

## **Supplementary materials**

### **Supplementary methods**

#### ***Animals, immunization and antibodies***

Chicken and rabbit antibodies directed against citrullinated or carbamylated vimentin were produced by Davids Biotechnology (Regensburg, Germany) by immunizing the animals according to the manufacturer's immunization protocol. To isolate specific antibodies, IgG fractions of rabbit or human antibodies were first prepared with a Protein G column (GE healthcare) according to the manufacturer's instruction. In case of chicken antibodies, IgY was prepared by Davids Biotechnology with the established 12 % polyethylenglycol precipitation method. To eliminate anti-MCV and anti-vimentin antibodies, the corresponding recombinant proteins were conjugated to CNBr-sepharose (GE Healthcare) (1 mg protein/ml equilibrated sepharose) according to the manufacturer's instructions and stored at 4°C in PBS with 0.1%BSA. An equal volume of animal antibodies and MCV/vimentin affinity sepharose was incubated over night at 4°C. The mixture was applied to an empty column and the flow through immunoglobulins were characterized and used as anti-carbamylated vimentin specific antibodies. Additional, the bound anti-MCV and anti-vimentin antibodies were eluted with 3.5 M MgCl<sub>2</sub> (pH 7.5). The buffer of antibodies containing fractions was exchanged against 1X PBS/ 20 % glycerine using a column (Zeba Spin; Pierce).

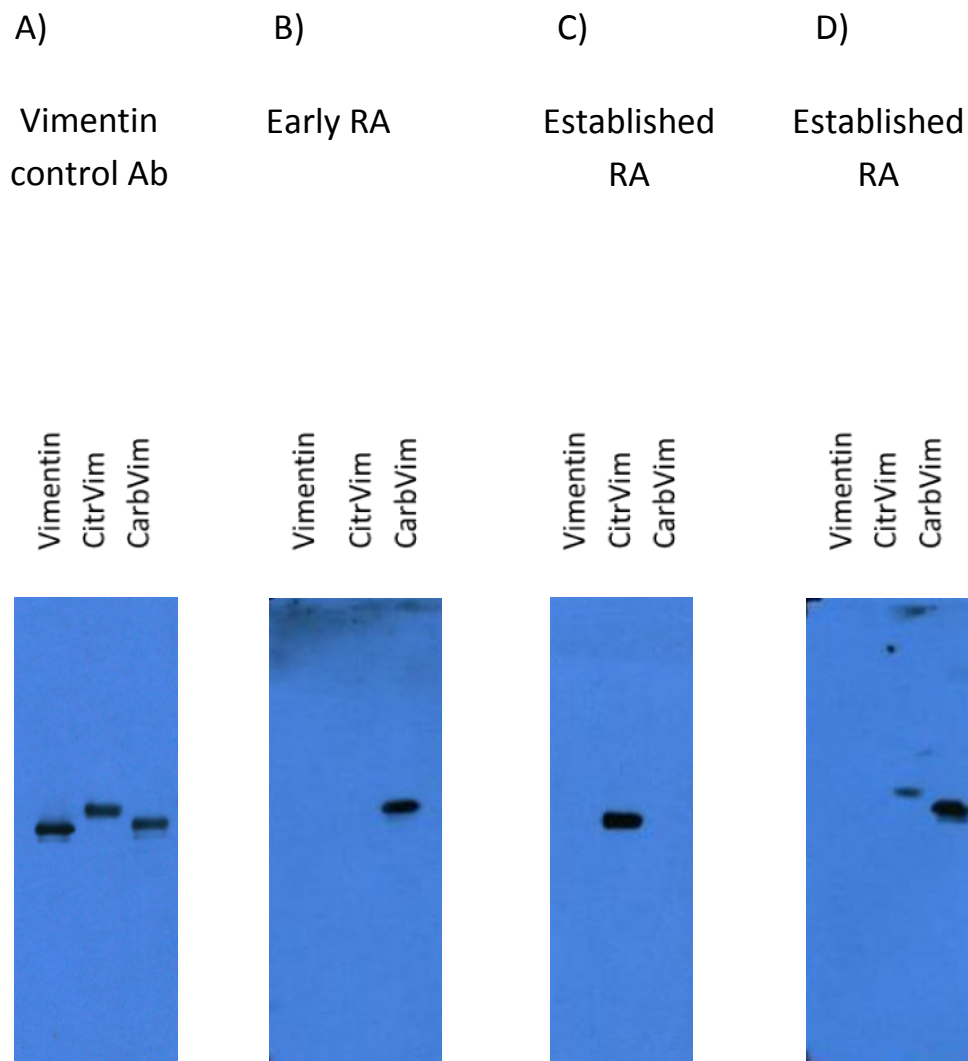
#### ***Cell fractions as source for modification of antigens***

HeLa cell (CILBIOTECH s.a., MONS, Belgium) extraction starts with gentle sedimentation and washing of the cells. To prepare cell fractions (F1 = cytosol, F2 = membranes, F3 = nuclear fraction and F4 = cytoskeleton fraction), a ProteoExtract® Subcellular Proteome Extraction Kit was used (Calbiochem, Cat. No. 539790). Briefly, 1 x 10<sup>7</sup>HeLa cells were incubated with Extraction Buffer I and cytosolic

proteins were released (fraction 1). Subsequently, membranes and membrane organelles were solubilized with Extraction Buffer II, without impairing the integrity of nucleus and cytoskeleton (fraction 2). Next, nucleic proteins were enriched with Extraction Buffer III (fraction 3). Components of the cytoskeleton were finally solubilized with Extraction Buffer IV (fraction 4). Samples of each fraction containing approximately 50 µg protein were mixed with an equal volume of carbamylation buffer (200 mM KOCN, 50 mM Hepes, 4 M urea, pH 7.5) and incubated for 2 h at 56°C. Additionally, a corresponding amount of protein from each fraction was citrullinated in peptidylarginine deiminase (PAD)-buffer (50 mM Tris, 2 M Urea, 5 mM CaCl<sub>2</sub>, 2 mM DTT, 0.5 mM EDTA, pH 7.4) with 1 U PAD enzyme (specific activity 0.11 U / µl, Sigma Aldrich, Germany). Excess of chemical reagents was removed with Zeba Spin desalting mini columns. Modified and untreated fractions were mixed with equal volumes of 2 × Laemmli sample buffer, boiled, and separated by SDS-PAGE (4-12 % NuPAGE, Invitrogen). Proteins were transferred to nitrocellulose and unspecific surface binding was blocked by incubation with 1 % BSA in 1X PBS.

## Supplementary figures and tables

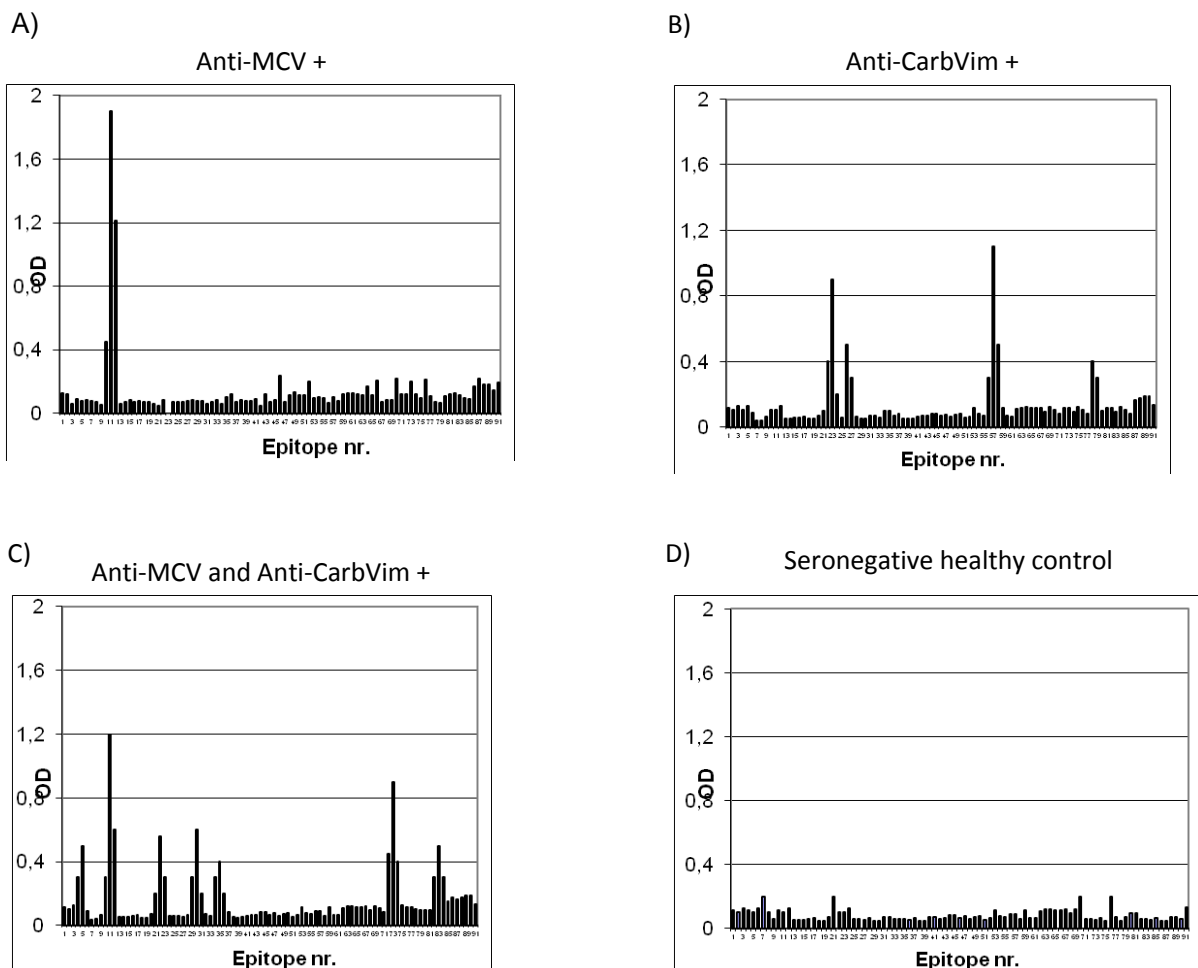
**Figure S1.**



**Figure S1. Recognition patterns against vimentin, citrullinated and carbamylated variants of vimentin using representative sera.** 20  $\mu$ g of vimentin and its citrullinated (CitrVim) and carbamylated (CarbVim) isoforms were mixed with an equal volume of 2  $\times$  Laemmli sample buffer, boiled, and separated by SDS-PAGE (4-12 % NuPAGE, Invitrogen). Proteins were transferred to nitrocellulose membranes, which were blocked using blocking buffer (PBS containing 5% non-fat dried milk and 0.1% NP-40) for one hour at room temperature and incubated for at

least 8 hours at 4°C with the diluted sera (1:1000) **A)** Anti-vimentin control antibodies, **B)** Patient with positive anti-CarbVim antibodies, **C)** Patient with positive ACPAs and **D)** Patients with positive ACPAs as well as anti-CarbVim antibodies. After washing with blocking buffer, bound antibodies were detected by incubation with horseradish-peroxidase conjugated secondary antibodies anti-human IgG (Dianova, Germany) or anti-rabbit (Santa Cruz) and the blots were developed using the ECL system (Amersham Biosciences).

**Figure S2.**



**Figure S2. Epitope recognition patterns using citrullinated peptides of vimentin.** A) Patients single positive for ACPAs, B) Patients single positive for anti-

CarbVim or C) Patients sera double positive for ACPAs and anti-CarbVim in comparison to D) healthy controls (with n=10 different serum samples per group).

**Table S1**

Sequence	Name	Code designation
GGVYATXSSAVR	Native vimentin peptide	
GRVYATRSSAVR	Mutated vimentin peptide	
GRVYATXSSAVR	Mutated citrullinated peptide (X = citrulline)	P18
GRVYATXSSAVR	Mutated carbamylated peptide (X = Homocitrulline)	HC52

**Table S1.** Amino acid sequence of used vimentin peptides. The native unmodified vimentin was used to synthesize modified peptides of vimentin. To increase the sensitivity of the citrullinated peptide, the glycine residue in position 2 was mutated into an arginine amino acid residue. The designated position X was substituted either with the citrulline to synthesize CitrVim (P18) or with the homocitrulline to synthesize CarbVim (HC52).

**Table S2.**

<b>Smokers</b>		<b>A MCV IgG positive patients (n = 27)</b>	<b>A MCV IgG negative patients (n = 15)</b>
A P18 IgG	positive (n, %)	24, (88.89 %)	3, (20 %)
	negative (n, %)	3, (11.11 %)	12, (80 %)
A HC52 IgG	positive (n, %)	15, (55.55 %)	0.00
	negative (n, %)	12, (44.44 %)	15, (100 %)
RF IgM	positive (n, %)	18, (66.67 %)	3, (20 %)
	negative (n, %)	9, (33.33 %)	12, (80 %)
<b>Non-smokers</b>		<b>A MCV IgG positive patients (n = 25)</b>	<b>A MCV IgG negative patients (n = 43)</b>
A P18 IgG	positive (n, %)	22, (88 %)	6, (13.95 %)
	negative (n, %)	3, (12%)	37, (86.05%)
A HC52 IgG	positive (n, %)	12, (48%)	5, (11.63%)
	negative (n, %)	13, (52 %)	38, (88.37 %)
RF IgM	positive (n, %)	20, (80 %)	7, (16.28 %)
	negative (n, %)	5, (20 %)	36, (83.72 %)
<b>Ex-smokers</b>		<b>A MCV IgG positive patients (n = 18)</b>	<b>A MCV IgG negative patients (n = 16)</b>
A P18 IgG	positive (n, %)	16, (88.89 %)	1, (6.25 %)
	negative (n, %)	2, (11.11 %)	15, (93.75 %)
A HC52 IgG	positive (n, %)	7, (38.89 %)	1, (6.25 %)
	negative (n, %)	11, (61.11 %)	15, (93.75 %)
RF IgM	positive (n, %)	15, (83.33 %)	3, (18.75 %)
	negative (n, %)	3, (16.67 %)	13, (81.25 %)

**Table S2.** Coincidence of anti citrullinated peptide of vimentin IgG (A P18 IgG), anti carbamylated peptide of vimentin IgG (A HC52 IgG) and rheumatoid factor IgM (RF IgM) antibodies with anti mutated citrullinated vimentin IgG (A MCV IgG) positive and negative rheumatoid arthritis (RA) patients classified according to the smoking status.