RAPID COMMUNICATION

# Identification of galectin-3 as a novel potential prognostic/predictive biomarker and therapeutic target for cerebral cavernous malformation disease

Cerebral cavernous malformation (CCM) is a major cerebrovascular disease of genetic origin characterized by abnormally dilated capillaries and a wide spectrum of symptoms, including headaches, seizures, neurological deficits, and intracerebral hemorrhage. Its unpredictable clinical course and the current lack of therapies make the identification of prognostic and predictive biomarkers an imperative research challenge.[1] Herein, we provide evidence that galectin-3 (Gal-3), a major tissue and circulating biomarker of oxidative stress and inflammation, is significantly up-regulated both in CCM patients and experimental models. Specifically, whole transcriptome sequencing, qRT-PCR, and Western blotting studies demonstrated a significant up-regulation of Gal-3 expression levels both in surgical CCM specimens and in blood samples of CCM patients. Moreover, immunohistochemical analyses showed strong Gal-3 immunoreactivity in CCM lesion endothelial cells and infiltrating leukocytes. Furthermore, using cellular and animal models, we found that Gal-3 expression levels are inversely correlated with those of *KRIT1*, the major causative gene for CCM disease,[2] implying a functional relationship. Overall, our findings demonstrate for the first time that Gal-3 up-regulation occurs in CCM disease and is linked to a causative gene, suggesting that it may serve as a useful biomarker of prognostic and predictive value for risk stratification and treatment of CCM patients.

A growing body of evidence indicates that Gal-3 plays a prominent role in chronic inflammation and is implicated in the development of many disease conditions, including cerebrovascular diseases.[3] However, the potential association between Gal-3 and CCM disease remains uncharacterized so far. To address such putative association, we first carried out a re-examination of RNA-sequencing (RNA-seq) datasets from our previous next-generation sequencing (NGS)-based comparative study of gene expression profiles in surgically resected human CCM lesions (n = 10) and control brain tissues (n = 4).[4] Specifically, a total of 4,928 protein-coding genes (PCGs) were identified to be significantly differentially expressed in human CCM versus control brain tissue samples, including 2,290 up-regulated and 2,638 down-regulated PCGs. An absolute log-fold change cutoff of 1.5 and a corrected *P*-value ($P < 0.01$) were applied to select significantly differentially expressed transcripts. The mRNA expression levels of the Gal-3 gene (*LGALS3*), one of the 2,290 up-regulated PCGs significantly increased in human CCM versus control tissue samples (log-fold change: 4.1; $P < 0.001$) (Fig. 1A), suggesting that an association between Gal-3 expression levels and CCM disease may indeed exist.

To verify and extend the RNA-seq results, we performed quantitative real-time PCR (qRT-PCR) analysis of Gal-3 mRNA levels in an independent set of surgically resected human CCM lesions and control brain tissues, as well as in plasma samples from CCM patients and healthy controls. The outcomes of these comparative analyses confirmed the strong up-regulation of Gal-3 mRNA levels in human CCM versus control brain tissue samples (Fig. 1B) and showed that significant up-regulation of Gal-3 mRNA levels can be also detected in plasma samples of CCM patients as compared to healthy controls (Fig. 1C).
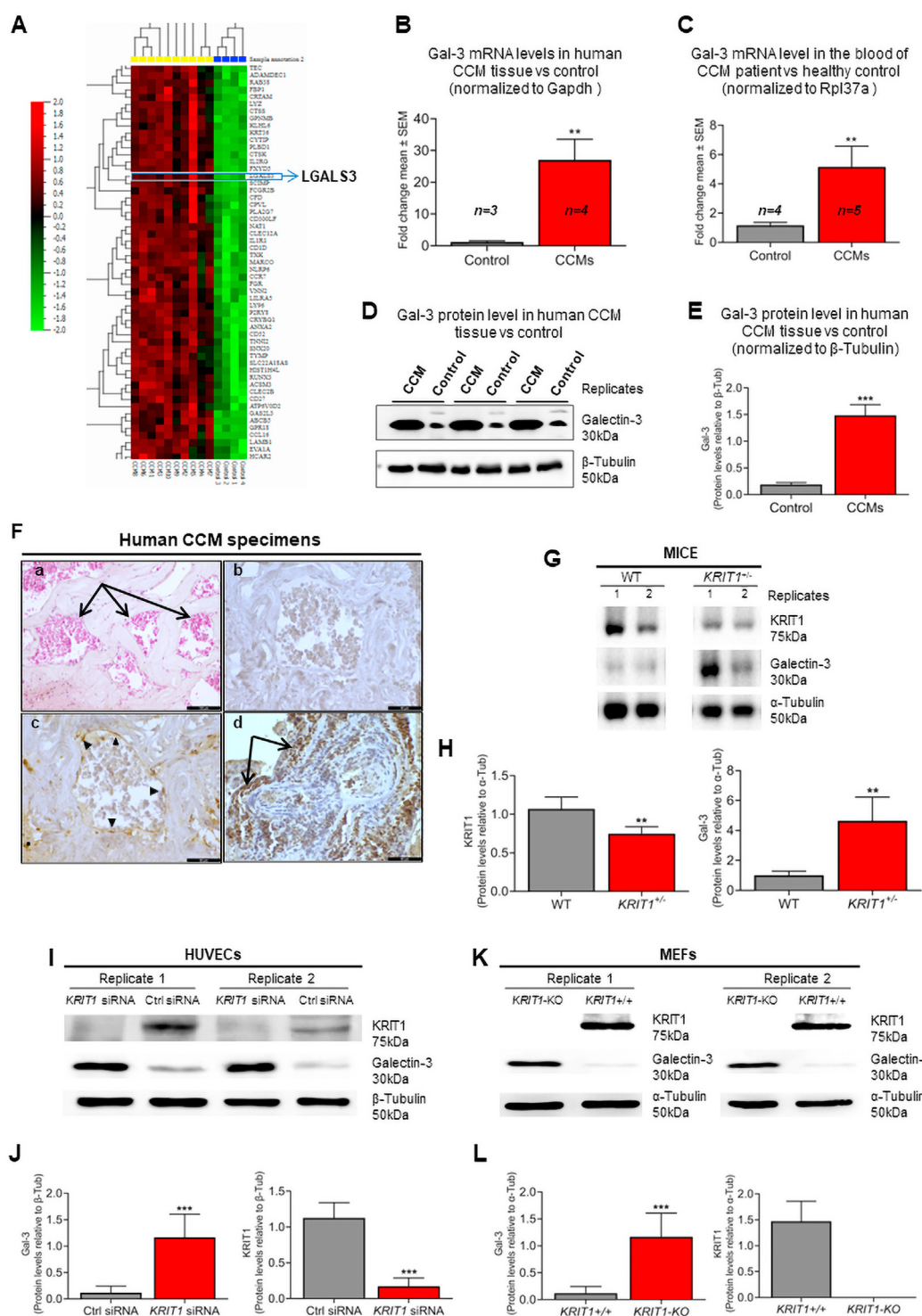
Western blot analysis was then performed to determine whether the significant difference in Gal-3 mRNA expression levels detected in surgically resected human CCM lesions versus control brain tissues was reflected at the protein level. The outcomes of this analysis demonstrated a significant up-regulation of Gal-3 protein levels in distinct

**Figure 1** Gal-3 expression levels were significantly up-regulated both in surgical CCM specimens and in blood samples of CCM patients. Moreover, they were inversely correlated with the expression levels of *KRIT1* in both cellular and animal models. **(A)** Transcriptome profiles showing 4928 protein-coding genes (PCGs) significantly differentially expressed in human CCM tissues. The heatmap shows the differential expression of PCGs between human CCM specimens ($n = 10$) and control brain tissues ($n = 4$) (GEO accession number: GSE137596). Up-regulated and down-regulated expressions are indicated in red and green colors, respectively. Galectin-3 (*LGALS3*) was identified among the significantly up-regulated PCGs in human CCM tissues, as highlighted in the inset image. Statistical significance was calculated by a two-tailed Student's *t*-test, and a log-fold change cutoff of $\pm 1.5$ and a *P* value of $<0.01$ were applied to select the most significantly differentially expressed PCGs. **(B—E)** A significant increase in Gal-3 mRNA expression levels occurred both in CCM lesions and blood samples of CCM patients and was reflected at the protein level. (B, C) Bar diagrams representing the fold change mean ± SEM in Gal-3 mRNA levels in human brain CCM tissue samples (CCM; $n = 4$) *vs.* paired

surgically resected human CCM lesions ($n = 3$) as compared to control brain tissues (Fig. 1D, E), suggesting that the increased expression of Gal-3 associated with CCM disease occurs both at the mRNA and protein levels.

To assess the localization of Gal-3 expression in human CCM lesions, histological sections were prepared from paraffin-embedded surgically resected CCM specimens and analyzed by hematoxylin and eosin (H&E) staining (Fig. 1F, panel a) and immunohistochemistry (IHC) (Fig. 1F, panels b–d). H&E staining showed the presence of multiple CCM caverns lined by a thin endothelium layer (Fig. 1F, panel a, arrows), whereas IHC with a specific Gal-3 antibody showed a positive staining both in endothelial cells lining CCM lesions (Fig. 1F, panel c, arrowheads) and in associated infiltrating leukocytes (Fig. 1F, panel d, arrows). Serial histological sections incubated with non-immune serum instead of primary antibody showed no staining, serving as negative controls (Fig. 1F, panel b). Remarkably, while the presence of infiltrating leukocytes within CCM lesions is consistent with previous reports, our finding that Gal-3 is significantly expressed in such infiltrating leukocytes raises the possibility that it may contribute to long-term neuro-inflammatory responses associated with CCM disease progression and severity. Accordingly, Gal-3 up-regulation has been clearly implicated in neuroinflammation,[5] and shown to facilitate the binding of inflammatory leukocytes to the endothelium by cross-linking carbohydrates on the respective cells.

controls (Control; $n = 3$) (B), and in blood samples of CCM patients (CCM; $n = 5$) vs. healthy controls (Control; $n = 4$) (C), as determined by qRT-PCR gene expression assays. The housekeeping genes GAPDH (B) and Rpl37a (C) were used as internal reference controls for normalizing the relative Gal-3 mRNA expression levels. **$P < 0.001$, Student's $t$-test of three independent assays. (D) Representative Western blot analysis of Gal-3 protein ($\sim$30 kDa) expression levels in three distinct surgically resected human CCM lesions (CCM) and control brain tissues (Control). $\beta$-tubulin ($\sim$52 kDa) was used as internal loading control for normalizing the relative Gal-3 protein expression levels. (E) Bar diagram representing the relative Gal-3 protein expression levels in human CCM lesions (CCM) vs. control brain tissues (Control). Semiquantitative densitometric analysis of protein bands on Western blots was performed with the ImageJ software, and relative quantification values were expressed as a ratio of the mean optical density (OD) of each Gal-3 band in CCM vs. control samples upon normalization with the OD of corresponding $\beta$-tubulin bands ($n = 3$ per group). Note that Gal-3 mRNA levels were significantly up-regulated both in CCM lesions (B) and blood samples (C) of CCM patients. Moreover, the significant increase in Gal-3 mRNA expression detected in CCM lesions (B) was clearly reflected at the protein level (D, E) (**$P < 0.0005$). Statistical significance was calculated by a two-tailed Student's $t$-test, and a $P$-value $\leq 0.05$ was considered statistically significant. **(F)** Immunohistochemical analysis showing that Gal-3 was highly expressed both in endothelial cells lining human CCM lesions and in infiltrating leukocytes. (F, panel a) Representative hematoxylin and eosin (H&E) staining of a histological section from a surgically resected human CCM lesion showing a cluster of abnormally enlarged capillaries (caverns) filled with blood cells and lined by a thin endothelium layer (arrows). (F, panel b) Negative immunohistochemical control, incubated with non-immune serum, showing no staining. (F, panel c) Representative immunohistochemical analysis of Gal-3 expression in histological sections obtained from surgically resected human CCM lesions. Note that there was positive Gal-3 staining in endothelial cells lining a large CCM cavern (solid arrowheads). Focal Gal-3 staining was also observed within the perilesional brain tissue (star). (F, panel d) Human CCM lesion showing infiltrating leukocytes as dark brown dots positive for Gal-3 antibody (arrows). **(G, H)** Gal-3 expression is up-regulated in the brain of *KRIT1* heterozygous mice. (G) Representative Western blot analysis of KRIT1 and Gal-3 expression levels in brain hemispheres of 26-week-old *KRIT1* heterozygous (*KRIT1*$^{+/-}$) mice and wild-type (WT) littermates. The housekeeping $\alpha$-tubulin protein was used as an internal reference control for normalizing the relative KRIT1 and Gal-3 protein expression levels. Two biological replicates are shown for each sample and are representative of three independent experiments. **(H)** Bar diagrams representing the densitometric analysis of Gal-3 and KRIT1 Western blot bands (left and right panels, respectively) upon normalization with the OD of corresponding $\alpha$-tubulin bands ($n = 6$ per group). Semiquantitative densitometric analysis of Western blot bands was performed with the ImageJ software, and relative quantification values were expressed as a normalized ratio of Gal-3 and KRIT1 OD mean with respect to $\alpha$-tubulin. Note that the down-regulation of KRIT1 protein levels in the brain of *KRIT1*$^{+/-}$ mice vs. WT littermates was associated with a significant upregulation of Gal-3 protein levels (**$P < 0.05$). Statistical significance was calculated by a two-tailed Student's $t$-test, and a $P$-value $\leq 0.05$ was considered statistically significant. **(I–L)** Gal-3 expression was strongly up-regulated in both *KRIT1*-silenced endothelial cells and *KRIT1*-knockout MEF cells. (I) Representative Western blot analysis of KRIT1 and Gal-3 expression levels in human umbilical vein endothelial cells (HUVECs) subjected to two rounds of transfection with short interfering RNAs (siRNAs) against KRIT1 (*KRIT1* siRNA) or scrambled control siRNAs (Ctrl siRNA). The housekeeping $\beta$-tubulin protein was used as an internal reference control for normalizing the relative KRIT1 and Gal-3 protein expression levels. (J) Bar diagrams representing the densitometric analysis of Gal-3 and KRIT1 Western blot bands (left and right panels, respectively) upon normalization with the OD of corresponding $\beta$-tubulin bands ($n = 6$ per group). (K) Representative Western blot analysis of KRIT1 and Gal-3 expression levels in genetically homogeneous MEF cells either *KRIT1*-knockout (*KRIT1*-KO) or re-expressing KRIT1 at high levels (*KRIT1*$^{+/+}$). The housekeeping $\alpha$-tubulin protein was used as an internal reference control for normalizing the relative KRIT1 and Gal-3 protein expression levels. (L) Bar diagrams representing the densitometric analysis of Gal-3 and KRIT1 Western blot bands (left and right panels, respectively) upon normalization with the OD of corresponding $\alpha$-tubulin bands ($n = 6$ per group). Two representative Western blot replicates are shown for both HUVEC (I) and MEF (K) cells, along with the densitometric quantification of all biological replicates (J, L). Semiquantitative densitometric analysis of Western blot bands was performed with the ImageJ software, and relative quantification values were expressed as a normalized ratio of Gal-3 and KRIT1 OD mean with respect to either $\beta$-tubulin or $\alpha$-tubulin. Note that there was a highly significant inverse correlation between KRIT1 and Gal-3 protein expression levels (***$P < 0.0001$). Statistical significance was calculated by a two-tailed Student's $t$-test, and a $P$-value $\leq 0.05$ was considered statistically significant.

To assess whether Gal-3 was also up-regulated in an *in vivo* model of CCM disease, we studied the brain of mice with the heterozygous deletion of *KRIT1*, the major causative CCM gene.[2] Specifically, we investigated Gal-3 protein expression levels in brain hemispheres of 26-week-old heterozygous ($KRIT1^{+/-}$) mice and wild-type (WT) littermates by Western blotting. The experimental outcomes showed that the down-regulation of KRIT1 protein levels detected in the brain of $KRIT1^{+/-}$ mice versus WT littermates was paralleled by a significant up-regulation of Gal-3 protein levels (Fig. 1G, H), suggesting a potential relationship between KRIT1 deficiency and molecular mechanisms underlying the control of Gal-3 expression, which deserves further investigation.

To provide further support to the emerged inverse relationship between KRIT1 and Gal-3 protein expression levels, we performed Western blot analysis of Gal-3 expression in established cellular models of CCM disease, including *KRIT1*-silenced human umbilical vein endothelial cells (HUVECs) and *KRIT1*-knockout mouse embryonic fibroblast (MEF) cells, which previously allowed the identification of new molecules and mechanisms involved in CCM pathogenesis and severity.[2] The outcomes of our Western blotting assays showed that Gal-3 expression was significantly higher in *KRIT1*-silenced (*KRIT1*-siRNA) versus control (Ctrl-siRNA) HUVECs (Fig. 1I, J) as well as in *KRIT1*-knockout (*KRIT1*-KO) versus *KRIT1*-expressing ($KRIT1^{+/+}$) MEF cells (Fig. 1K, L), thereby confirming and extending the inverse correlation between KRIT1 and Gal-3 protein expression levels identified in the $KRIT1^{+/-}$ mouse model (Fig. 1G, H). While the molecular mechanisms underlying such an inverse correlation remain to be elucidated, these findings open novel avenues for future studies aiming at a better understanding of KRIT1 loss-of-function effects associated with CCM disease pathogenesis, and the development of new prognostic and therapeutic strategies.

Overall, our study demonstrates for the first time that Gal-3 is significantly up-regulated both in clinical samples from CCM patients and in experimental models of CCM disease, suggesting that it could represent a pro-inflammatory determinant of CCM pathogenesis and severity as well as a novel therapeutic target and useful serological biomarker of prognostic and predictive value for improved clinical management and treatment of CCM patients. Indeed, despite some limitations, such as a small number of clinical samples, our findings provide new insights into the identification of novel molecular determinants and biomarkers of CCM disease, paving the way for future studies in animal models and large cohorts of CCM patients that might advance current knowledge towards the development of safe and effective precision medicine strategies.

## Ethics declaration

The study protocol was strictly performed in accordance with the Declaration of Helsinki and with permission from the local ethical committee of the Hannover Medical School, Germany (Approval Number 6960) and the Ethics Committee of University Bonn Medical Center (Approval Number 076/08 and 042/08). Written informed consent was collected from all the patients involved in this study. The animal protocol was approved by the local Animal Use and Care Committee of the University of Torino (Torino, Italy) and was in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes as well as the Guide for the Care and Use of Laboratory Animals.

## Author contributions

Study conception and planning: S.F.R.; Experimental design: S.F.R. and S.K.; Methodology: S.F.R., K.K.B., S.K.; Investigation on blood and tissue samples from CCM patients: S.K., K.K.B., A.K., A.N., B.K., L.G.; Investigation on cellular and mouse models: A.P. and R.M.; Data curation and interpretation: S.K. and S.F.R.; Writing-original draft preparation: S.K., A.P., S.F.R.; Writing-review and editing: S.R.F.; Providing experimental tools and materials (patient clinical data and Gal-3 protein expression analysis on CCM tissue samples, CCM tissue samples, and control TLE tissues): C.H., H.B., A.S., W.S.K. Supervision: S.K. and S.F.R. All authors read and approved the manuscript in its current form.

## Conflict of interests

The authors declare no conflict of interests.

## Data availability

The RNA-seq data used in this study have been deposited at the GEO repository with the accession number GSE137596 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE137596).[4]

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.02.045.

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18 November 2022

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