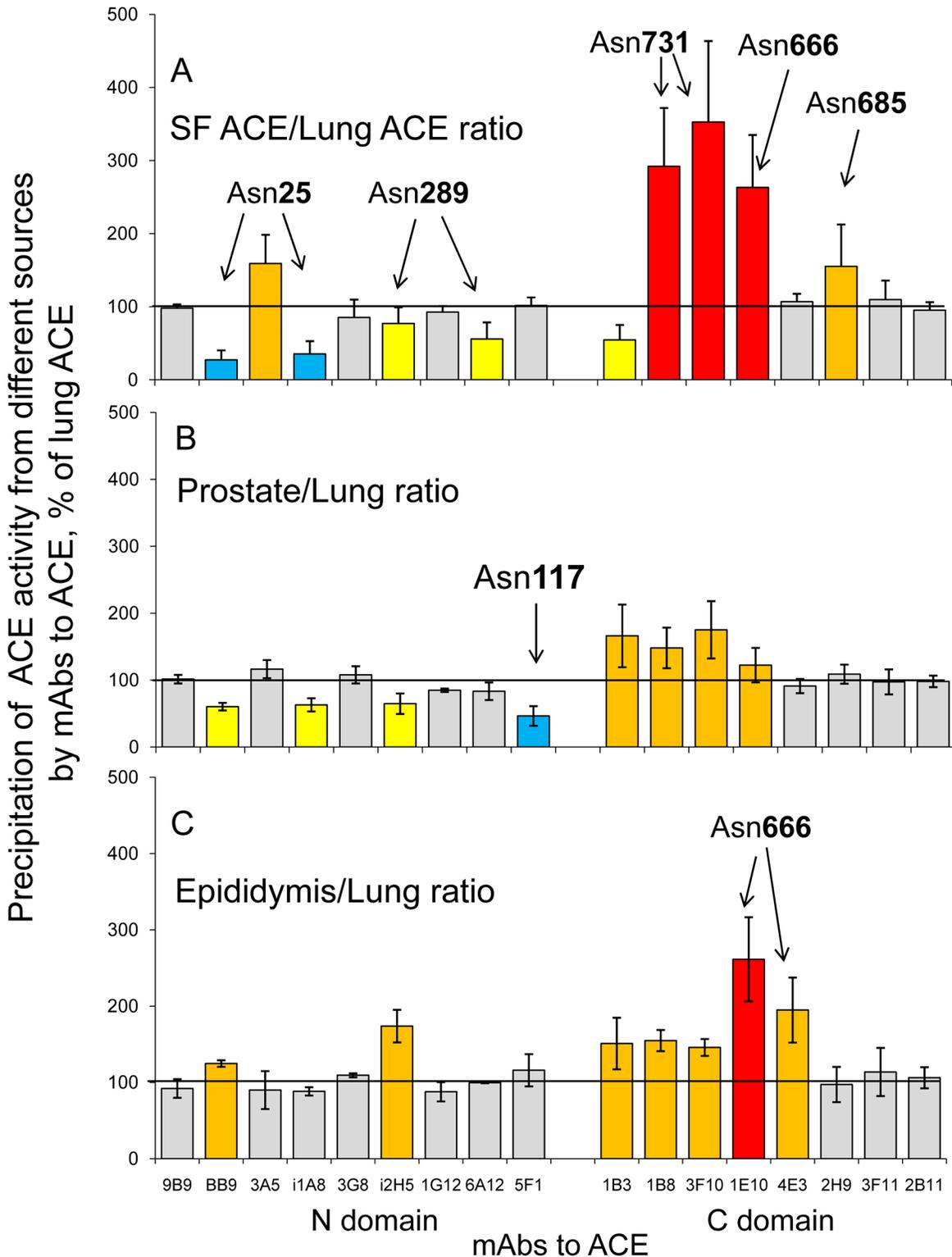
## Protein digestions and mass spectrometry

The lung ACE and seminal fluid ACE were subjected to electrophoresis in denaturing conditions. For each ACE sample, two protein bands on an electrophoretic gel were obtained, which were further subjected to mass spectrometry peptide fingerprinting as follows. A piece of ACE band from electrophoretic gel was transferred into a test tube, washed from the dye, and then the protein was digested by trypsin as is described elsewhere [26]. Peptides were extracted with 30% acetonitrile containing 0.1% trifluoroacetic acid and subjected to MALDI-TOF mass spectrometry. All mass spectra were obtained on a time-of-flight MALDI-TOF UltrafleXtreme mass spectrometer (Bruker Daltonics, Germany) using 2,5-dihydroxybenzoic acid as a matrix. The data were analyzed by MASCOT program ([www.matrixscience.com](http://www.matrixscience.com)) with the use of SWISS-PROT database, and then using the ExpASY online tools, FindPept ([www.expasy.org/findpept](http://www.expasy.org/findpept)) and GlycoMod ([web.expasy.org/glycomod](http://web.expasy.org/glycomod)). The FindPept tool was used to find additional matching to mass spectrometry data, peptides, such as C-terminal (unspecific) tryptic peptides which could not be discriminated by the MASCOT program. Non-matched peaks from the mass spectra were further analyzed with the GlycoMod tool in order to find possible glycopeptides and N-linked glycans in the ACE. The possibility of cysteine modification with acrylamide and methionine oxidation, which are typical variable modifications in mass spectrometry of proteins, was included in the analysis as well as the possibility for one or two missed cleavages with trypsin. Since a search with the GlycoMod tool is based on a comparison of experimental masses of glycopeptides with theoretical ones, its results sometimes contain false positive hits (displaying rather exotic glycan structures). So, finally only those N-linked glycan structures were taken into consideration and further manual analysis that matched the oligosaccharide compositions contained in the UniCarbKB database.

## Results and Discussion

### Immunological characterization of ACEs purified from human lung and seminal fluid

We characterized the conformation of pure lung and seminal fluid ACE, using a panel of mAbs directed against 17 different epitopes located on the N and C domains of catalytically active human ACE - "conformational fingerprint of ACE"[19]. As apparent in Fig 1A, the immunoprecipitation profile of pure ACEs from seminal fluid and lung dramatically differed. Because



**Fig 1. Immunological characterization of different ACEs.** Conformational fingerprinting of different ACEs with a set of mAbs to ACE. Seventeen monoclonal antibodies (mAbs) were used to precipitate ACE from different sources. Immunoprecipitated ACE activity is presented as a normalized value ("binding ratio" expressed as a percentage of the activity of ACE from one source to that from another source) to highlight differences in immunoprecipitation pattern ("conformational fingerprint") among different ACE variants. (A). The ratio of precipitated activity of pure seminal fluid ACE to that of pure lung ACE. B-C. The ratio of precipitated activity of ACE from prostate homogenate (B) or epididymis homogenate (C) to that of lung homogenate. Data are presented as

a mean of at least 3 independent experiments. Ratios increased more than by 20% are highlighted in orange and more than by 100% are highlighted in red. Ratios decreased more than by 20% are highlighted in yellow, more than by 50% in deep blue.

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we mapped the epitopes for all these mAbs to human ACE [27–31] we can make a conclusion that seminal fluid ACE (originated from epithelial cells of epididymis and prostate) and lung ACE (originated from endothelial cells) exhibit different conformations of their surface. Most probable, these differences are caused by different glycosylation of ACE from different cells.

Tissue- and cell-specific post-translational modifications (PTM) are common for various proteins [32–36]. PTMs precisely regulate the functions of proteins by inducing conformational changes which subtly or dramatically alter the protein surface or its overall tertiary structure. The most common PTM is glycosylation, however, phosphorylation, methylation, acetylation, deamidation, etc. are also widespread [32, 35–37].

Somatic ACE represents a characteristic N-glycosylated glycoprotein, exact glycan structures of which, as well as exact locations of actually present oligosaccharide chains on the surface of the protein globule, can vary with different protein sources. The sequence of human somatic ACE contains 17 potential sites for N-glycosylation [38]. The structure and exact positions of glycan moiety in human somatic ACE from different tissues are still poorly investigated. It was reported that seven of the 17 potential sites are glycosylated in human seminal plasma ACE (Asn9, in particular), the majority of glycans belonging to biantennary structures [39]. Human kidney ACE was reported to have at least six N-glycosylated sites, residues 9, 25, 82, 117, 480, and 913 [26]. Human plasma proteomics revealed three glycosylation sites in blood ACE, Asn480, 666, and 685 [40].

It was shown previously that somatic ACE contains mostly complex-type oligosaccharides [26, 39]. Modeling of the structure of the common sialylated biantennary complex oligosaccharide and subsequent molecular dynamics of single-domain ACE with attached oligosaccharide allowed us to show that the contact of the oligosaccharide chain with the surface of the enzyme can occur within the area about 200–300 Å. As epitopes for mAbs are usually 400–750 Å [27], it is obvious that the presence or absence of the oligosaccharide (as well as its definite structure, i.e. the number of antennae, sialylation, fucosylation, etc.) within an epitope can hugely influence on mAbs binding.

Seminal fluid ACE and lung ACE possess rather equal masses as was shown by SDS-PAGE (not shown) which indicates on the absence of major differences in the degree of glycosylation of these ACEs. However, some evidence of different modes of glycosylation of lung ACE and seminal fluid ACE was obtained by the analysis of the action of neuraminidase on both enzymes. It was found that while desialylation of seminal fluid ACE did not result in the appearance of any neuraminic acid on the ion-exchange chromatogram, desialylation of lung ACE resulted in the appearance of 5 neuraminic acid residues per molecule of the enzyme (not shown). It is known that human lung ACE is characterized by a lower pI value (5.1) compared to that of kidney ACE (5.2) which was attributed to a bigger number of neuraminic acid residues in lung ACE glycans [41]. On the other hand, the number of neuraminic acid residues per molecule of kidney ACE was reported to be equal to 5.9 [42]. So, based on a lower pI value of lung enzyme, we might expect much larger content of neuraminic acid residues per molecule of lung ACE than 5 observed in our work. Moreover, we did not find any neuraminic acid residues for ACE from kidney as well (not shown). It seems, that desialylation in experimental conditions was not complete which resulted in underestimated values, if any, of neuraminic acid residues per ACE molecule. Thus, the difference in neuraminic acid residues content between seminal fluid ACE and lung ACE can not be considered as quantitative finding but rather an indication that the degree of sialylation of seminal fluid ACE is much less than that of

lung ACE. The molecular basis for this phenomenon could be that, while the composition of the oligosaccharide chains in lung ACE is still unknown, seminal fluid ACE contains five complex-type glycans, which can contain sialic acid residues, while two other glycans in this ACE represent oligomannose-type glycans [39]. In addition, it is interesting that from 5 sialic acid residues found for lung ACE, three residues corresponded to N-acetyl-neuraminic acid whereas two residues represented N-glycolyl-neuraminic acid, which was reported earlier to be abundant in testicular ACE but not found in kidney ACE [39].

An analysis of the conformational fingerprint of lung ACE and seminal fluid ACE (Fig 1A) allowed us to suggest that different glycosylation of ACEs from different cells might occur in the following glycosylation sites on ACE protein globule: Asn25 in the epitopes for mAbs BB9, 3A5 and i1A8, Asn289 in the epitopes for mAbs i2H5 and 6A12 on the N domain, Asn666 in the epitopes for 1E10 and 4E3, and Asn685 in the epitope for 2H9 on the C domain.

As for the dramatic difference in binding of mAbs 1B8 and 3F10 to seminal fluid ACE and lung ACE, it might be explained by the difference in glycosylation of Asn731, which is in the center of the epitopes for these two mAbs on the C domain of ACE [30] or, alternatively, by the presence of transmembrane anchor in some molecules of lung ACE, which influences the binding of these very mAbs to ACE [19, 30]. The presence of such molecules was demonstrated by MALDI TOF analysis which revealed in lung ACE digested with trypsin a presence of not only the C-terminal peptide 1190–1203 but also peptides 1204–1213 and even 1214–1227 (Table 1 and Fig 2). To estimate possible contamination of lung ACE with molecules carrying hydrophobic transmembrane anchor, we analyzed lung ACE preparation by phase separation in Triton X-114. About 90% of the enzyme was determined in water phase, while only 10% appeared in organic phase. Therefore, the proportion of ACE molecules with transmembrane-anchor in the pure lung ACE preparation is not high and we can conclude that the presence of anchor-containing molecules in lung enzyme unlikely contributes to the observed difference in the binding of mAbs to the C-region of ACE molecule.

In order to analyze the reasons for the differences in mAbs 1B8 and 3F10 binding with lung and seminal fluid ACE further we compared conformational fingerprint of ACE from lung homogenate with that from epididymis and prostate homogenates (Fig 1B and 1C), which should have similar proportion of anchor-containing ACE molecules. This comparison clearly demonstrates remarkable differences in conformation of ACE from lung and ACE from both prostate and epididymis. Better binding of mAbs 1B8 and 3F10 to prostate and epididymal ACE, compared to lung ACE, reproduces better binding of these mAbs to pure seminal fluid ACE compared to pure lung ACE and most probably indicates different glycosylation (extent or type) of Asn731 in ACE from the epithelial cells of prostate and epididymis than in lung ACE from endothelial cells (Fig 1B and 1C).

The enhanced binding of these two mAbs, as well as mAb 1B3 (directed to the epitope on the stalk region [43–44]), to epididymal and prostate ACE allows us to suggest that better access of the stalk region to these mAbs may indicate on the better access of the stalk region in ACE on the surface of epithelial cells of prostate and epididymis for ACE secretase as well and may result in higher rate of shedding

Moreover, a comparison of the conformational fingerprints of epididymal and prostate ACE with their seminal fluid ACE counterpart indicates that seminal fluid ACE could represent a mixture of ACE originated from epididymis and ACE originated from prostate. In support of this suggestion, we discovered by MALDI-TOF analysis of seminal fluid ACE digested with trypsin (Table 1) a presence of two C-terminal peptides, a peptide with molecular mass 1533.8 (found among matching peptides for unspecific cleavage by FindPept tool) which corresponds to the peptide 1190–1202 (calculated mass 1533.7) reported previously [45] and a peptide with molecular mass 1689.8 which corresponds to the peptide 1190–1203 (calculated mass

Table 1. Observed [M+H]<sup>+</sup> ions of unglycosylated peptides in the mass spectra of human ACE tryptic digests.

Lung ACE			Seminal fluid ACE		
Peptide	Observed m/z	Calculated m/z	Peptide	Observed m/z	Calculated m/z
53–71 <sup>c</sup>	2191.1	2191.1	53–71 <sup>c</sup>	2191.1	2191.1
54–71	2035.0	2035.0	54–71	2035.0	2035.0
97–107	1084.6	1084.6	97–107	1084.7	1084.6
<b>127–132<sup>a</sup></b>	744.4	744.4	<b>127–132<sup>a</sup></b>	744.4	744.4
133–151 <sup>a</sup>	2135.1	2135.0	133–151 <sup>a</sup>	2135.1	2135.0
152–187	4117.1	4117.0	152–187	4117.2	4117.0
152–187 <sup>b</sup>	4133.0	4133.0	152–187 <sup>b</sup>	4132.9	4133.0
188–199	1416.6	1416.6	188–199	1416.6	1416.6
200–230	3837.9	3837.9	200–230	3837.8	3837.9
231–236 <sup>c</sup>	808.4	808.5	231–236 <sup>c</sup>	808.5	808.5
241–245	678.4	678.4	241–245	678.4	678.4
296–326	3526.7	3526.7	296–326	3526.7	3526.7
296–326 <sup>b</sup>	3542.7	3542.6	296–326 <sup>b</sup>	3542.7	3542.6
327–340 <sup>a</sup>	1767.8	1767.8	327–340 <sup>a</sup>	1767.8	1767.8
327–341 <sup>a,c</sup>	1895.9	1895.9	327–341 <sup>a,c</sup>	1895.9	1895.9
342–346 <sup>c</sup>	678.4	678.4	342–346 <sup>c</sup>	678.4	678.4
351–373	2821.4	2821.3	351–373	2821.3	2821.3
351–373 <sup>b</sup>	2837.4	2837.3	351–373 <sup>b</sup>	2837.3	2837.3
374–380	799.5	799.5	374–380	799.5	799.5
381–407 <sup>c</sup>	2852.5	2852.5	381–407 <sup>c</sup>	2852.5	2852.5
382–407	2696.4	2696.4	382–407	2696.4	2696.4
408–413	686.4	686.4	408–413	686.5	686.4
433–446	1724.9	1724.9	433–446	1724.9	1724.9
447–453	808.4	808.4	447–453	808.4	808.4
459–467	1362.6	1362.6	459–467	1362.7	1362.6
468–479 <sup>c</sup>	1362.7	1362.7	468–479 <sup>c</sup>	1362.7	1362.7
470–479	1133.6	1133.6	470–479	1133.6	1133.6
<b>490–500</b>	1342.7	1342.7	<b>490–500</b>	1342.8	1342.7
			<b>490–517<sup>c</sup></b>	3429.5	3429.8
501–532 <sup>c</sup>	3837.9	3837.8	501–532 <sup>c</sup>	3837.8	3837.8
518–532 <sup>a</sup>	1821.8	1821.8	518–532 <sup>a</sup>	1821.8	1821.8
542–557 <sup>c</sup>	1826.0	1826.0	542–557 <sup>c</sup>	1826.0	1826.0
543–557	1697.9	1697.9	543–557	1697.9	1697.9
558–572	1598.9	1598.8	558–572	1598.9	1598.8
623–629	957.4	957.4	623–629	957.5	957.4
<b>661–677<sup>c</sup></b>	1973.9	1974.0	<b>661–677<sup>c</sup></b>	1973.8	1974.0
671–676	695.4	695.3	671–676	695.4	695.3
694–700 <sup>c</sup>	887.5	887.5	694–700 <sup>c</sup>	887.5	887.5
701–713	1475.8	1475.7	701–713	1475.8	1475.7
750–762 <sup>c</sup>	1751.9	1751.9	750–762 <sup>c</sup>	1751.9	1751.9
751–762	1623.8	1623.8	751–762	1623.8	1623.8
751–767 <sup>c</sup>	2151.1	2151.0	751–767 <sup>c</sup>	2151.1	2151.0
768–775	979.6	979.6	768–775	979.6	979.6
776–785	1176.7	1176.6	776–785	1176.7	1176.6
786–797	1352.6	1352.6	786–797	1352.6	1352.6
798–811	1697.9	1697.8	798–811	1697.9	1697.8

(Continued)

Table 1. (Continued)

Lung ACE			Seminal fluid ACE		
Peptide	Observed <i>m/z</i>	Calculated <i>m/z</i>	Peptide	Observed <i>m/z</i>	Calculated <i>m/z</i>
798–811 <sup>b</sup>	1713.9	1713.8	798–811 <sup>b</sup>	1713.8	1713.8
812–828	2117.2	2117.2	812–828	2117.2	2117.2
834–883	5562.0	5561.7	834–883	5561.3	5561.7
884–889	744.4	744.4	884–889	744.4	744.4
915–924	1133.6	1133.6	915–924	1133.6	1133.6
940–944 <sup>c</sup>	678.4	678.4	940–944 <sup>c</sup>	678.4	678.4
945–971 <sup>a</sup>	3305.6	3305.6	945–971 <sup>a</sup>	3305.5	3305.6
972–978	783.5	783.5	972–978	783.5	783.5
979–1001	2309.3	2309.2	979–1001	2309.2	2309.2
1002–1025	2693.3	2693.3	1002–1025	2693.3	2693.3
1031–1044	1754.9	1754.9	1031–1044	1755.0	1754.9
1045–1077 <sup>c</sup>	4066.1	4066.1	1045–1077 <sup>c</sup>	4066.0	4066.1
1047–1054	866.5	866.5	1047–1054	866.5	866.5
1055–1065	1524.7	1524.7	1055–1065	1524.7	1524.7
1068–1077	1129.6	1129.6	1068–1077	1129.6	1129.6
1078–1087	1035.5	1035.5	1078–1087	1035.5	1035.5
1088–1098	1315.7	1315.7	1088–1098	1315.7	1315.7
1099–1125	3089.5	3089.5	1099–1125	3089.4	3089.5
1181–1189	1056.5	1056.5	1181–1189	1056.5	1056.5
			<b>1190–1202</b>	1533.7	1533.8
<b>1190–1203</b>	1689.8	1689.8	<b>1190–1203</b>	1689.8	1689.8
1204–1213	1014.5	1014.5			
1214–1227	1532.8	1532.8			

<sup>a</sup> Acrylamide adduct on cysteine.

<sup>b</sup> Oxidized methionine.

<sup>c</sup> Contains one or two missed cleavage(s) by trypsin.

Peptides that contain potential N-glycosylation sites are shown in bold.

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1689.8). So, it is possible that the seminal fluid contains two somatic ACE forms—shorter form of ACE (1202 residues) and common form (1203 residues), which can be produced differently by epididymis and prostate or, alternatively, the shorter form could be the result of the secondary carboxypeptidase processing as suggested in [45].

Measurement of ACE activity in tissues homogenates prepared in equal conditions (tissue: buffer = 1:9) revealed that prostate exhibited much lower content of ACE (44 mU/g), than epididymis (720 mU/g), which ACE activity was comparable to that in lung—1063 mU/g with ZPHL as a substrate. These values are in accordance with lower ACE protein content in prostate than in epididymis reported earlier [11–12] and confirmed recently in the Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)), which demonstrates that epididymis exhibits at least 6-fold more ACE expression than prostate. However, higher content of ACE in epididymis is likely compensated by higher mass of prostate which leads to pretty equal content of ACE in both organs [11] and perhaps explains why ACE in seminal fluid is considered to originate equally from both organs [16]. This conclusion is supported by the comparison of conformational fingerprints of prostate, epididymis and seminal fluid ACEs (Fig 1). It seems that the pattern of mAbs binding to pure seminal fluid ACE represents a mixture of patterns of mAbs binding to

Lung ACE		Seminal fluid ACE	
1	LDPGLQPGFSADEAGAQLFAQSYSSAEQVLFQSVAAASHADHTITAEN 50	1	LDPGLQPGFSADEAGAQLFAQSYSSAEQVLFQSVAAASHADHTITAEN 50
51	ARRQEEAALLSQEFABAWGQKAKELYEPIWQFTDPQLRRIIGAVRTLGS 100	51	ARRQEEAALLSQEFABAWGQKAKELYEPIWQFTDPQLRRIIGAVRTLGS 100
101	ANLPLAKRQOYNALLSNMSRIYSTAKVCLPNKTATCWSLDPDLTNILASS 150	101	ANLPLAKRQOYNALLSNMSRIYSTAKVCLPNKTATCWSLDPDLTNILASS 150
151	RSYAMLLFAWEGWHNAAGIPLKPLYEDFTALSNEAYKQDGFDTGAYWRS 200	151	RSYAMLLFAWEGWHNAAGIPLKPLYEDFTALSNEAYKQDGFDTGAYWRS 200
201	WYNSPTFEDDLEHLYQOLEPLYLNLHAFVRRALHRRYGDRYINLRGPIPA 250	201	WYNSPTFEDDLEHLYQOLEPLYLNLHAFVRRALHRRYGDRYINLRGPIPA 250
251	HLLGDMWAQSWENIYDMVVPFDPKPNLDVTSTMLQQGWATHMFRVAEEF 300	251	HLLGDMWAQSWENIYDMVVPFDPKPNLDVTSTMLQQGWATHMFRVAEEF 300
301	FTSLELSPMPPEFWEWSMLEKPADGREVVCHASAWDFYNRKDFRIKQCTR 350	301	FTSLELSPMPPEFWEWSMLEKPADGREVVCHASAWDFYNRKDFRIKQCTR 350
351	VTMDQLSTVHHEMGIQYYLQYKDLFVSLRRGANPGFHEAIGDVLALSVS 400	351	VTMDQLSTVHHEMGIQYYLQYKDLFVSLRRGANPGFHEAIGDVLALSVS 400
401	TPEHLHKIGLLDRTNTESDINYLKMALEKIAFLPFYGILVDQWRWGVF 450	401	TPEHLHKIGLLDRTNTESDINYLKMALEKIAFLPFYGILVDQWRWGVF 450
451	SGRTPPSRYNFDWWYLRTKYQIGICPPVTRNETHFDAGAKFHVNVTPYIR 500	451	SGRTPPSRYNFDWWYLRTKYQIGICPPVTRNETHFDAGAKFHVNVTPYIR 500
501	YFVSVFLQFQFHEALCKEAGYEGPLHQCDIYRSTKAGAKLRKVLQAGSSR 550	501	YFVSVFLQFQFHEALCKEAGYEGPLHQCDIYRSTKAGAKLRKVLQAGSSR 550
551	PWQEVKDMVGLDALDAQPLLYFQPVTVQWLQEQNQNGEVLGWPEYQWH 600	551	PWQEVKDMVGLDALDAQPLLYFQPVTVQWLQEQNQNGEVLGWPEYQWH 600
601	PPLPDNYPEGIDLVTDEAEASKFVEEYDRTSQVVWNEYAEANWNYNTNIT 650	601	PPLPDNYPEGIDLVTDEAEASKFVEEYDRTSQVVWNEYAEANWNYNTNIT 650
651	TETSKILLQKNMQIANHTLKYGTQARKFDVNQLQNTTIKRIIKVQDLER 700	651	TETSKILLQKNMQIANHTLKYGTQARKFDVNQLQNTTIKRIIKVQDLER 700
701	AALPAQELEEYNKILLDMETTYSVATVCHPNSGCLQLEPDLTNVMATSRK 750	701	AALPAQELEEYNKILLDMETTYSVATVCHPNSGCLQLEPDLTNVMATSRK 750
751	YEDLLWAWEGWRDKAGRILQFYPKYVELINQAARLNGYVDAGDSWRSMY 800	751	YEDLLWAWEGWRDKAGRILQFYPKYVELINQAARLNGYVDAGDSWRSMY 800
801	ETPSLEQDLERLFOELQPLYLNLHAYVRRALHRRHYGAQHINLEGPIPAHL 850	801	ETPSLEQDLERLFOELQPLYLNLHAYVRRALHRRHYGAQHINLEGPIPAHL 850
851	LGNMWAQTWSNIYDLVVPFAPSMDTTEAMLKQGWTPRRMFKEADDFFT 900	851	LGNMWAQTWSNIYDLVVPFAPSMDTTEAMLKQGWTPRRMFKEADDFFT 900
901	SLGLLPVPPEFWKSMLEKPTDGRVCHASAWDFYNGKDFRIKQCTTVN 950	901	SLGLLPVPPEFWKSMLEKPTDGRVCHASAWDFYNGKDFRIKQCTTVN 950
951	LEDLVVAHHEMGIQYFMQYKDLFVALREGANPGFHEAIGDVLALSVSSTP 1000	951	LEDLVVAHHEMGIQYFMQYKDLFVALREGANPGFHEAIGDVLALSVSSTP 1000
1001	KHLHSLNLLSSEGGSDHDIINFLMKMALDKIAFIPIFSYLVDQWRWRVFDG 1050	1001	KHLHSLNLLSSEGGSDHDIINFLMKMALDKIAFIPIFSYLVDQWRWRVFDG 1050
1051	SITKENYNQEWWSLRKLYQGLCPPVPRQTQGFDPGAKFHIPSSVPIRYF 1100	1051	SITKENYNQEWWSLRKLYQGLCPPVPRQTQGFDPGAKFHIPSSVPIRYF 1100
1101	VSFIIQFQFHEALCQAAGHTGPLHKCDIYQSKEAGQRLATAMKLGFSRPW 1150	1101	VSFIIQFQFHEALCQAAGHTGPLHKCDIYQSKEAGQRLATAMKLGFSRPW 1150
1151	PEAMQLITGQPMASAMLSYFKPLLDWLRTENELHGEKLGWPOYNWTPN 1200	1151	PEAMQLITGQPMASAMLSYFKPLLDWLRTENELHGEKLGWPOYNWTPN 1200
1201	SARSEGPLPDSGRVSVFLGLDLDAQARVGQWLLFLGLIALLVATLGLSQR 1250	1201	SARSEGPLPDSGRVSVFLGLDLDAQARVGQWLLFLGLIALLVATLGLSQR 1250
1251	LFSIRHRSLHRHSHGPFQFGEVELRHS 1277	1251	LFSIRHRSLHRHSHGPFQFGEVELRHS 1277

**Fig 2. Amino acid sequences of the lung and seminal fluid ACEs.** Peptides identified by MALDI TOF MS are shown in bold; potential sites of trypsin cleavage are underlined; potential glycosylation sites are marked by green; zinc-recognizing motives are marked by red; putative glycopeptides are shaded.

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prostate and epididymal ACE. Thus, epididymal ACE, but not prostate ACE, exhibited enhanced binding of mAb 1E10 to the C domain of ACE, which is also characteristic for seminal fluid ACE. As opposite, prostate ACE is characterized by lowered binding of mAbs BB9, i1A8, and i2H5 to the N domain of ACE, which is characteristic for seminal fluid ACE as well (Fig 1).

ACE in prostate homogenate, however, is also characterized by a very low binding of mAb 5F1, while the binding of this mAb to ACE in epididymal homogenate and to pure seminal fluid ACE are rather high. The epitope for this mAb contains potential site of glycosylation, Asn117 [29]. Moreover, another site of glycosylation, Asn480, is located close to the epitope for mAb5F1 [29]. It was shown earlier [40] that, at least, in serum ACE Asn480 is glycosylated. So, we could suggest that glycosylation of Asn117 and/or Asn480 is dramatically different in prostate ACE in comparison to epididymal and lung ACEs (Fig 1B and 1C).

As a whole, the results allow us to assume that seminal fluid ACE originates likely equally from epididymis and prostate glandular epithelial cells.

## Identification of N-linked glycosylation sites in lung and seminal fluid ACEs

Somatic ACEs isolated from human lung and seminal fluid were digested with trypsin and the resulting peptides were analyzed using MALDI TOF MS. After a primary search of matching tryptic peptides with the MASCOT program, the protein sequence coverage was about 50% in both cases. After finding additional matching peptides using the FindPept tool, the sequence coverage became higher, 61% for the lung ACE and 59% for seminal fluid enzyme. Unglycosylated peptides identified by the FindPept tool in the tryptic digest of the lung ACE are listed in [Table 1](#) and shown in [Fig 2](#); four of these peptides contain potential glycosylation sites, Asn131, 494, 666, and 1196. The same four peptides were found in the trypsin digest of seminal fluid ACE ([Table 1](#)). It should be noted, however, that the intensity of the MS peak corresponding to the peptide 661–677, containing Asn666 as a potential glycosylation site, was extremely small, especially in the case of the lung ACE. It casts doubt on the classification of this peptide to the unglycosylated ones.

Analysis of the mass spectra data with the GlycoMod tool allowed revealing several peaks matching by mass potential glycopeptides in the lung ACE and seminal fluid ACE. After expert revision, we limited the number of putative glycopeptides by several ones with the most favorable structures reported in UniCarbKB. The results are presented in [Table 2](#). It is seen that in some cases the interpretation could be rather equivocal, as the data could be attributed to different peptides within amino acid sequence of ACE. In a few cases, an observed peak could be related to the glycopeptides with the same peptide structure but with different glycan moieties.

Nevertheless, we could state that asparagins 82, 117, 480, 648, 666, and 731 could be glycosylated in human lung ACE. The glycosylated Asn82, 117, and 480 have been found previously in human kidney ACE [\[26\]](#), while the glycosylated Asn480, 666, and 685 have been reported to be present in human plasma ACE [\[40\]](#). As the source of plasma ACE is mainly lung ACE [\[4–5\]](#), we could assume that Asn685 in lung ACE may also be glycosylated. It is worth noting that the peptide 661–677 containing Asn666 was also found among unglycosylated peptides in the tryptic digest of the lung ACE ([Table 1](#)), although the intensity of the corresponding MS peak was very small. Thus, we could not consider this definite N-glycosylation site Asn666 as fully glycosylated, but rather as partially glycosylated. The peptides containing Asn82, 117, 480, 648, and 731 were not found among unglycosylated peptides. So, these sites could be fully glycosylated. There is still some uncertainty in referring the peptides with observed  $m/z$  2468.1 to the peptides containing Asn480 or Asn666, as well as peptide with  $m/z$  5885.4 to the peptides containing either Asn648 or Asn731. The putative N-glycosylation site Asn648 is not attributed to any known epitope for mAbs to ACE, while the N-glycosylation site Asn731 is a part of the epitopes for mAbs 1B8 and 3F10 to the C domain of ACE ([Fig 2](#)). The remarkable difference in the efficiency of these mAbs binding to the lung ACE and seminal ACE ([Fig 1](#)) convincingly shows that the glycosylation of this definite Asn731 is different in the lung ACE and seminal fluid ACE.

Similarly, asparagins in positions Asn117, 416, 648, 666, and 685 could be glycosylated in human seminal fluid ACE. The peptide 661–677 containing Asn666 was also found among unglycosylated peptides ([Table 1](#)), although the intensity of the corresponding MS peak was very small (as in the case of the lung ACE), therefore, this site could be partially glycosylated. Other Asn residues can be considered as fully glycosylated.

**Table 2. N-glycosylated peptides and glycan structures found by the GlycoMod tool in ACE from lung and seminal fluid using MS data.**

<b>Lung ACE</b>				
N-Glycosylation site	Peptide	Observed <i>m/z</i>	Calculated <i>m/z</i>	Putative glycan structures
Asn82	Ala72-Arg89	3853.9	3853.7	(HexNAc) <sub>2</sub> (NeuAc) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
Asn117	Gln109-Arg120	3030.4	3030.3	(HexNAc) <sub>2</sub> (NeuAc) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
Asn480	Asn480-Lys489		2468.0	(Hex) <sub>3</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
and/or		2468.1		
Asn666	Asn661-Lys670		2468.1	(HexNAc) <sub>2</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
				(Hex) <sub>5</sub> (HexNAc) <sub>4</sub> (NeuAc) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub> and/or
Asn648	Thr630-Lys655		5885.4	(Hex) <sub>4</sub> (HexNAc) <sub>4</sub> (Deoxyhexose) <sub>1</sub> (NeuAc) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
and/or		5885.4		
Asn731	Ile714-Arg749		5885.6	(Hex) <sub>4</sub> (HexNAc) <sub>2</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub> and/or
				(Hex) <sub>3</sub> (HexNAc) <sub>2</sub> (Deoxyhexose) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
<b>Seminal fluid ACE</b>				
N-Glycosylation site	Peptide	Observed <i>m/z</i>	Calculated <i>m/z</i>	Putative glycan structures
Asn117	Gln109-Arg120	5469.1	5469.1	(Hex) <sub>5</sub> (HexNAc) <sub>5</sub> (Deoxyhexose) <sub>1</sub> (NeuAc) <sub>4</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
Asn416	Val414-Lys427	3288.5	3288.5	(Hex) <sub>1</sub> (HexNAc) <sub>3</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
Asn648	Thr630-Lys655	5885.2	5885.4	(Hex) <sub>5</sub> (HexNAc) <sub>4</sub> (NeuAc) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub> and/or
				(Hex) <sub>4</sub> (HexNAc) <sub>4</sub> (Deoxyhexose) <sub>1</sub> (NeuAc) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
Asn666	Asn661-Lys670	3245.4	3245.3	(Hex) <sub>3</sub> (HexNAc) <sub>2</sub> (NeuAc) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub> and/or
				(Hex) <sub>2</sub> (HexNAc) <sub>2</sub> (Deoxyhexose) <sub>1</sub> (NeuAc) <sub>1</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
Asn685	Lys677-Lys689	2765.3	2765.3	(Hex) <sub>2</sub> +(Man) <sub>3</sub> (GlcNAc) <sub>2</sub>

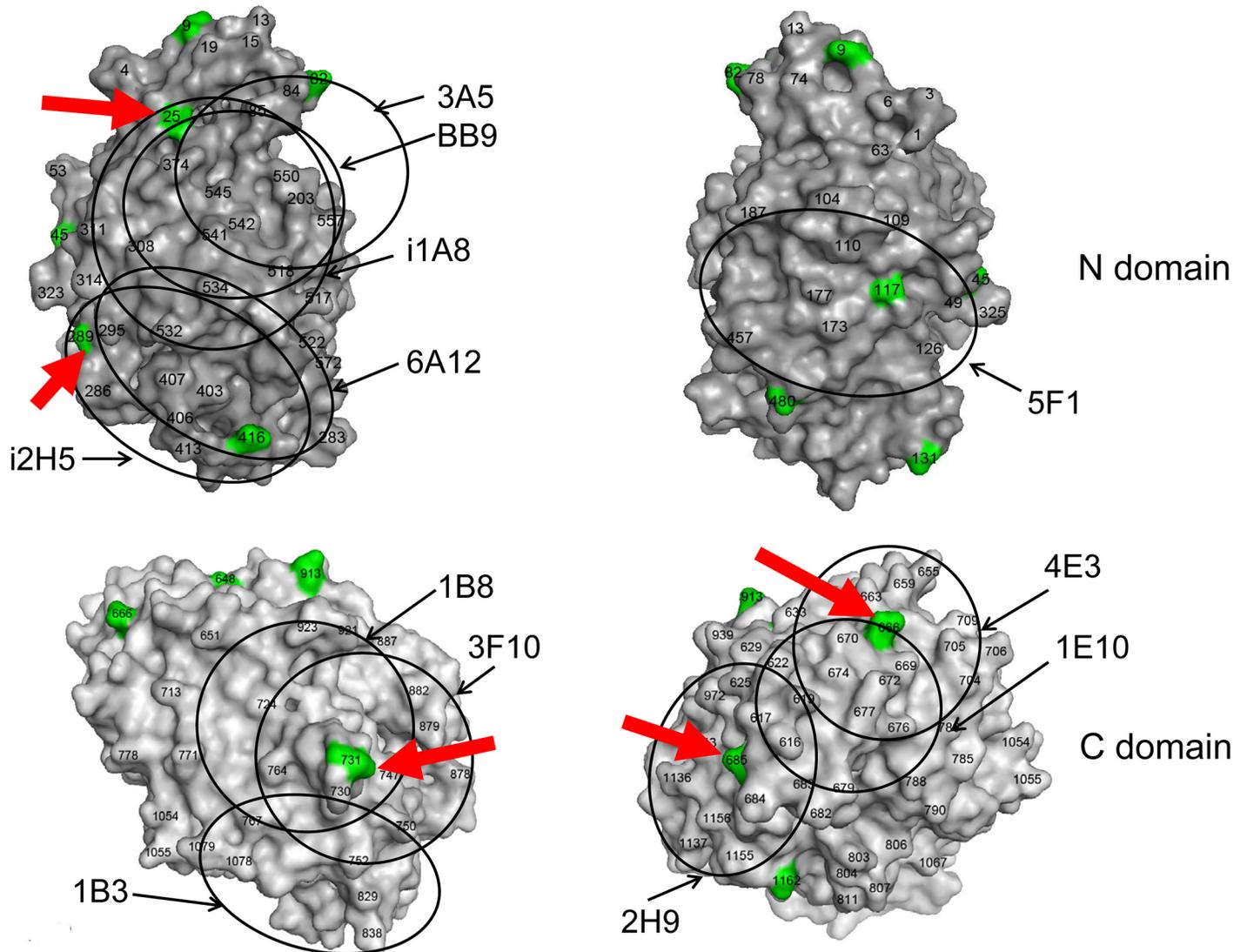
doi:10.1371/journal.pone.0143455.t002

Comparison of the data obtained for lung and seminal fluid ACEs shows that both enzymes contain fully glycosylated Asn117, however, the structure of putative glycans is different, glycan from seminal fluid ACE exhibiting more branches. Both ACEs could contain fully glycosylated Asn648, the suggested glycans being similar in both cases. And, again, both ACEs could contain partially glycosylated Asn666, but the structures of suggested glycans in these ACE forms remarkably differ, glycans in seminal fluid ACE being more complicated. In addition, analysis of the tryptic digest of lung ACE revealed also Asn82, 480, and 731 as occupied N-glycosylation sites, while for seminal fluid ACE occupied N-linked glycosylation sites were found to be 416, and 685. The difference in N-glycosylation of ACE from different sources can be the reason for the remarkable difference in the efficacy of binding of some mAbs to these ACEs (Fig 1), i.e. differences in local ACE conformation.

The N-glycosylation sites on the N and C domains of human ACE are shown in Fig 3. In addition to the putative difference in ACE N-glycosylation revealed by the mass spectra analysis, different N-glycosylation in ACE originated from lung endothelial cells and in ACE from epithelial cells of prostate and epididymis can include Asn25, and 289 on the N domain, as indicated by the mAbs binding.

### Inhibition of seminal fluid and lung ACEs

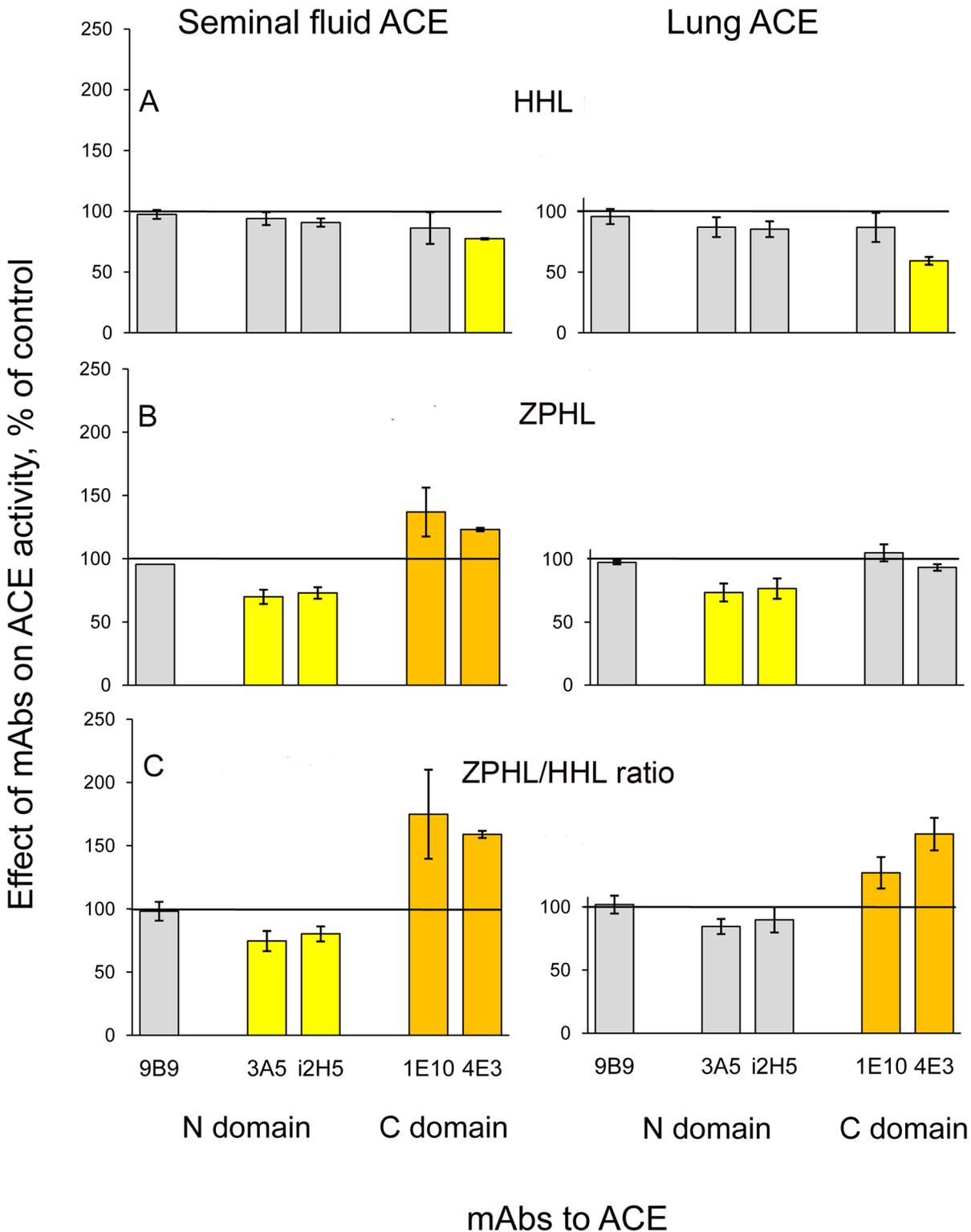
We examined the effect of ACE inhibitors, enalaprilat (analogue of tripeptide) and teprotide (nonapeptide), on enzymatic activity of pure lung and seminal fluid ACEs. Both ACEs were equally inhibited by ACE inhibitors with IC<sub>50</sub> about 6 nM for enalaprilat and 10 nM for teprotide. We also tested the inhibitory effects of mAbs 3A5 and i2H5, anti-catalytic mAb to the N domain [24, 27], and mAbs 1E10 and 4E3, anti-catalytic mAbs to the C-domain [30] on ACE activity (Fig 4). Inhibitory effect of both mAbs to the N domain, 3A5 and i2H5, at tested concentrations was significant towards both ACEs only with a substrate ZPHL (Fig 4A and 4B).



**Fig 3. The structures of N and C domains of ACE with potential glycosylation sites and epitopes for mAbs.** Human N domain structure was based on PDB P2C6N and C domain structure—based on PDB 1O86. The epitopes were marked on the N and C domains according to [27–31, 41]. The positions of the epitopes for some mAbs (12 out of 17) are shown by circles on both sides of domain globule. The potential sites of N-glycosylation, 9 on the N domain and 6 on the C domain, are marked by green; Asn494 on the N domain is not seen while Asn1196 is not present in structure of the C domain. The glycosylation sites which might be differently glycosylated in seminal fluid ACE and lung ACE are shown by arrows. Some amino acid residues are shown by numbers according to [38] for orientation.

doi:10.1371/journal.pone.0143455.g003

However, these mAbs can be considered as more effective towards seminal fluid ACE, as reflected by a more prominent decrease in the sensitive parameter ZPHL/HHL ratio (Fig 4C), this decrease indicating preferable inhibition of the N domain of ACE [22]. Inhibitory effect of mAbs, anti-catalytic to the C domain, was epitope-, substrate-, and tissue-specific. Both mAbs, 1E10 and 4E3, more effectively inhibited ACEs with HHL as a substrate, mAb 4E3 causing more noticeable effect than mAb 1E10 and more effectively inhibiting lung ACE activity (Fig 4A and 4B). However, an effect of these mAbs on ACE activity with ZPHL as a substrate was rather paradoxical: the binding of anti-catalytic mAbs to the C domain of seminal fluid ACE, but not to the lung ACE, caused reliable enhancement of ACE activity (Fig 4B). We can consider this fact as an indication of the putative conformational changes in the N domain of



**Fig 4. Effect of anti-catalytic mAbs on the activity of pure seminal fluid and lung ACEs.** Pure seminal fluid and lung ACEs (5 mU/ml with ZPHL as a substrate) were incubated with mAbs (10 ug/ml), which are anti-catalytic for the N-domain active center, i2H5 and 3A5 [27], and for the C domain active center, 1E10 and 4E3 [30], of ACE. Residual ACE activity was determined with substrates HHL (A) and ZPHL (B) and is presented as the ratio of ACE activity in the presence of mAbs to that without mAbs. Data are also presented as ZPHL/HHL ratio of ACE activity in the presence of mAbs to that without mAbs (C). Results are the mean  $\pm$  SD of 2–4 experiments, made in duplicates.

doi:10.1371/journal.pone.0143455.g004

seminal fluid ACE, which are induced by the binding of mAbs to the adjacent C domain and accompanied by the increase of ACE N domain activity.

## Effect of potential ACE-binding partners on seminal and lung ACEs

How these remarkable differences in local conformation of seminal fluid and lung ACEs may be converted into different regulation of the functions of these ACEs, including the regulation of their shedding? We demonstrated recently that the rate of recombinant human ACE shedding from the surface of CHO-ACE, HEK-ACE and endothelial cells in the presence of bovine and human serum was dramatically diminished, up to 2-times. However, the extent of the serum effect varied depending on the type of serum and cells (unpublished observation).

Recently, it was found that albumin may bind to ACE [46–47]. Bearing in mind that albumin concentration in the seminal fluid (1 mg/ml) is 50-fold less than in the blood [48] it was very tempting to suggest that 50-fold increase in ACE concentration in seminal fluid is solely explained by 50-fold decrease in albumin concentration. Therefore, we estimated the effect of inactivated human plasma, inactivated seminal fluid, as well as pure human and bovine albumins, on mAbs binding to pure lung and seminal fluid ACEs (Fig 5).

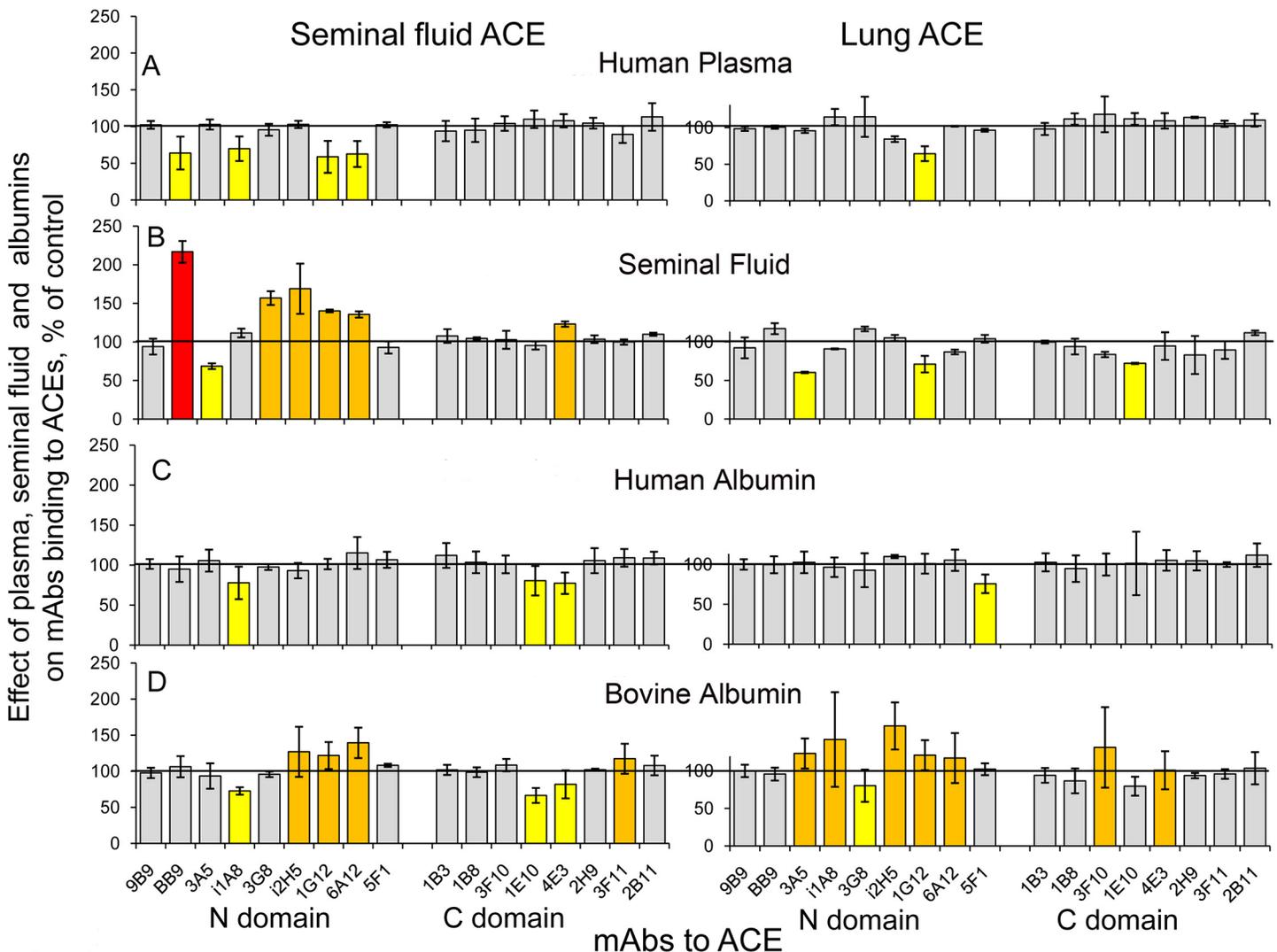
Effect of inactivated human plasma on mAbs binding to two types of ACE differed significantly (Fig 5A), with seminal fluid ACE being more sensitive to the presence of plasma. Effect of inactivated seminal fluid on mAbs binding to two types of ACE also showed more prominent effect for seminal fluid ACE (Fig 5B), strengthening our suggestion that different conformations of these ACEs can participate in different regulation of the functions of ACEs on endothelial and epithelial cells. It is worth noting that only a small percentage of the overall effect of plasma on mAbs binding to ACE could be attributed to albumin (Fig 5C), namely, a decrease in binding of mAb i1A8, which is seen only for seminal fluid ACE. We also should state that an effect of pure albumins is strongly species specific (Fig 5C and 5D). While the effects of human and bovine albumins were rather equal on mAbs i1A8, 1E10, and 4E3 binding to seminal fluid ACE, only bovine albumin caused the increase of mAbs i2H5, 1G12, and 6A12 (with overlapping epitopes) binding to this enzyme. Alternatively, only human albumin caused the decrease of mAb 5F1 binding to lung ACE (without effect on the binding of other mAbs to lung ACE), whereas bovine ACE caused multiple conformational changes in this ACE.

## LMW component from blood also binds to ACE

We found that Low Molecular Weight (LMW) component from human plasma binds to human ACE (unpublished observation). Therefore, we tested an effect of human plasma 3kDa filtrate, containing LMW component (-s), on mAbs binding to both structurally different ACEs and showed that this filtrate had a strong (and different) effect on both types of ACE (Fig 6).

Comparison of the effect of 3 kDa human plasma filtrate (Fig 6B) with the effect of heat-inactivated human plasma (Fig 6A) clearly demonstrates that LMW components of human plasma exhibited more significant effect on mAbs binding to ACEs than proteins within this plasma.

We identified recently a bilirubin as one of the components of 3 kDa plasma filtrate which is able to bind to ACE in the region of the overlapping epitopes 1G12/6A12 (unpublished observation). Therefore, we tested an effect of this novel ACE-binding partner, bilirubin, on two different, tissue specific ACE types (alone or in combination with human albumin), seen on Fig 6C and 6D. Again, the effect of bilirubin on ACE was strongly tissue specific: significant effect of bilirubin on mAbs binding to the N domain of lung ACE, while less pronounced for



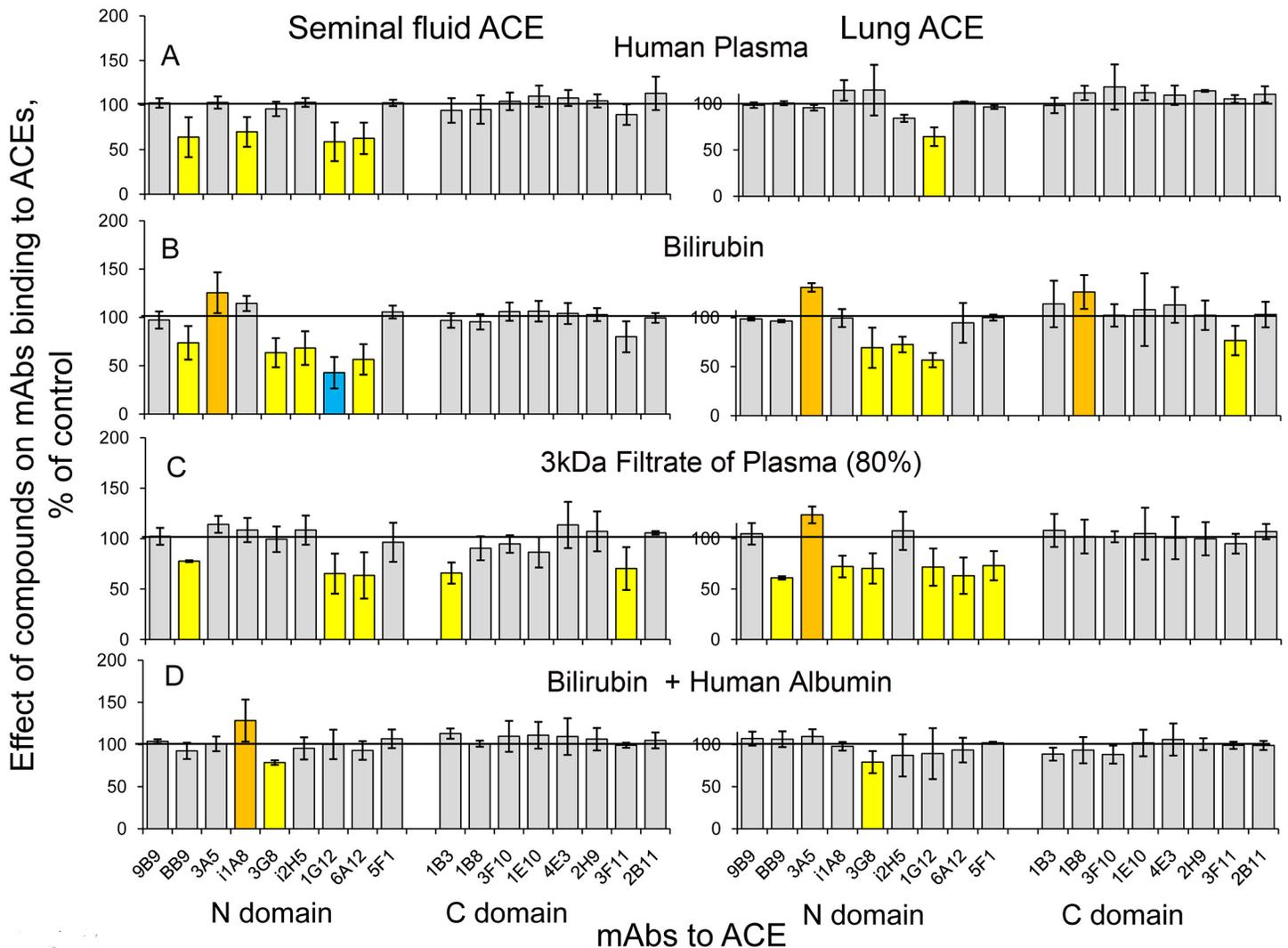
**Fig 5. Effect of human plasma, seminal fluid and albumins on mAbs binding to ACEs.** ACE activity immunoprecipitated by 17 mAbs to ACE (as in Fig 1) was presented as a normalized value (“binding ratio”) to highlight differences in immunoprecipitation pattern (“conformational fingerprint”) after adding heat-inactivated human citrated plasma, heat-inactivated seminal fluid, as well as human and bovine albumins to purified seminal fluid and lung ACEs with that without additives. **A-B.** Effect of 20% of heat-inactivated human plasma (**A**) and heat-inactivated seminal fluid (**B**); **C-D.** Effect of human (**C**) and bovine (**D**) albumins at concentrations of 8 mg/ml (similar to albumin concentration in 20% serum). Data are presented as in Fig 1.

doi:10.1371/journal.pone.0143455.g005

seminal fluid ACE, and visible effect on the mAbs binding to the C domain of seminal fluid ACE without any effect on the C domain of the lung ACE (Fig 6C).

The fact that human albumin practically abolished an effect of bilirubin on mAbs binding to both ACEs (Fig 6D) shows that only unbound bilirubin (which represents only minor part (about 10%) of total bilirubin content in the blood [49] can bind to ACE.

Thus, significant differences in local conformation of lung ACE (originated from endothelial cells) and seminal fluid ACE (originated from epithelial cells of prostate and epididymis) allow us to suggest that possible regulation of ACE shedding from epithelial cells of male reproductive tract and from endothelial cells of capillaries (blood vessels) by the constituents of blood and seminal fluid may be different due to different effect of possible regulators of ACE shedding on the structurally different ACEs in these cells.



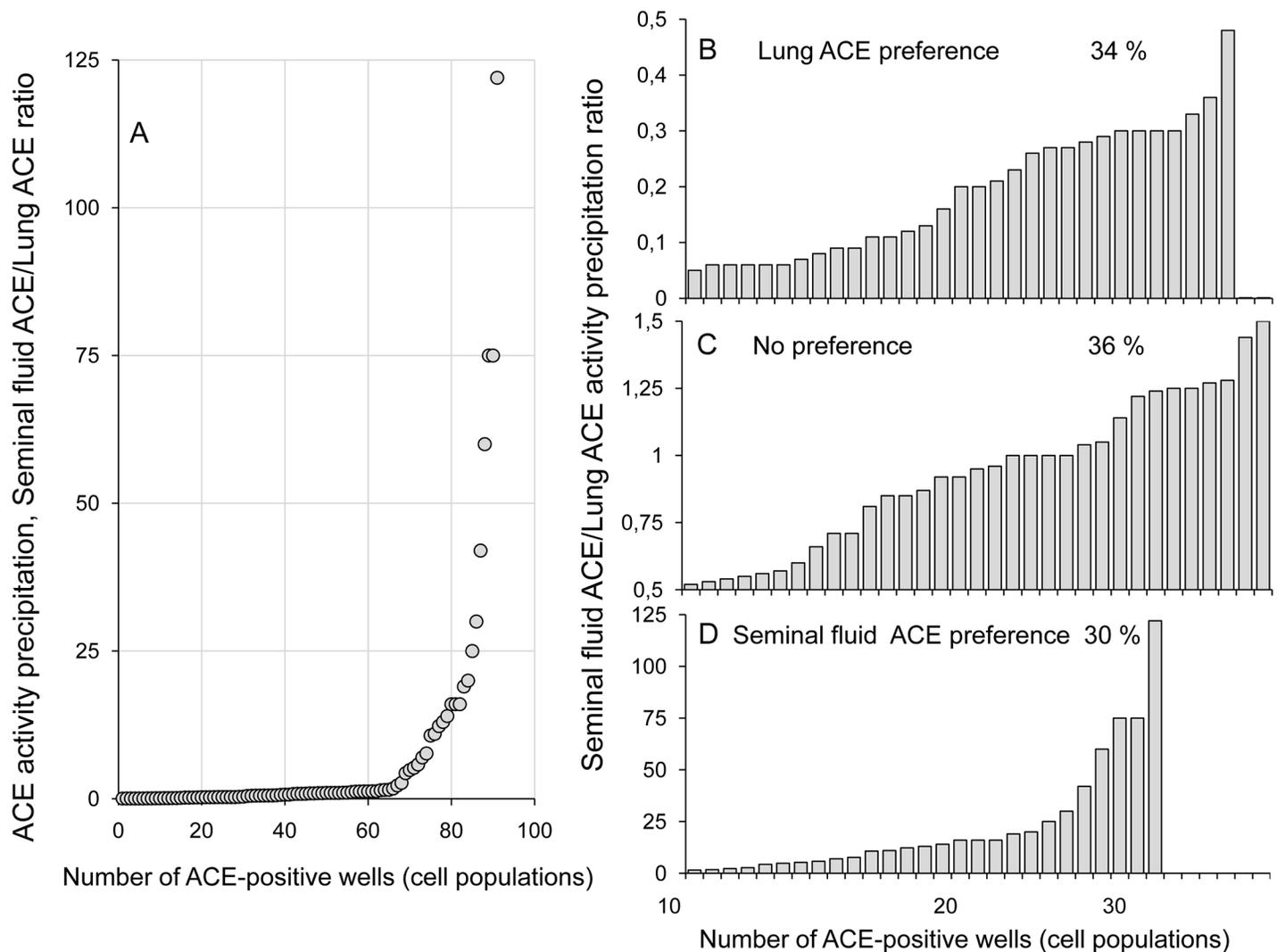
**Fig 6. Effect of different additives on mAbs binding to seminal fluid and lung ACEs.** ACE activity immunoprecipitated by 17 mAbs to ACE (as in Fig 1) was presented as a normalized value (“binding ratio”) to highlight differences in immunoprecipitation pattern (“conformational fingerprint”) after adding of tested compounds to purified seminal fluid and lung ACEs with that without additives. (A) Effect of 20% of human heat-inactivated plasma. (B) Effect of 80% 3 kDa filtrate of human citrated plasma. C-D. Effect of bilirubin (150 ug/ml) in the absence (C) or presence (D) of human albumin at 8 mg/ml concentration (which correspond to its concentration in 20% serum). Data are presented as in Fig 1.

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In addition, membrane-bound glycoproteins (including ACE) may often be shed into blood circulation as a by-product of the disease process. Recently an increase in blood ACE level was demonstrated for patients with epithelial ovarian carcinoma [50]. Likely, such an increase may be explained by an increase of local vascular permeability induced by the increased VEGF production by tumor tissues.

Significant structural differences in ACE from endothelial cells (major source of blood ACE) and ACE from epithelial cells, particularly prostate ACE (demonstrated in this study) and, likely, ovary ACE, may be the base for the development of specific mAbs, which can be used to identify the tissue- or cell source of increased ACE in blood in different diseases. In order to estimate the possibility of generating monoclonal antibodies which would discriminate between endothelial and epithelial ACEs we attempted immunization of mice with pure

somatic ACE from seminal fluid which is the mixture of prostate and epididymal ACEs [15–16]. We used the hybridoma fusion technology to dissect the immune response based on primary screening of post-fusion populations. This would estimate the possibility of generation of tissue ACE-specific antibodies and, eventually, establish antibody-producing hybridoma clones. The results of primary screening of 670 post-fusion populations are shown in Fig 7. The positive response was detected in 91 wells (Fig 7A). The 36% of positive populations reacted rather equally with seminal fluid and lung ACEs (SF/Lung ratio equals to 0.5–1.5, i.e. no significant preference, Fig 7C). The 34% of positive populations demonstrated preference of different degree towards seminal fluid ACE (Fig 7B), while 30% showed preference of different degree to lung ACE (Fig 7D). Therefore, even at the level of polyclonal immune response against seminal



**Fig 7. Primary immune response in mice to pure somatic ACE from seminal fluid.** Culture fluids from 670 post-fusion cell populations grown in 96-well plates (primary screening) were analyzed for the presence of antibodies to seminal fluid ACE and lung ACE in parallel in plate precipitation assay (as in Fig 1). Presence of antibodies to seminal fluid and/or lung ACE was detected in 91 wells by precipitated ACE activity and the data are presented as the ratio of ACE activity precipitation from seminal fluid ACE to that from lung ACE (SF/Lung ratio). Discrimination of these two ACEs by antibodies from these positive wells was observed in a wide range (A). Besides expected antibodies, recognizing both ACEs (C) with SF/Lung ratio in the region from 0.5 to 1.5, we identified significant proportions of antibodies which preferentially recognized seminal fluid ACE (B) with SF/Lung ratio more than 1.5, and antibodies which preferentially recognized lung ACE (D) with SF/Lung ratio less than 0.5, correspondingly.

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fluid ACE it was possible to detect spectrum of antibodies with different preferences to tissue specific ACEs.

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## Author Contributions

Conceived and designed the experiments: OAK SMD. Performed the experiments: OVK VET GFK INT SMD. Analyzed the data: OVK IVU OAK INT AVG SMD. Contributed reagents/materials/analysis tools: EZG DES ROD SMD VVE. Wrote the paper: SMD OAK INT.

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