Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda,
Polskiej Akademii Nauk

Piotr Łacina

Czynniki związane z metabolizmem energetycznym jako potencjalne biomarkery w nowotworach hematologicznych

Rozprawa doktorska

Promotor:

prof. dr hab. Katarzyna Bogunia-Kubik

Laboratorium Immunogenetyki Klinicznej i Farmakogenetyki

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Polish Academy of Sciences

Piotr Łacina

Factors associated with energy metabolism as potential biomarkers in haematological malignancies

Doctoral Thesis

Supervisor:

Prof. dr hab. Katarzyna Bogunia-Kubik

Laboratory of Clinical Immunogenetics and Pharmacogenetics

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Składam serdeczne podziękowania

Prof. dr hab. Katarzynie Boguni-Kubik, promotorowi niniejszej pracy,

za opiekę merytoryczną, cenne uwagi, cierpliwość i motywację.

Współpracownikom i doktorantom

za codzienną życzliwość, dzielenie się swoim doświadczeniem

oraz okazaną pomoc.

Wszystkim,

którzy przyczynili się do powstania tej pracy.

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STRESZCZENIE

Metabolizm energetyczny oznacza ogół procesów mających na celu wytwarzanie oraz przechowywanie energii niezbędnej komórkom do ich prawidłowego funkcjonowania. W komórkach nowotworowych zachodzi nietypowa zmiana metabolizmu energii, zwana efektem Warburga. Efekt ten związany jest ze zwiększonym wykorzystaniem przez komórki nowotworowe procesu glikolizy do produkcji energii, zamiast bardziej wydajnej fosforylacji oksydacyjnej. Glikoliza jest związana z powstawaniem jonów mleczanowych, a ich nadmiar może prowadzić do kwasicy (acydemii) poprzez obniżenie pH. Przeciwdziałają temu transportery monokarboksylowe (ang. *monocarboxylate transporters*, MCT), białka wyspecjalizowane w transporcie mleczanu przez błonę komórkową. Do prawidłowego funkcjonowania muszą one jednak oddziaływać z basiginą. Basigina (BSG, CD147) jest transbłonową immunoglobuliną i charakteryzuje się podwyższonym poziomem ekspresji w wielu nowotworach. Badania wskazują, że działanie pro-nowotworowe BSG jest głównie wynikiem jej udziału w transporcie mleczanu, chociaż ma ona również udział w innych procesach pro-nowotworowych, np. indukcji angiogenezy. BSG jest opisywana jako marker wielu chorób nowotworowych, natomiast jej rozpuszczalna forma (ang. *soluble BSG*, sBSG) jest niezależnie uznawana za nowy potencjalny biomarker. Niestety niewiele wiadomo na temat BSG w nowotworach hematologicznych. Dotychczasowe badania wykazały jej podwyższoną ekspresję w szpiczaku plazmocytowym (ang. *multiple myeloma*, MM), uważa się również, że może mieć znaczenie w ostrej białaczce szpikowej (ang. *acute myeloid leukaemia*, AML).

Celem niniejszej rozprawy doktorskiej było: 1) sprawdzenie czy wybrane warianty genetyczne BSG i MCT1 mają związek z przebiegiem MM; 2) potwierdzenie nadekspresji BSG i MCT1 w AML i sprawdzenie czy warianty genetyczne BSG/MCT1 oraz ekspresja formy rozpuszczalnej BSG (sBSG) mogą być potencjalnymi biomarkerami w AML; 3) wskazanie, czy sBSG może być potencjalnym biomarkerem w MM. Wyniki badań zostały przedstawione w trzech kolejnych publikacjach naukowych.

W pierwszej publikacji opisane zostały polimorfizmy pojedynczego nukleotydu (ang. *single nucleotide polymorphism*, SNP) w genach kodujących BSG i MCT1 u pacjentów z MM. W oparciu o analizę *in silico* wybrano po cztery SNP w obu genach. Polimorfizmy te zostały przeanalizowane w grupie 135 pacjentów z MM oraz 135 osób zdrowych. Allele rs4919859 C oraz rs8637 G w BSG były związane z lepszym przeżyciem wolnym od wznowy u pacjentów z MM, natomiast allele rs1049434 A, rs7556664 A i rs7169 T w genie kodującym MCT1 były

związane z lepszym przeżyciem ogólnym. Ponadto allele rs4919859 C, rs8637 G i rs8259 A były częstsze u pacjentów z wyższym stadium choroby w momencie diagnozy. Badanie to było pierwszym opisującym polimorfizm BSG rs4919859 i potwierdzającym jego przewidywany efekt na chorobę.

W drugiej publikacji podjęte zostało zagadnienie znaczenia BSG i MCT1 w AML. Badanie przeprowadzone na sześciu liniach komórkowych AML oraz zdrowych komórkach pełniących funkcję kontroli potwierdziło, iż oba te geny ulegają nadekspresji w większości linii AML na poziomie mRNA. W pracy tej przeanalizowanych zostało również 37 pacjentów z AML i 25 osób zdrowych pod kątem ekspresji rozpuszczalnej formy BSG (sBSG). Badanie wykazało zwiększoną ekspresję tej formy w surowicy pacjentów. Zauważono ponadto, że wysoki poziom sBSG był związany z gorszym przeżyciem ogólnym, wyższym odsetkiem blastów i wyższym poziomem leukocytów we krwi pacjentów z AML. Zaobserwowano korelację ekspresji czynnika pro-angiogennego VEGF z ekspresją BSG na poziomie białka, co może potwierdzać pro-angiogenną rolę BSG w AML. Natomiast nie stwierdzono korelacji poziomu sBSG ani z ekspresją BSG na poziomie mRNA, ani z jego poziomem w komórce, co może wynikać ze skomplikowanej natury procesów wydzielania tego białka. W ramach kontynuacji badań nad wariantami genetycznymi BSG i MCT1 uprzednio wybrane SNP przeanalizowano w grupie 92 pacjentów z AML oraz 135 osób zdrowych. Allele BSG 4919859 C oraz rs4682 C, a także genotyp MCT1 rs1049434 AA korelowały z gorszym przeżyciem całkowitym u pacjentów z AML. Wyniki te potwierdzają negatywne działanie allelu BSG rs4919859 C.

Trzecia publikacja obejmuje badania nad ekspresją sBSG w MM. W badaniu wykorzystane zostały próbki krwi 62 pacjentów z MM oraz 25 osób zdrowych. Poziom sBSG był wyższy u pacjentów z MM, a także wyższy u pacjentów w bardziej zaawansowanym stadium choroby. Wykazano również, że poziom ten spada wraz z remisją choroby. Wysoki poziom sBSG związany był z gorszym przeżyciem wolnym od wznowy. Zbadana została również ekspresja na poziomie mRNA w podgrupie pacjentów z MM. Podobnie jak w przypadku pacjentów z AML, badanie to wykazało brak korelacji między poziomem sBSG i ekspresją mRNA BSG. Potwierdziło ono jednak korelację między ekspresją mRNA BSG z MCT1 i VEGF.

Wyniki badań opisanych w powyższych publikacjach potwierdziły znaczenie BSG i MCT1 w patogenezie ostrej białaczki szpikowej i szpiczaka plazmocytowego. Sugerują one również, że polimorfizmy w genach kodujących te białka, a także poziom rozpuszczalnej formy BSG w surowicy, mogą być potencjalnymi biomarkerami w nowotworach hematologicznych.

ABSTRACT

Energy metabolism refers to the processes used by cells to generate and store the energy needed to support their functioning and growth. In cancer cells, an unusual rearrangement of energy metabolism, known as the Warburg effect, is observed. It is characterized by increased reliance of cancer cells on glycolysis, rather than the much more efficient oxidative phosphorylation. Lactate ions are produced as a by-product of glycolysis, and cancer cells need special lactate removal mechanisms to prevent acidosis. Monocarboxylate transporters (MCTs) are proteins responsible for the transport of lactates across the membrane; however, they must interact with basigin to function properly. Basigin (BSG, CD147), a membrane-bound immunoglobulin, is significantly overexpressed in many cancers, and studies show that it supports cancer cell survival mostly by maintaining efficient lactate transport. It is also implicated in other processes associated with cancer, e.g. induction of angiogenesis. BSG is considered a diagnostic and prognostic biomarker in many cancers, and some studies suggest that its soluble form (sBSG) may be an interesting marker on its own. However, little is known about BSG in haematological malignancies. It has been established that it is overexpressed in multiple myeloma (MM) and may have a role in acute myeloid leukaemia (AML).

The aim of this doctoral thesis was to 1) verify if selected genetic variants of BSG and monocarboxylate transporter 1 (MCT1) can affect MM; 2) confirm that BSG and MCT1 are overexpressed in AML and verify if BSG/MCT1 genetic variants, as well as soluble BSG expression, can be potential biomarkers in AML; 3) establish if soluble BSG can be a potential biomarker in MM. Results were described in three corresponding scientific papers.

The first paper describes single nucleotide polymorphisms (SNPs) in the genes coding for BSG and MCT1 in MM patients. Four SNPs were selected in each gene based on an *in silico* analysis. These SNPs were then analysed in a group of 135 MM patients and 135 healthy individuals. BSG alleles rs4919859 C and rs8637 G were significantly associated with better progression-free survival in MM patients, while MCT1 alleles rs1049434 A, rs7556664 A and rs7169 T were associated with better overall survival. Additionally, rs4919859 C, rs8637 G and rs8259 A were more common in patients with more advanced disease at diagnosis. This was the first study that described the BSG rs4919859 SNP and confirmed its predicted effect on disease.

The second paper deals with BSG and MCT1 in AML. Using six model AML cell lines and healthy cells as a control, it confirmed that both genes are overexpressed in most AML models

on the mRNA level. In a study on 37 AML patients and 25 healthy individuals, sBSG was found to be overexpressed in serum of AML patients. Furthermore, high sBSG was associated with worse overall survival, higher blast percentage and higher white blood cell count in AML. Expression of VEGF, a pro-angiogenic factor, correlated with BSG protein expression, potentially confirming the pro-angiogenic role of BSG in AML. Interestingly, sBSG expression correlated neither with BSG mRNA level, nor with total BSG protein production, which can result from the complicated nature of sBSG secretion. Continuing the study on BSG and MCT1 genetic variants in MM, the previously selected SNPs were tested on a group of 92 AML patients and 135 healthy individuals. BSG alleles rs4919859 C and rs4682 C, as well as MCT1 genotype rs1049434 AA were found to correlate with worse overall survival, confirming the adverse effect of allele rs4919859 C.

The third paper describes sBSG expression in MM. For the purpose of the study, samples of 62 MM patients and 25 healthy individuals were used. sBSG level was found to be higher in serum of MM patients and in a subgroup of patients with more advanced disease. Furthermore, its level dropped after positive response to treatment. High sBSG level was associated with worse progression-free survival. mRNA expression was also analysed in a subgroup of MM patients. Similarly to AML, sBSG level did not correlate with BSG mRNA expression in MM patients. However, BSG mRNA expression was confirmed to correlate with MCT1 and VEGF expression.

Results of these studies confirm the involvement of BSG and MCT1 in AML and MM pathogenesis and suggest that their genetic variants, as well as soluble BSG level, may be potential biomarkers in haematological malignancies.

List of Publications

1. **Łacina P**, Butrym A, Mazur G, Bogunia-Kubik K. BSG and MCT1 Genetic Variants Influence Survival in Multiple Myeloma Patients. Genes. 2018; 9(5):226.

2. **Łacina P**, Butrym A, Turlej E, Stachowicz-Suhs M, Wietrzyk J, Mazur G, Bogunia-Kubik K. BSG (CD147) Serum Level and Genetic Variants Are Associated with Overall Survival in Acute Myeloid Leukaemia. Journal of Clinical Medicine. 2022; 11(2):332.

3. **Łacina P**, Butrym A, Frontkiewicz D, Mazur G, Bogunia-Kubik K. Soluble CD147 (BSG) as a Prognostic Marker in Multiple Myeloma. Current Issues in Molecular Biology. 2022; 44(1):350-359.

Co-authors' Declarations

Piotr Łacina

Hirszfeld Institute of Immunology and Experimental Therapy,

Polish Academy of Sciences, ul. Rudolfa Weigla 12, 53-114 Wrocław, Poland

27.01.2022

Declaration

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Aleksandra Butrym	10 %	Recruitment of patients, collection of clinical data, supervision, review and editing
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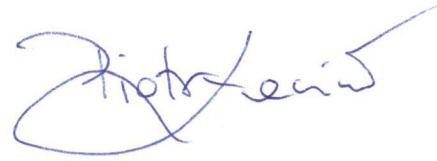

Aleksandra Butrym
Wroclaw Medical University
ul. Borowska 213, 50-556 Wrocław, Poland

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Aleksandra

Uniwersytet Medyczny we Wrocławiu
ZAKŁAD PROFILAKTYKI I LECZENIA
CHORÓB NOWOTWOROWYCH
kierownik
dr hab. Aleksandra Butrym, prof. nadzw.

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Grzegorz Mazur

Wroclaw Medical University

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Kierownik
Prof. dr hab. Grzegorz Mazur

Katarzyna Bogunia-Kubik
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Polish Academy of Sciences, ul. Rudolfa Weigla 12, 53-114 Wrocław, Poland

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Aleksandra Butrym
Wroclaw Medical University
ul. Borowska 213, 50-556 Wrocław, Poland

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kierownik
dr hab. Aleksandra Butrym, prof. nadzw.

27.01.2022

Eliza Turlej

Hirszfeld Institute of Immunology and Experimental Therapy,

Polish Academy of Sciences, ul. Rudolfa Weigla 12, 53-114 Wrocław, Poland

Wrocław University of Environmental and Life Sciences

ul. Norwida 27b, 50-375 Wrocław, Poland

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Turlej Eliza.

Martyna Stachowicz-Suhs

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Polish Academy of Sciences, ul. Rudolfa Weigla 12, 53-114 Wrocław, Poland

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Stachowicz-Suhs

Joanna Wietrzyk

Hirszfeld Institute of Immunology and Experimental Therapy,

Polish Academy of Sciences, ul. Rudolfa Weigla 12, 53-114 Wrocław, Poland

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Piotr Łacina, Aleksandra Butrym, Eliza Turlej, Martyna Stachowicz-Suhs, Joanna Wietrzyk, Grzegorz Mazur, Katarzyna Bogunia-Kubik. BSG (CD147) Serum Level and Genetic Variants Are Associated with Overall Survival in Acute Myeloid Leukaemia. Journal of Clinical Medicine. 2022; 11(2):332.

is correctly characterized in the table below.

Contributor	Contribution [%]	Description of main tasks
Piotr Łacina	60 %	Conceptualization, methodology and investigation – gene expression and sBSG measurement and genotyping, analysis of experimental and clinical data, data visualization, project administration, funding acquisition, original draft preparation
Aleksandra Butrym	10 %	Recruitment of patients, collection of clinical data, supervision, review and editing
Eliza Turlej	5 %	Methodology and investigation – growing cell cultures, review and editing
Martyna Stachowicz-Suhs	5 %	Methodology and investigation – western blot, review and editing
Joanna Wietrzyk	5 %	Resources – instrumentation, supervision, review and editing
Grzegorz Mazur	5 %	Recruitment of patients, supervision, review and editing
Katarzyna Bogunia-Kubik	10 %	Conceptualization, resources – instrumentation, project administration, supervision, review and editing

Piotr Łacina

Hirszfeld Institute of Immunology and Experimental Therapy,

Polish Academy of Sciences, ul. Rudolfa Weigla 12, 53-114 Wrocław, Poland

27.01.2022

Declaration

I hereby declare that my contribution to the following manuscript:

Piotr Łacina, Aleksandra Butrym, Diana Frontkiewicz, Grzegorz Mazur, Katarzyna Bogunia-Kubik. Soluble CD147 (BSG) as a Prognostic Marker in Multiple Myeloma. Current Issues in Molecular Biology. 2022; 44(1):350-359.

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Contributor	Contribution [%]	Description of main tasks
Piotr Łacina	65 %	Conceptualization, methodology and investigation – sBSG measurement and gene expression, analysis of experimental and clinical data, data visualization, project administration, funding acquisition, original draft preparation
Aleksandra Butrym	10 %	Recruitment of patients, collection of clinical data, supervision, review and editing
Diana Frontkiewicz	5 %	Recruitment of patients, collection of clinical data, review and editing
Grzegorz Mazur	5 %	Recruitment of patients, supervision, review and editing
Katarzyna Bogunia-Kubik	15 %	Conceptualization, resources – instrumentation, project administration, supervision, review and editing

Aleksandra Butrym
Wroclaw Medical University
ul. Borowska 213, 50-556 Wrocław, Poland

27.01.2022

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Uniwersytet Medyczny we Wrocławiu
ZAKŁAD PROFILAKTYKI I LECZENIA
CHORÓB NOWOTWOROWYCH
Kierownik
dr hab. Aleksandra Butrym, prof. nadzw.

Diana Frontkiewicz

Sokołowski Specialist Hospital

ul. Sokołowskiego 4, 58-309 Wałbrzych, Poland

27.01.2022

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Frontkiewicz Diana

Grzegorz Mazur
Wroclaw Medical University
ul. Borowska 213, 50-556 Wrocław, Poland

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KATEDRA I KLINIKA CHORÓB WEWNĘTRZNYCH
ZAWODOWYCH, NADCIŚNIENIA TĘTNICZEGO
I ORGANOWYCH CHORÓB KLINICZNEJ
Prof. dr hab. Grzegorz Mazur

Katarzyna Bogunia-Kubik
Hirszfeld Institute of Immunology and Experimental Therapy,
Polish Academy of Sciences, ul. Rudolfa Weigla 12, 53-114 Wrocław, Poland

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Publications

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Article

BSG and *MCT1* Genetic Variants Influence Survival in Multiple Myeloma Patients

Piotr Łacina [1,*], Aleksandra Butrym [2], Grzegorz Mazur [2] and Katarzyna Bogunia-Kubik [1]

[1] Laboratory of Clinical Immunogenetics and Pharmacogenetics, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wrocław, Poland; bogunia@iitd.pan.wroc.pl
[2] Department of Internal, Occupational Diseases, Hypertension and Clinical Oncology, Wroclaw Medical University, 50-556 Wrocław, Poland; aleksandra.butrym@gmail.com (A.B.); grzegorz.mazur@umed.wroc.pl (G.M.)
* Correspondence: piotr.lacina@iitd.pan.wroc.pl; Tel.: +48-713-709-961

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Abstract: Multiple myeloma (MM) is a haematologic malignancy characterized by the presence of atypical plasma cells. Basigin (BSG, CD147) controls lactate export through the monocarboxylic acid transporter 1 (MCT1, SLC16A1) and supports MM survival and proliferation. Additionally, BSG is implicated in response to treatment with immunomodulatory drugs (thalidomide and its derivatives). We investigated the role of single nucleotide polymorphisms (SNPs) in the gene coding for BSG and SLC16A1 in MM. Following an in silico analysis, eight SNPs (four in *BSG* and four in *SLC16A1*) predicted to have a functional effect were selected and analyzed in 135 MM patients and 135 healthy individuals. Alleles rs4919859 C, rs8637 G, and haplotype CG were associated with worse progression-free survival ($p = 0.006$, $p = 0.017$, $p = 0.002$, respectively), while rs7556664 A, rs7169 T and rs1049434 A (all in linkage disequilibrium (LD), $r^2 > 0.98$) were associated with better overall survival ($p = 0.021$). Similar relationships were observed in thalidomide-treated patients. Moreover, rs4919859 C, rs8637 G, rs8259 A and the CG haplotype were more common in patients in stages II–III of the International Staging System ($p < 0.05$), while rs8259 A correlated with higher levels of β-2-microglobulin and creatinine ($p < 0.05$). Taken together, our results show that *BSG* and *SLC16A1* variants affect survival, and may play an important role in MM.

Keywords: basigin; monocarboxylic acid transporter 1; single nucleotide polymorphisms; multiple myeloma; survival

1. Introduction

Multiple myeloma (MM) is the second most common human haematologic malignancy. It is characterized by presence of atypical plasma cells (myeloma cells) in the bone marrow, impaired immunoglobulin production, and presence of monoclonal protein in serum and urine [1]. The number of new MM cases is estimated at ~20,000 per year in the United States, and this number is forecast to rise above 30,000 by 2032 [2].

Basigin (BSG), also known as CD147 and extracellular matrix metalloproteinase inducer (EMMPRIN), is a transmembrane glycoprotein and a member of the immunoglobulin superfamily [3]. It is widely expressed on many cells and also carries the Ok blood group in humans [4]. BSG is of major importance in myeloma cells, due to its participation in the transport of energy metabolism products, most importantly lactate anions. Lactates are produced in the glycolytic pathway and need to be removed from the cell because they decrease intracellular pH [5]. Lactate production is increased in cancer cells, as their reliance on the glycolytic pathway for energy production is much higher than that of normal cells—this is known as the Warburg effect. BSG is essential for lactate

transport, although it is proteins of the monocarboxylate transporter (MCT) family that are directly responsible for it. MCTs are dependent on BSG as they are bound to it in the cell membrane, and BSG also functions as a chaperone for MCT1 and MCT4. This tight association is best seen by the fact that an MCT inhibitor was found to actually target BSG directly [3,6]. BSG overexpression was shown for many tumours, and has also been proved in multiple myeloma. Additionally, it has been demonstrated that an increase in BSG expression accompanies MM progression [7]. It was also found that MCT1 and MCT4 are overexpressed in MM, but that only MCT1 downregulation leads to decreased myeloma cell proliferation [5]. Other studies showed increased lactate export in myeloma cells, and a correlation of *BSG* gene expression with key regulators of Warburg effect, further confirming the role of BSG in lactate transport in MM [5,8].

A recent study showed that BSG is also implicated in MM treatment with immunomodulatory drugs (IMiDs), i.e., thalidomide and its derivatives [9]. IMiDs are known to exert their effect by binding to a protein called cereblon (CRBN) [10]. CRBN seems to promote maturation and activations of BSG and MCT1 through a chaperone-like mechanism. This process is independent of ubiquitination of proteins Ikaros and Aiolos, in which CRBN is involved, and which was shown earlier to lead to myeloma cell cycle arrest. This chaperone-like mechanism is abrogated following IMiD therapy, leading to accumulation of lactate in myeloma cells [9]. Additionally, another study showed that *BSG* mRNA expression is higher in patients responding to IMiD treatment than in non-responders [11].

These findings prompted us to analyse whether *BSG* and *SLC16A1* (*MCT1*) gene polymorphism might affect risk, survival, or outcome of treatment in multiple myeloma patients. In our previous studies, we showed that single nucleotide polymorphisms (SNPs) located in genes associated with IMiD metabolism do affect response to therapy [12,13]. In the present work, following an in silico analysis using the National Institute of Environmental Health Sciences SNP Function Prediction tool, we picked SNPs located within the *BSG* and *SLC16A1* genes that had minor allele frequency (MAF) in European populations higher than 0.15, and that were expected to have a functional effect [14]. Using the above criteria, eight SNPs were selected: *BSG* rs4919859—located in a potential transcription factor binding site, *BSG* rs8259—located in a potential microRNA binding site, *BSG* rs4682—located in an exonic splicing enhancer/silencer (ESE/ESS), *BSG* rs8637—located in an ESE/ESS and a potential microRNA binding site, *SLC16A1* rs9429505—located in a potential microRNA binding site, *SLC16A1* rs7556664—located in a potential transcription factor binding site, *SLC16A1* rs7169—located in a potential microRNA binding site and *SLC16A1* rs1049434—a missense Asp to Glu mutation.

2. Materials and Methods

2.1. Patients and Controls

The study included a group of 135 Polish MM patients and 135 healthy blood donors that served as controls. The group was also investigated in our previous study on *CTNNB1* (β-catenin) and *CRBN* variants; detailed information is included there [12]. In brief, the group of patients consisted of 70'males and 65 females, median age on diagnosis was 61 years. Among patients, 35% were in stage I, 34% in stage II, and 31% in stage III of the disease, according to the International Staging System (ISS) criteria. 74.1% were administered thalidomide as part of the first-line treatment, mostly together with cyclophosphamide and dexamethasone.

2.2. Genotyping

DNA was extracted from samples of peripheral blood taken on EDTA using Maxwell 16 blood DNA purification kit (Promega Corp., Madison, WI, USA) or silica membranes (Qiagen, Hilden, Germany), following the recommendations of the manufacturers. *BSG* and *SLC16A1* polymorphic variants were determined using the Taqman (Thermo Fisher, Waltham, MA, USA) and LightSNiP (TIB MOLBIOL, Berlin, Germany) assays. PCR was performed on a LightCycler 480 II device (Roche Diagnostics, Rotkreuz, Switzerland), according to the manufacturer's recommendations.

2.3. Statistical Analysis

Linkage disequilibrium and Hardy-Weinberg equilibrium analyses were performed with the Haploview 4.2 software [15]. The null hypothesis that there is no difference between allele and genotype frequencies between patients and controls was tested with the Fisher's exact test [16]. Survival was assessed using the Kaplan-Meier method, and associations with clinical parameters were calculated using the *U* Mann-Whitney test. Both of these analyses were performed with the real statistics resource pack for Microsoft excel 2013 (version 15.0.5023.1000, Microsoft, Redmont, Washington, DC, USA). $p < 0.05$ were considered statistically significant, and those between 0.05 and 0.10 as indicative of a trend. Genotypes were tested for deviations from Hardy-Weinberg equilibrium using the χ^2 test.

3. Results

3.1. BSG *and* SLC16A1 *Allele and Genotype Frequencies*

Patients and healthy donors were genotyped for all eight SNPs, and allele and genotype frequencies were calculated for both MM patients and controls. Frequencies for all the SNPs were in accordance with Hardy-Weinberg equilibrium in both groups. Linkage disequilibrium (LD) analysis revealed that all the *BSG* SNPs were in low-to-medium LD (r^2 range between 0.15 and 0.57, see Figure 1), while three of the four *SLC16A1* SNPs (rs7169, rs1049434, rs7556664) were in perfect or near perfect LD ($r^2 \geq 0.98$, see Figure 1). Because of this, rs7556664 was used as a tag SNP for rs7169 and rs1049434, and no further analyses were performed for the two latter SNPs.

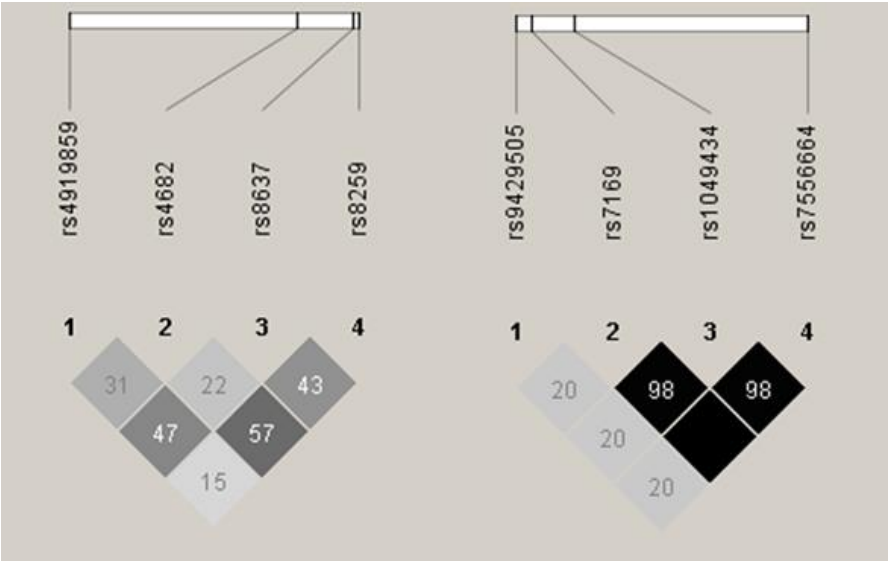

Figure 1. Linkage disequilibrium (LD) between single nucleotide polymorphisms (SNPs) under study. Basigin (*BSG)* SNPs are shown to the left, and monocarboxylic acid transporter 1 (*SLC16A1*, MCT1) to the right. Darker colour shows higher r^2 values, while the value shown inside the squares is $r^2 \times 10^2$. Results as presented by the Haploview 4.2 software [15].

We did not observe any statistically significant difference in allele distribution between MM patients and healthy controls in any of the SNPs tested. Distribution of genotypes in patients and controls is presented in Table 1.

Table 1. Distribution of *BSG* and *SLC16A1* genotypes in multiple myeloma (MM) patients (entire patient cohort and thalidomide-treated patients only) and healthy controls.

	MM Patients (All)	MM Patients (Thalidomide-Treated)	Controls		MM Patients	MM Patients (Thalidomide-Treated)	Controls
BSG rs4919859				*SLC16A1* rs9429505			
CC	15 (11.2%)	13 (13.1%)	19 (14.1%)	AA	77 (57.0%)	59 (59.0%)	67 (49.6%)
CG	65 (48.5%)	47 (47.5%)	54 (40.0%)	AG	51 (37.8%)	34 (34.0%)	60 (44.4%)
GG	54 (40.3%)	39 (39.4%)	62 (45.9%)	GG	7 (5.2%)	7 (7.0%)	8 (5.9%)
BSG rs4682				*SLC16A1* rs7169			
CC	3 (2.2%)	3 (3.0%)	4 (3.0%)	CC	22 (16.3%)	15 (15.0%)	23 (17.0%)
CT	45 (33.6%)	35 (35.4%)	34 (25.4%)	CT	63 (46.7%)	46 (46.0%)	58 (43.0%)
TT	86 (64.2%)	61 (61.6%)	96 (71.6%)	TT	50 (37.0%)	39 (39.0%)	54 (40.0%)
BSG rs8637				*SLC16A1* rs1049434			
AA	44 (32.8%)	32 (32.3%)	47 (34.8%)	AA	51 (37.8%)	39 (39.0%)	54 (40.0%)
AG	63 (47.0%)	45 (45.5%)	61 (45.2%)	AT	62 (45.9%)	46 (46.0%)	58 (43.0%)
GG	27 (20.2%)	22 (22.2%)	27 (20.0%)	TT	22 (16.3%)	15 (15.0%)	23 (17.0%)
BSG rs8259				*SLC16A1* rs7556664			
TT	77 (57.0%)	57 (57.0%)	78 (57.8%)	AA	50 (37.0%)	39 (39.0%)	54 (40.0%)
TA	49 (36.3%)	35 (35.0%)	49 (36.3%)	AT	63 (46.7%)	46 (46.0%)	58 (43.0%)
AA	9 (6.7%)	8 (8.0%)	8 (5.9%)	TT	22 (16.3%)	15 (15.0%)	23 (17.0%)

Numbers of patients and controls analysed for various SNPs are slightly different. One person was not included in calculations for rs4919859, rs4682, rs8637 (patients) and rs4682 (controls).

3.2. *Associations of* BSG *and* SLC16A1 *Polymorphism with Survival*

We performed survival analyses for the SNPs regarding both progression-free (PFS) and overall survival (OS), in the whole MM patient group, and in a subgroup of patients treated with thalidomide (first-line treatment), constituting 74.1% of all patients. The analysis showed that alleles rs4919859 C and rs8637 G were particularly associated with adverse PFS ($p = 0.006$ and $p = 0.017$, for the C and G allele, respectively) (see Figure 2). Similar relationship with rs4919859 C and rs8637 G were observed in a subgroup of patients treated with thalidomide ($p = 0.042$ and $p = 0.065$, for the C and G allele, respectively). Given the similarity of rs4919859 C and rs8637 G regarding PFS and their relatively high LD, we investigated the CG haplotype and found it to be an even better predictor of adverse PFS ($p = 0.002$ and $p = 0.017$, for all patients and the thalidomide-treated subgroup respectively). Furthermore, a trend towards better PFS was observed for the *BSG* rs8259 T allele, but not in the thalidomide-treated subgroup ($p = 0.102$, $p = 0.203$, for all patients and the thalidomide-treated subgroup respectively, see Figure 2).

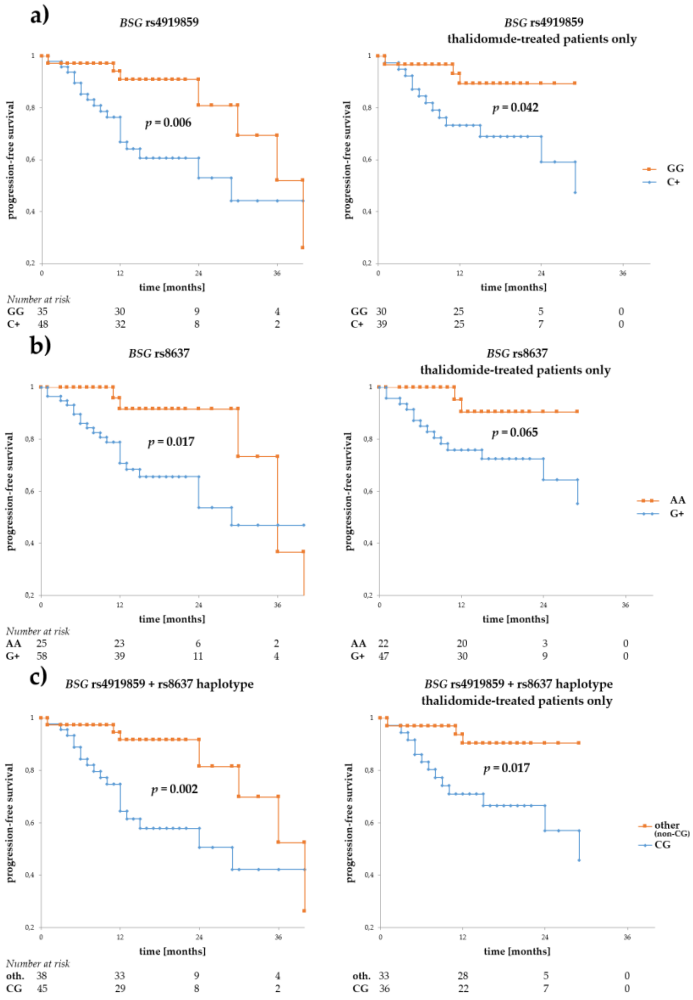

Figure 2. Progression-free survival in multiple myeloma (MM) patients with respect to *BSG* SNPs. Panels (**a**,**b**) show survival for rs4919859 C and rs8637 G, respectively. The two SNPs, located in a presumed transcription factor and microRNA binding sites, are in low/medium LD. Given their similar effect on progression-free (PFS), an rs4919859 C and rs8637 G (or CG, in short) haplotype was also tested for association with PFS, and is shown in panel (**c**). Curves to the left show PFS calculated for the entire group of patients, while those to the right show PFS calculated for the subgroup of thalidomide-treated patients. The numbers at risk show numbers of patients in the risk set (i.e., patients without disease progression, a number used to calculate survival) at a given time point on the *x*-axis. Survival was calculated using the Kaplan-Meier method and the real statistics resource pack for Microsoft excel 2013 (Microsoft).

No statistically significant association was observed for either of the *BSG* rs4682 alleles. Additionally, no statistically significant associations were observed for the *BSG* SNPs regarding OS, although there was a trend for slightly better OS in patients with rs4682 C ($p = 0.097$ for all patients, and $p = 0.090$ for the thalidomide-treated subgroup), and those with rs8637 G ($p = 0.092$ for all patients).

Regarding *SLC16A1* SNPs, the rs7556664 A allele (and, in consequence, rs7169 T and rs1049434 A) was associated with better OS ($p = 0.021$ for all patients and $p = 0.065$ for the thalidomide-treated subgroup, see Figure 3), but no such association was observed for either of the rs9429505 alleles. However, there was a trend for better PFS in patients with rs9429505 G ($p = 0.078$, for all patients). No statistically significant association regarding PFS was observed for rs7556664.

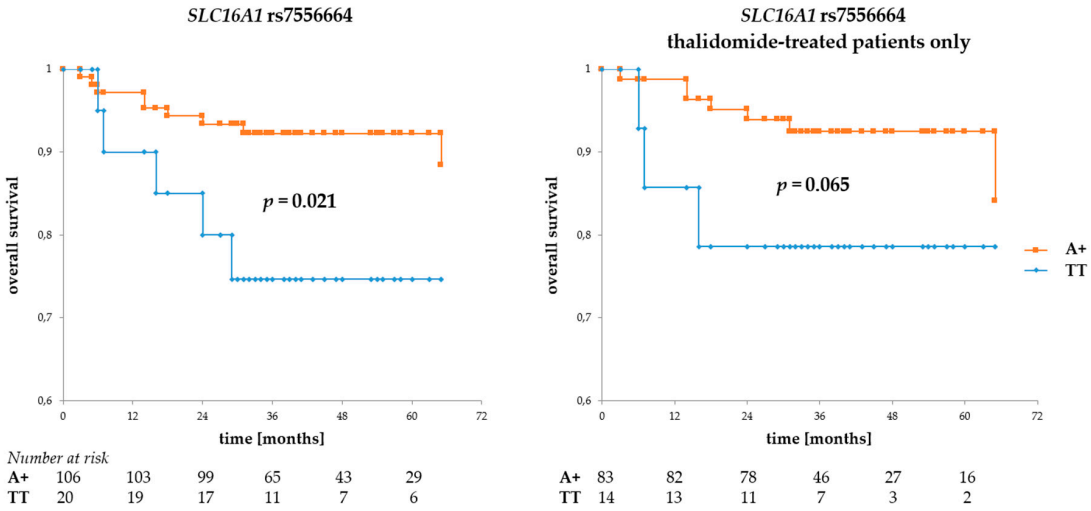

Figure 3. Overall survival in MM patients with respect to *SLC16A1* rs7556664 A. This SNP is in near-perfect LD with two other *SLC16A1* SNPs described in this study. Allele rs7556664 A (orange curve) also corresponds to rs1049434 A and rs7169 T; however, for the sake of simplicity, only rs7556664 A is shown in the figure above. The left panel shows OS calculated for the entire group of patients, while that to the right shows overall survival (OS) calculated for thalidomide-treated patients only. The numbers at risk show numbers of patients in the risk set (i.e., patients still living, a number used to calculate survival) at a given time point on the *x*-axis. Survival was calculated using the Kaplan-Meier method and the real statistics resource pack for Microsoft excel.

3.3. *Influence of* BSG *and* SLC16A1 *Polymorphism on Response to Treatment*

Keeping in mind the importance of *BSG* and *SLC16A1* in CRBN-mediated response to IMiD therapy, we looked for associations between the SNPs under study and response to first-line treatment. Interestingly, we only observed a borderline significant association with *SLC16A1* rs9429505 (the G allele was more common in patients achieving complete or very good partial remission than in patients achieving only partial remission or no remission, $p = 0.048$), and a trend towards better response with *BSG* rs8259 (allele A was more common in patients achieving complete or very good partial remission, $p = 0.082$). Both were seen in patients administered the cyclophosphamide, thalidomide, dexamethasone (CTD) regimen.

3.4. *BSG and SLC16A1 Polymorphism and Other Clinical Parameters*

We compared the occurrence of *BSG* and *SLC16A1* variants in patients with various MM stages on diagnosis according to the international staging system (ISS). Interestingly, we found alleles rs4919859 C, rs8259 A, and rs8637 G and the CG haplotype (as defined in the Section 3.2. dealing with survival) to be more common in patients in stages II–III than in those in stage I ($p = 0.024$, $p = 0.001$, $p < 0.001$, $p = 0.001$, respectively). A similar, although not statistically significant, relationship was also observed

for allele rs4682 C (p = 0.053). However, no association between ISS stages and *SLC16A1* variants was observed.

Regarding other clinical parameters, we found rs8259 A to be associated with higher β-2-microglobulin and creatinine levels (p = 0.022 and p = 0.017, respectively).

4. Discussion

In recent years, there have been many significant discoveries regarding the processes occurring during response to MM therapy with immunomodulatory drugs. First, CRBN was found to play a central role, and further studies showed importance of proteins Ikaros and Aiolos, as well as BSG and MCT1 [9,10,17]. Additionally, overexpressed BSG and MCT1 were demonstrated to be involved in energy metabolism in MM by the means of supporting the export of toxic lactate anions in myeloma cells [5,7].

Our present study aimed at elucidating the role played by genetic variability in *BSG* and *SLC16A1* (MCT1) in myeloma cells. Three SNPs located in the *SLC16A1* gene turned out to be in near-perfect LD; therefore, only one of them was chosen for further analysis (rs7556664). It should, however, be noted that the observed effect of rs7556664 could also be equally attributed to either rs7169 or rs1049434.

Of the eight SNPs under study, rs4682, rs4919859, rs7169, and rs7556664 have, to the best of our knowledge, never been studied before. *BSG* rs8637 was mentioned in the context of carotid atherosclerotic plaques, but the study found the SNP not to be associated with risk for this disease [18]. rs8259 was described in three different non-haematologic diseases in which allele T was associated with lower mRNA *BSG* expression and lower plasma levels of soluble BSG [19–21]. This appears to agree with our findings, as rs8259 T seems to positively affect progression-free survival.

SLC16A1 rs1049434 (in near-perfect LD with rs7169 and rs7556664) was studied the most due to its exonic location, and the fact that it affects MCT1 protein structure [22–25]. It has been shown that the mutated variant (containing Glu in the protein sequence and T in the DNA sequence) exhibits increased lactate transport via SLA16A1, as compared to the wild type Asp/A [26]. This seems to be in line with our results showing that rs1049434 A is correlated with better survival (increased lactate transport is favourable for myeloma cells, and therefore detrimental for patient's survival).

We do not know how the other SNPs described in this study affect BSG, but given their presumed functions (as described in the introduction), we expect them to affect *BSG* and *SLC16A1* expression (both on mRNA and protein level, depending on whether or not they are located in a potential transcription factor or miRNA binding site), or splicing (especially in the case of rs4682).

In the present study, we observed an interesting association with *BSG* and *SLC16A1* SNPs and progression-free and overall survival. These associations show that the selected *BSG* and *SLC16A1* alleles may influence survival in MM patients in general. However, it should be noted that some of the relationships (e.g., SLC16A1 SNPs and survival) cannot be ascertained definitively due to the relatively small size of one of the subgroups analysed. Furthermore, because many of the associations were no longer statistically significant after limiting the group to patients treated with thalidomide, we cannot decisively conclude that the various alleles affected CRBN-mediated response to thalidomide treatment. Indeed, it is possible that the observable effect might have been exclusively due to the *BSG* and *SLC16A1* alleles variously affecting BSG-governed lactate export. As described in the results section, we found correlations with response to thalidomide treatment, although only in the case of rs9429505 was the relationship statistically significant. This relative lack of correlations between *BSG* SNPs and response to treatment seems to be at odds with results of Bolomsky et al., as they showed that *BSG* mRNA expression was significantly higher in patients responding to IMiD therapy [11]. Interestingly though, a more recent study showed no association between *BSG* mRNA expression and response to IMiD treatment [27]. However, it should be noted that comparison of such studies might be elusive, as the exact therapies used (e.g., IMiD chosen, other drugs used together with IMiDs, dosages etc.) vary between studies. In addition, some studies suggest that CRBN-mediated response to IMiD is controlled largely in a post-translational manner [9,28]. Regardless of molecular

explanations for the survival associations, it is interesting to note that Krönke et al. also reported an association between *BSG* expression levels and PFS, albeit only in patients with standard (and not high) cytogenetic risk [27].

In conclusion, our study shows that selected *BSG* and *SLC16A1* genetic variants may affect progression-free and overall survival in multiple myeloma, and correlate with various clinical parameters of MM, such as ISS stage, β-2-microglobulin and creatinine levels. These results should be further confirmed on larger cohorts of MM patients. More studies are necessary to elucidate the precise mechanism of these variants and the means by which they affect BSG/SLC16A1 levels and functions.

Author Contributions: P.Ł. and K.B.-K. conceived and designed the experiments; P.Ł. performed the experiments; P.Ł. and K.B.-K. analysed the data; A.B., G.M. and K.B.-K. contributed materials; P.Ł. and K.B.-K. wrote the paper.

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BSG (CD147) Serum Level and Genetic Variants Are Associated with Overall Survival in Acute Myeloid Leukaemia

Piotr Łacina [1,*], Aleksandra Butrym [2], Eliza Turlej [3,4], Martyna Stachowicz-Suhs [3], Joanna Wietrzyk [3], Grzegorz Mazur [5] and Katarzyna Bogunia-Kubik [1]

1 Laboratory of Clinical Immunogenetics and Pharmacogenetics, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland; katarzyna.bogunia-kubik@hirszfeld.pl
2 Department of Cancer Prevention and Therapy, Wroclaw Medical University, 50-556 Wroclaw, Poland; aleksandra.butrym@umw.edu.pl
3 Department of Experimental Oncology, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland; eliza.turlej@upwr.edu.pl (E.T.); martyna.stachowicz@hirszfeld.pl (M.S.-S.); joanna.wietrzyk@hirszfeld.pl (J.W.)
4 Department of Experimental Biology, Wrocław University of Environmental and Life Sciences, 50-375 Wroclaw, Poland
5 Department of Internal, Occupational Diseases, Hypertension and Clinical Oncology, Wroclaw Medical University, 50-556 Wroclaw, Poland; grzegorz.mazur@umw.edu.pl
* Correspondence: piotr.lacina@hirszfeld.pl; Tel.: +48-713-709-960-236

Abstract: Basigin (BSG, CD147) is a multifunctional protein involved in cancer cell survival, mostly by controlling lactate transport through its interaction with monocarboxylate transporters (MCTs) such as MCT1. Previous studies have found that single nucleotide polymorphisms (SNPs) in the gene coding for BSG and MCT1, as well as levels of the soluble form of BSG (sBSG), are potential biomarkers in various diseases. The goal of this study was to confirm BSG and MCT1 RNA overexpression in AML cell lines, as well as to analyse soluble BSG levels and selected BSG/MCT1 genetic variants as potential biomarkers in AML patients. We found that BSG and MCT1 were overexpressed in most AML cell lines. Soluble BSG was increased in AML patients compared to healthy controls, and correlated with various clinical parameters. High soluble BSG was associated with worse overall survival, higher bone marrow blast percentage, and higher white blood cell count. BSG SNPs rs4919859 and rs4682, as well as MCT1 SNP rs1049434, were also associated with overall survival of AML patients. In conclusion, this study confirms the importance of BSG/MCT1 in AML, and suggests that soluble BSG and BSG/MCT1 genetic variants may act as potential AML biomarkers.

Keywords: basigin; CD147; monocarboxylate transporter 1; acute myeloid leukemia; single nucleotide polymorphism; survival

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1. Introduction

Acute myeloid leukaemia (AML) is a haematologic malignancy associated with uncontrolled proliferation of immature leukemic blasts that can lead to bone marrow failure [1]. It is the most commonly diagnosed acute leukaemia in adults and its prognosis remains poor, with the 5-year overall survival rate being only 25% [2]. While various prognostic markers exist, the highly heterogeneous nature of AML means that treatment outcomes can vary widely between patients [3]. Therefore, new diagnostic and prognostic markers are needed.

Basigin (BSG), also known as cluster of differentiation 147 (CD147) and extracellular matrix metalloproteinase inducer (EMMPRIN), is a glycoprotein that belongs to the immunoglobulin superfamily. It is a transmembrane protein commonly expressed on many human cell types and first identified as a stimulator of matrix metalloproteinase (MMP) production, although it is now known to also be involved in many other cellular

pathways [4,5]. It was recently found to be an alternative entry receptor for the SARS-CoV-2 virus, contributing to its spread [6,7]. BSG expression is increased in many cancers, and it is considered to be a biomarker for cancer diagnosis and prognosis [8–10]. It is known to promote tumour invasiveness and metastasis by stimulating MMP production [11]. However, it was shown that its pro-tumour function is mostly due to its interaction with monocarboxylate transporters (MCTs), such as MCT1 (also known as SLC16A1) and MCT4 (also known as SLC16A3), rather than MMPs [12]. MCTs are membrane-bound proteins that mediate transport of lactic acid across the membrane [13]. MCTs are especially important in cells using glycolysis for energy generation, as increased lactate production through glycolysis can drastically decrease cytosolic pH and lead to cell death through acidosis [14]. This is particularly true for cancer cells, which, unlike most normal cells, continually rely on glycolysis [15]. BSG acts as a chaperone to MCT1 and is necessary for its proper functioning, as BSG downregulation leads to disruption of lactate transport and decreases proliferation of tumour cells [16–18]. Another function of BSG that is important in cancer is its stimulatory effect on angiogenesis by inducing the expression of the pro-angiogenic vascular endothelial growth factor (VEGF) [19].

While many studies exist on BSG in solid tumours [20–23], little is known about its role in haematological malignancies. Studies on multiple myeloma (MM) proved that BSG expression is increased and this increase accompanied disease progression [24]. While both MCT1 and MCT4 were overexpressed in MM, only MCT1 downregulation led to a decrease in myeloma cell proliferation [16]. In our own study, BSG and MCT1 single nucleotide polymorphisms (SNPs) were also shown to affect MM survival [25]. In AML, BSG was shown to be co-expressed with VEGF in an immunohistochemical staining experiment, which showed it to be mostly present in the cytoplasm and cell membrane. This BSG–VEGF co-expression turned out to be a prognostic marker of overall survival in AML [26]. BSG overexpression was found to promote AML cell proliferation, and BSG inhibitor AC-73 had a potent antiproliferative effect on AML cells [27].

Apart from its membrane-bound form, BSG can also be secreted in soluble form. This can be done either by cleavage of its extracellular domain by MMPs/ADAM12, or by release of microvesicles [28–30]. Soluble BSG (sBSG) can be easily detected in various body fluids such as serum or plasma, and was found to be a biomarker in some diseases [31–33], although it was never studied in haematological malignancies. sBSG is thought to bind to membrane-bound BSG and stimulate proliferation. sBSG internalization was also shown to stimulate cells to produce more sBSG through MMP-14-mediated cleavage [34].

In the present study, we aimed to confirm the expression status of BSG and associated proteins (MCT1 and VEGF) in AML. Furthermore, we wanted to determine whether soluble BSG, as well as BSG and MCT1 genetic variants, can be used as potential markers of AML susceptibility, survival, and progression.

2. Materials and Methods

2.1. Culture of Cell Lines

Six established human AML cell lines, as well as normal human primary bone marrow CD34+ cells, were cultured in vitro for the analysis of BSG, MCT1, and VEGF expression (as previously described [35]). The normal CD34+ cells were used as a control. AML cell lines used in the study were Kasumi-3, Kasumi-1, NB4, HT93, MUTZ-3, and U937. All the cell lines were purchased from either the American Type Culture Collection (ATCC, Rockville, MD, USA) or Leibniz Institute DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and maintained at the Cell Culture Collection of the Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland). The HT93, NB4, Kasumi-1, Kasumi-3, and U937 lines were grown in RPMI-1640 (Gibco, Carlsbad, CA, USA) medium supplemented with 10% (NB4, U937) or 20% (HT-93, Kasumi-1, −3) FBS (Gibco, Carlsbad, CA, USA) and antibiotics. NB4 was additionally supplemented with GlutaMax (Gibco, Carlsbad, CA, USA). Normal CD34+ cells were grown in PromoCell Hematopoietic Progenitor Medium (PromoCell GmbH,

Heidelberg, Germany) and antibiotics, while MUTZ-3 was grown in MEMalfa medium (Gibco, Carlsbad, CA, USA) supplemented with 20% FBS and 20% 5637 conditioned medium (CM). All cells were incubated with 5% CO_2 at 37 °C. Cells and cell culture media were collected for further analyses.

2.2. Patients and Controls

The study involved a group of 37 newly diagnosed AML patients and 25 healthy blood donors for the analysis of serum soluble BSG, as well as a larger group of 92 newly diagnosed AML patients and 135 healthy blood donors for the analysis of BSG and MCT1 genetic variants. The healthy blood donors served as a control group. Patients in the larger group were aged 20–93, and the median age was 61. The group included 54 male and 38 female patients. The smaller group used for serum analyses was aged 25–93, and the median age was 62; there were 18 males and 19 females. Some of the patients (n = 27) from the smaller serum group were also included in the larger genetic group. Blood samples for all patients were obtained at diagnosis. Approval from the Wroclaw Medical University Bioethical Committee (ethical approval code: 368/2019) was obtained for the study. Clinical data analysed in the study are included in Table 1 for both groups.

Table 1. Characteristics of AML patients included in the study.

Data	Serum Study Group Range and Median (n = 37) [1]	Genetic Study Group Range and Median (n = 92) [1]
age	25–93, med = 62	20–93, med = 61
bone marrow blasts (%)	4–95, med = 68	20–98, med = 65
white blood cell count (G/L)	0.8–510.5, med = 22.7	0.7–510.5, med = 21
haemoglobin (g/dL)	6.0–11.4, med = 8.8	6.1–19.5, med = 9.2
platelets (G/L)	5–393, med = 44.5	2–460, med = 52
lactate dehydrogenase (U/L)	168–2170, med = 446.5	26–2535, med = 369
C-reactive protein (mg/L)	2.3–115.4, med = 36.3	1.1–359, med = 25

[1] The serum study group (n = 37) was used for analysis of serum soluble BSG, while the larger genetic study group (n = 92) was used for analyses of BSG and MCT1 genetic polymorphism.

2.3. RNA Extraction and Gene Expression Analysis

Total RNA was extracted from dry cell culture pellets using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA purity and integrity were verified on the DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA) and by gel electrophoresis. Using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Waltham, MA, USA), 2000 ng of isolated RNA was then reverse transcribed into cDNA and an RNase Inhibitor (Applied Biosystems, Waltham, MA, USA) was added into the reaction mix. Reverse transcription was performed in a SimpliAmp Thermal Cycler (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. cDNA was then stored at −70 °C until further use.

Quantitative real-time PCR was used to evaluate BSG, MCT1, and VEGF gene expression, and the expression data were normalized to β-actin (ACTB), acting as an internal control. TaqMan Gene Expression assays and TaqMan Gene Expression Master Mix (Applied Biosystems, Waltham, MA, USA) were used for the PCR. The probes used for the reaction were: Hs00936295_m1 (BSG), Hs01560299_m1 (MCT1), Hs00900055_m1 (VEGF), and Hs01060665_g1 (ACTB). Samples were run in triplicate, and three independent experiments were performed. PCR was performed on a LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. ELISA Analysis

Peripheral blood was collected from 37 AML patients and 25 healthy individuals. Blood was allowed to clot and was centrifuged for 15 min at 1000× g. Serum was collected

and stored at −70 °C until further use. Likewise, cell culture supernatants were collected (from cells cultured as described in Section 2.1), aliquoted, and stored at −70 °C. Soluble BSG and VEGF protein concentration were measured using the Human EMMPRIN/CD147 Quantikine ELISA Kit and Human VEGF Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocols. Absorbance was measured in a Sunrise absorbance microplate reader with Magellan analysis software (Tecan Trading AG, Männedorf, Switzerland). All samples were run in duplicate.

2.5. Western Blot Analysis

Western blot was used to assess the total BSG level in the lysates of cell lines. RIPA buffer with a protease inhibitor cocktail (Merck, Darmstadt, Germany) was used for cell lysate preparation. The total protein concentration of the lysates was measured using the modified Lowry method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. Subsequently, 10–25 µg of protein samples were separated on polyacrylamide gel, and three repeats were performed, for a total of three polyacrylamide gel electrophoreses. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes with a pore size of 0.45 µm (Merck, Darmstadt, Germany). The membranes were then incubated for 1 h with 5% non-fat dry milk in 0.1% Tris-buffered saline/Tween-20 (TBST) (Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland; Merck, Darmstadt, Germany), washed four times with TBST, and incubated overnight at 4 °C with an anti-BSG primary mouse antibody (1:200, sc-21746; Santa Cruz Biotechnology, Dallas, TX, USA). Subsequently, the membranes were washed four times with TBST, incubated for 1 h with a horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibody, and then washed again four times with TBST. The Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) was used for chemiluminescence, and bands corresponding to BSG expression were visualized on a ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA). For β-actin detection, the membranes were first incubated for 40 min in 100% methanol to remove the remaining ECL signal, then washed four times with TBST, blocked for 1 h with 5% non-fat dry milk, and washed again four times with TBST. The membranes were then incubated with a mouse anti-β-actin-HRP monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h and washed four times with TBST. Detection was performed as described above. Densitometry was performed using ImageJ 1.48v software (U. S. National Institutes of Health, Bethesda, MD, USA) and the results were normalized to β-actin.

2.6. DNA Isolation, SNP Selection and Genotyping

Genomic DNA was extracted from peripheral blood taken on EDTA from 92 AML patients and 135 healthy individuals using a Qiagen DNA Isolation Kit (QIAGEN, Hilden, Germany). DNA was also isolated from AML cell lines. DNA purity and concentration were verified on the DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA). Isolated DNA was subsequently stored at −20 °C until further use.

BSG and MCT1 SNPs were selected based on three criteria: (1) minor allele frequency (MAF) in European populations higher than 0.15, (2) a functional effect on expression/protein structure predicted by the National Institute of Environmental Health Sciences SNP Function Prediction tool [36], and (3) lack of high linkage disequilibrium between the SNPs. Based on these criteria, six SNPs (four in the gene coding for BSG and two in the gene coding for MCT1) were chosen. BSG and MCT1 SNP were determined using TaqMan SNP Genotyping (Applied Biosystems, Waltham, MA, USA) and LightSNiP (TIB MOLBIOL, Berlin, Germany) assays. PCR was performed on a LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturers' protocols.

2.7. Statistical Analysis

A non-parametric Mann–Whitney U test was used for comparison of VEGF levels and clinical parameters (e.g., blasts, WBC count, haemoglobin, lactate dehydrogenase, and

C-reactive protein) among AML patients with high/low sBSG. The Mann–Whitney U test and logistical regression were used for comparison of sBSG levels between patients and healthy individuals. A one-way ANOVA with Dunnett's multiple comparison test and multiplicity adjusted *p*-value was used to compare differences between BSG/MCT1/VEGF expression/sBSG/total BSG level in AML cell lines vs. normal CD34+ cells. Survival was analysed using the Kaplan–Meier method, while Spearman's coefficient was used to assess the correlation between sBSG and clinical parameters and VEGF. The Mann–Whitney U test, Kaplan–Meier survival analysis, and Spearman's correlation analysis were performed in a Real Statistics Resource Pack for Microsoft Excel 2013 (version 15.0.5023.1000, Microsoft, Redmont, WA, USA), logistical regression was performed in RStudio (RStudio, PBC., Boston, Massachusetts, USA), and the ANOVA with Dunnett's test was performed in GraphPad Prism (version 8.0.1, GraphPad Software, San Diego, CA, USA). Linkage disequilibrium and Hardy–Weinberg equilibrium analyses were performed with the Haploview 4.2 software. Fisher's exact test was used to test if allele/genotype frequencies differed between patients and controls, as well as between subgroups of patients. This was calculated using the web-based tool http://vassarstats.net/tab2x2.html (accessed on 29 September 2021). *p*-values < 0.050 were considered statistically significant, while those between 0.050 and 0.100 were considered to be indicative of a trend.

3. Results

3.1. Expression of BSG, MCT1 and VEGF mRNA in AML and Control Cell Lines

To assess the general BSG expression level in AML, we analysed BSG mRNA levels in six AML cell lines (U937, Kasumi-1, NB4, HT93, MUTZ-3, and Kasumi-3), as well as in normal primary bone marrow CD34+ cells. Relative BSG expression was significantly higher in all AML lines compared to the normal CD34+ cells ($p < 0.001$; Figure 1A). Expression was the highest in Kasumi-1 (8.99 relative to CD34+ cells), and the lowest in MUTZ-3 (2.51 relative to normal CD34+ cells), while the median expression level of all AML lines was 3.14.

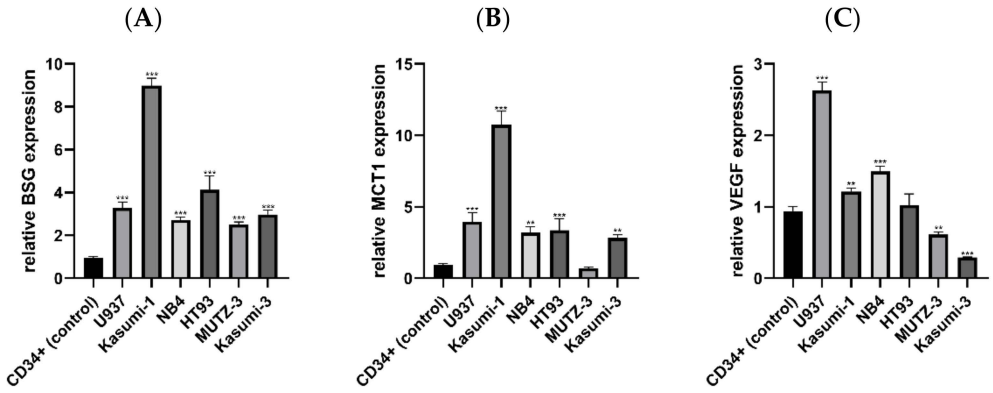

Figure 1. Relative expression of BSG (**A**), MCT1 (**B**), and VEGF (**C**) mRNA in acute myeloid leukaemia (AML) cell lines and normal primary bone marrow CD34+ cells. Expression of BSG is uniformly higher in all AML lines, while MCT1 is overexpressed in all lines except for MUTZ-3. Expression of VEGF is not consistent, although it is either higher than or similar to normal CD34+ cells in most AML lines. Each bar represents results from three independent experiments. The significance of the differences between individual lines and the normal CD34+ cells is indicated as: $p < 0.050$, ** $p < 0.010$, *** $p < 0.001$.

Additionally, we assessed the mRNA expression of MCT1 and VEGF, the two proteins associated with BSG function in cancer. MCT1 expression was higher in all AML lines excluding MUTZ-3 ($p < 0.010$), which had slightly lower MCT1 expression compared to normal CD34+ cells (0.70 relative to normal CD34+ cells; Figure 1B). As with BSG, Kasumi-1 was characterized by the highest level of MCT1 expression (10.76 relative to CD34+ cells), while the median expression level of all AML lines (including MUTZ-3) was

3.36. Unlike MCT1 and BSG, relative VEGF expression was not uniform among AML lines, although most AML lines had expression levels higher than or similar to normal CD34+ cells (Figure 1C). The highest expression was observed in U937 cells (2.63 relative to normal CD34+ cells). Two lines (Kasumi-1, NB4) had expression slightly higher than normal CD34+ cells. One was not significantly different from the control (HT93), while two lines (Kasumi-3 and MUTZ-3) had lower expression than the control.

3.2. Soluble BSG in Serum as a Marker of AML Susceptibility and Survival

Having proven the increased expression of BSG in AML, we attempted to analyse the expression of soluble BSG (sBSG) in the serum of AML patients (n = 37) and healthy individuals (n = 25). The median and interquartile range were 4186.45 pg/mL (IQR = 3658.70–5710.65) for the former and 3894.45 pg/mL (IQR = 2903.50–4544.15) for the latter group. Comparing sBSG levels in the two groups, we found that sBSG was higher in the serum of AML patients compared to healthy individuals (p = 0.039, Figure 2). Additionally, we performed an age- and sex-adjusted logistical regression analysis that included the level of sBSG as a potential factor differentiating cases from controls. This analysis seems to confirm the trend of higher sBSG in AML patients (p = 0.077). This indicates that serum sBSG may be a potential marker of AML.

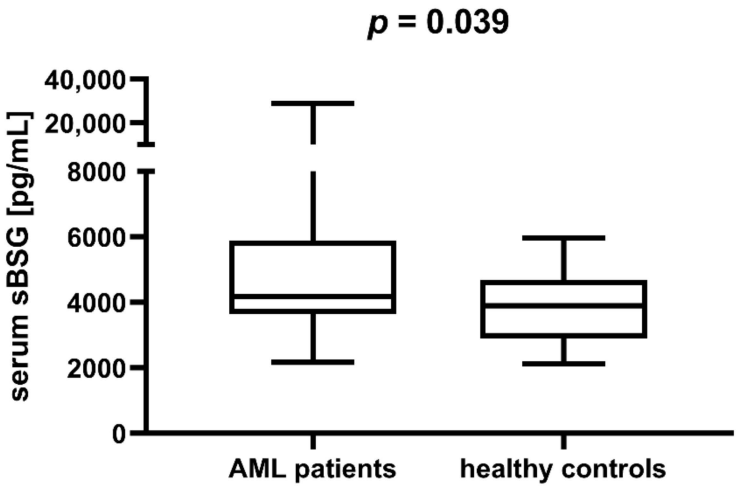

Figure 2. Serum soluble basigin (sBSG) in acute myeloid leukaemia (AML) patients is higher than in healthy individuals.

For further analyses, we divided our cohort of AML patients into two groups according to their sBSG level—those with high sBSG (values above the upper quartile, or 5710.65 pg/mL) and those with low sBSG (values below the upper quartile). Using the Kaplan–Meier method, we found that patients in the high sBSG group had significantly worse overall survival (OS) than patients in the low sBSG group (p = 0.028; Figure 3).

In the next step, we compared sBSG with various clinical parameters of AML. Soluble BSG was positively correlated with the percentage of blasts in the bone marrow (p = 0.017, R = 0.441), and negatively with haemoglobin (p = 0.049891, R = −0.339), and a trend for a positive correlation with white blood cell (WBC) count was observed (p = 0.061, R = 0.325). In terms of groups with different (high/low, as defined in the previous paragraph) sBSG expression, patients with high sBSG were characterized by a higher percentage of bone marrow blasts (p = 0.034; Figure 4A) and higher white blood cell count (p = 0.017; Figure 4B) compared to low sBSG patients. A trend for lower haemoglobin (p = 0.082; Figure 4C) was also observed. However, sBSG did not correlate with the serum levels of the angiogenic factor VEGF (p = 0.786, R = 0.048).

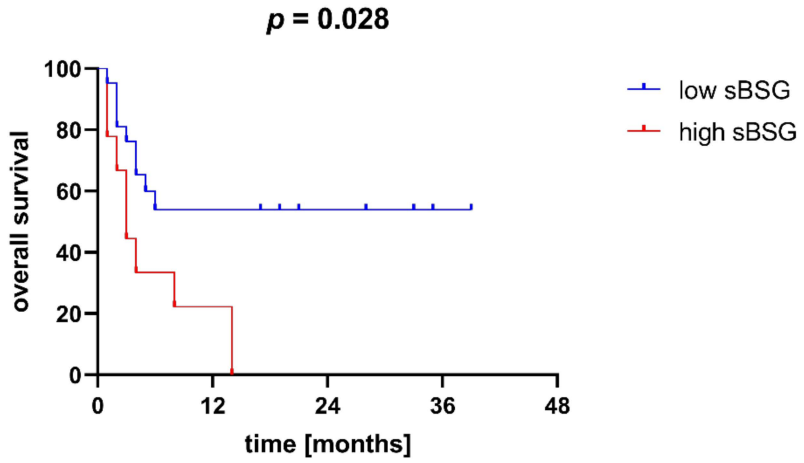

Figure 3. Overall survival (OS) of patients with high and low soluble BSG (sBSG) serum levels. Patients with high sBSG are characterized by significantly worse OS. The cut-off for high BSG was the upper quartile among the studied patients, or 5710.65 pg/mL.

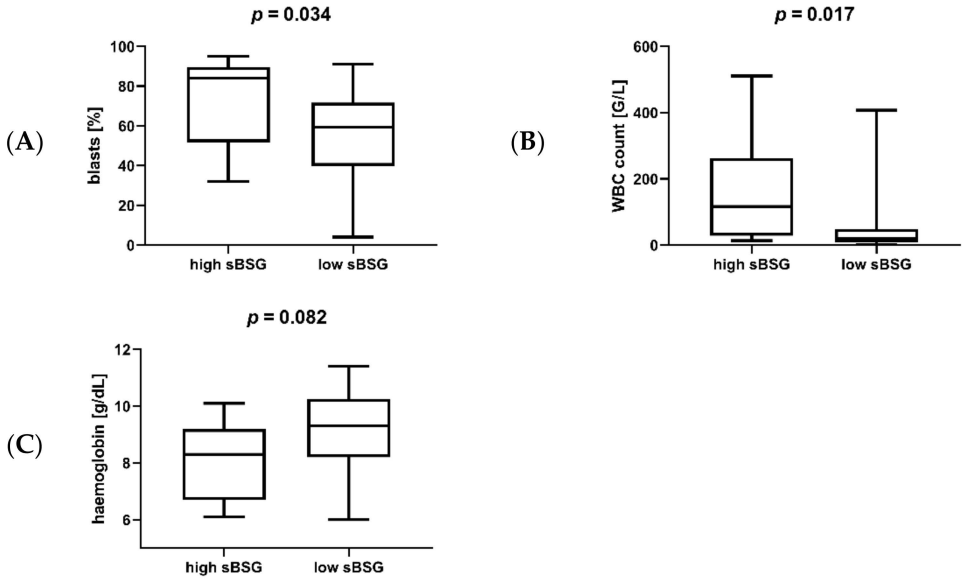

Figure 4. Percentage of blasts in the bone marrow (**A**), white blood cell (WBC) count (**B**), and haemoglobin levels (**C**) in AML patients with high and low sBSG. Patients with high sBSG were characterized by a higher percentage of blasts, higher WBC count, and lower haemoglobin levels, although the latter association was not statistically significant. Increased percentage of blasts, increased WBC count, and decreased haemoglobin are all hallmarks of more advanced AML. The cut-off for high sBSG was the upper quartile among the studied patients, or 5710.65 pg/mL.

Soluble BSG was also measured in supernatants of AML cell lines and normal CD34+ cells. All AML lines except for NB4 (2928.93 pg/mL) secreted lower sBSG levels than normal CD34+ cells (2129.42 pg/mL; Figure 5A). The median sBSG level for all AML lines was 687.06 pg/mL and two lines (Kasumi-3 and HT93) secreted sBSG at barely detectable levels (120.98 and 152.22 pg/mL, respectively). Similarly, we measured the total BSG in AML cells and normal CD34+ cells. Three cell lines (U937, Kasumi-1, and NB4) had higher BSG levels compared to normal CD34+ cells (corresponding to higher levels of their mRNA expression), but one had similar levels (HT93), and two had lower levels (MUTZ-3, Kasumi-3; Figure 5B). Interestingly, the total BSG was strongly correlated with VEGF mRNA expression in AML cell lines ($p = 0.005$, R = 0.943).

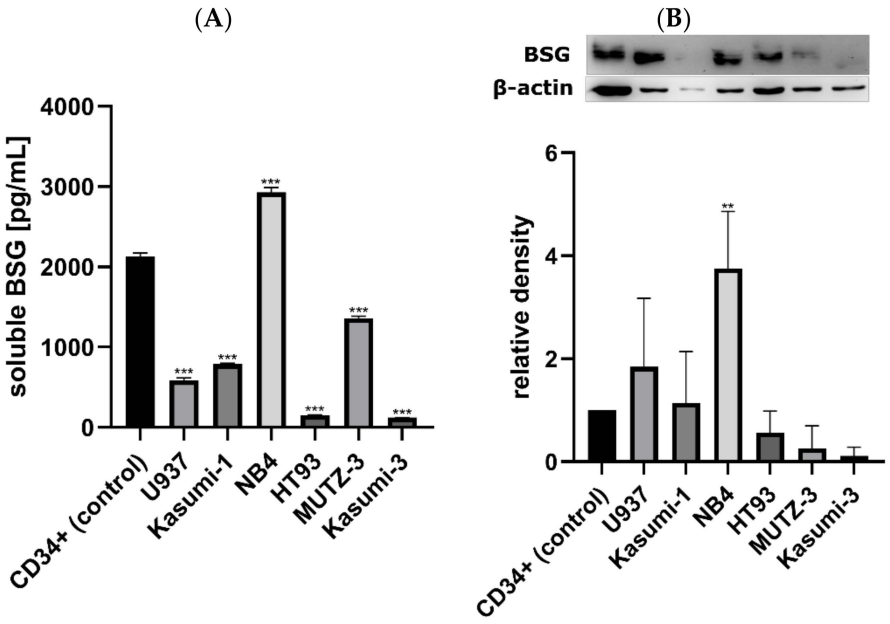

Figure 5. Protein expression of BSG in AML cell lines and normal CD34+ cells. (**A**) shows secreted soluble BSG in cell culture supernatants, while (**B**) shows the total BSG levels in cell lysates of AML cell lines and normal CD34+ cells normalized to β-actin. Protein expression of BSG in relation to normal CD34+ cells differs between various AML lines and is not consistent, although most cell lines exhibit lower soluble BSG levels than the control (**A**), and total BSG levels higher than or similar to the control (**B**). Each bar represents results from three technical repeats, and representative immunoblots for BSG and β-actin are shown above the plot (**B**). The significance of the differences between individual lines and the normal CD34+ cells is indicated as: $p < 0.050$, ** $p < 0.010$, *** $p < 0.001$.

3.3. BSG and MCT1 Genetic Variants Are Associated with Survival and Other Clinical Parameters of AML

We genotyped a group of AML patients ($n = 92$) and healthy individuals ($n = 135$) for four BSG (rs4919859, rs4682, rs8637, and rs8259) and two MCT1 (rs1049434 and rs9429505) SNPs. The frequencies for all the SNPs were in accordance with the Hardy–Weinberg equilibrium in both groups. No statistically significant differences between patients and healthy individuals were detected, suggesting no association of selected SNPs with the predisposition to AML. The distribution of genotypes is presented in Table 2.

The Kaplan–Meier method was used to assess the effects of BSG and MCT1 SNPs on overall survival. The analysis showed that BSG alleles rs4919859 C and rs4682 C were associated with significantly worse OS ($p = 0.014$ and $p = 0.048$, respectively; Figure 6A and 6B), while carriers of MCT1 allele rs1049434 T were characterized by better OS ($p = 0.043$; Figure 6C). Additionally, a trend towards better OS in patients with MCT1 allele rs9429505 A was observed ($p = 0.063$). No association between the SNPs and soluble BSG levels was found in AML patients.

Table 2. Distribution of basigin (BSG) and monocarboxylate transporter 1 (MCT1) genotypes in acute myeloid leukaemia (AML) patients and healthy individuals.

	AML Patients	Healthy Individuals
BSG rs4919859		
CC	18 (19.6%)	19 (14.1%)
CG	35 (38.0%)	54 (40.0%)
GG	39 (43.4%)	62 (45.9%)
BSG rs4682		
CC	7 (7.6%)	4 (3.0%)
CT	26 (28.3%)	35 (25.9%)
TT	59 (64.1%)	96 (71.1%)
BSG rs8637		
AA	30 (32.6%)	47 (34.8%)
AG	45 (48.9%)	61 (45.2%)
GG	17 (18.5%)	27 (20.0%)
BSG rs8259		
TT	50 (54.3%)	78 (57.8%)
TA	31 (33.7%)	49 (36.3%)
AA	11 (12.0%)	8 (5.9%)
MCT1 rs1049434		
AA	28 (30.4%)	54 (40.0%)
AT	45 (48.9%)	58 (43.0%)
TT	19 (20.7%)	23 (17.0%)
MCT1 rs9429505		
AA	52 (56.5%)	67 (49.6%)
AG	34 (37.0%)	60 (44.4%)
GG	6 (6.5%)	8 (5.9%)

We also analysed BSG and MCT1 SNPs in relation to various clinical parameters of AML. We observed that BSG alleles rs4682 C and rs8259 A were more common in AML subtypes M0–M2 (myeloblastic leukaemia with/without maturation) than in other subtypes ($p = 0.017$ and $p = 0.006$, respectively). Additionally, MCT1 allele rs9429505 G was associated with higher CRP ($p = 0.049$).

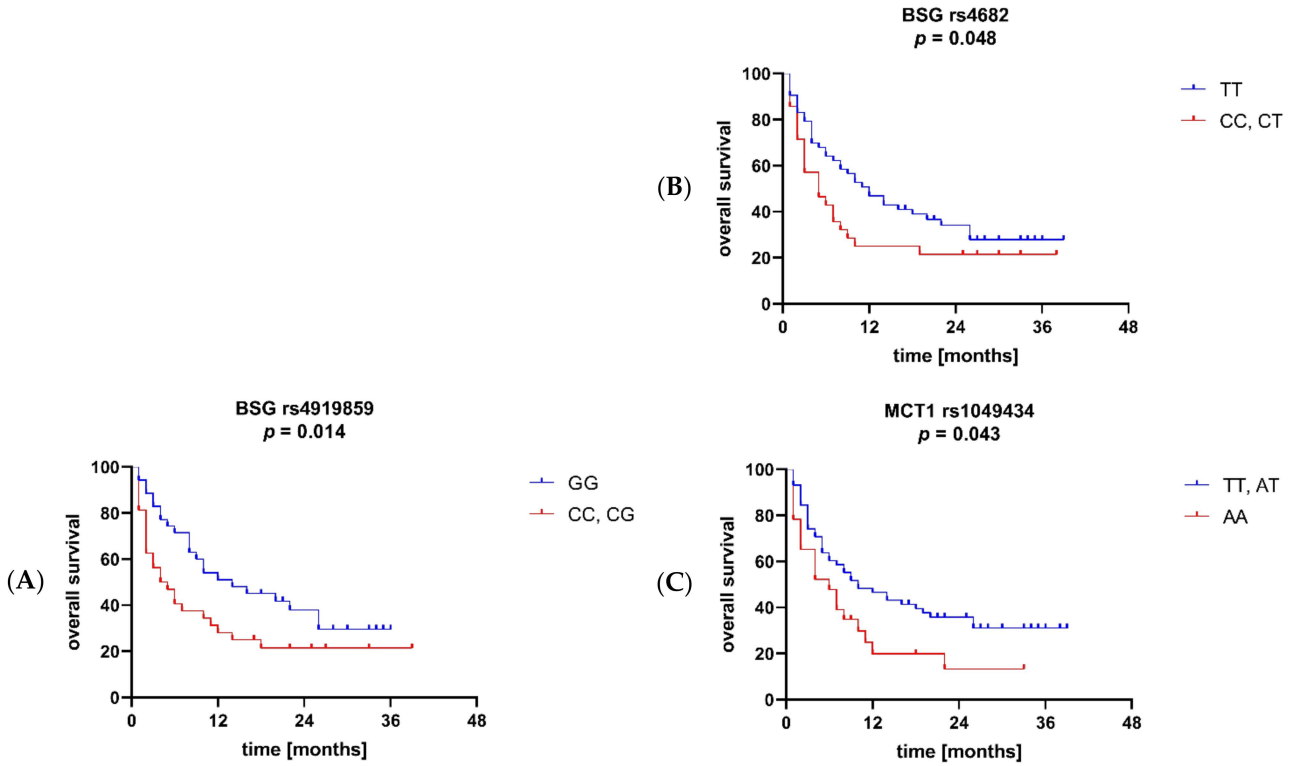

Figure 6. Overall survival (OS) in acute myeloid leukaemia (AML) patients with variants of basigin (BSG) and monocarboxylate transporter 1 (MCT1) SNPs. Patients with BSG alleles rs4919859 C (**A**) and 4682 C (**B**) were characterized by worse OS, while those with MCT1 allele rs1049434 T (**C**) had better OS.

4. Discussion

BSG (CD147) is a multifunctional glycoprotein involved in several regulatory pathways associated with cell adhesion, migration, proliferation, angiogenesis, ion transport, or drug efflux. Recent findings on its potential involvement in SARS Coronavirus 2 (SARS-CoV-2) infection increased the need to better describe BSG expression patterns in various tissues [6,7]. BSG was studied in many solid tumours, as well as recently in multiple myeloma and acute lymphoblastic leukaemia [20–25,37–40]. However, relatively little is known about its involvement in acute myeloid leukaemia (AML).

In the present study, we aimed to clarify the expression status of BSG and MCT1 RNA in AML cells and to study soluble serum BSG as well as BSG and MCT1 genetic variants as potential biomarkers of AML risk, survival, and progression. We found BSG and MCT1 mRNA expression to be significantly increased in almost all AML cell lines compared to normal primary bone marrow CD34+ cells. This confirms earlier reports regarding potential BSG and MCT1 overexpression in AML [26,27,41]. This pattern of BSG and MCT1 overexpression was also observed earlier in multiple myeloma [16,42]. Unusually, MCT1 showed decreased expression in one cell line, MUTZ-3, which is a model for M4 AML, or acute myelomonocytic leukaemia. The reason for this is unclear and requires further study. Nonetheless, the BSG level was still increased in this cell line.

It is established that angiogenesis plays a role in AML [43]. Since BSG was previously shown to stimulate expression of the pro-angiogenic factor VEGF [19], and VEGF was shown to be co-expressed with BSG in bone marrow samples from AML patients [26], we decided to assess VEGF expression in AML cell lines. However, the pattern of VEGF mRNA expression differed from that of BSG: some lines exhibited overexpression (NB4, U937, Kasumi-1), some had similar expression levels to the normal cells (HT93), while others had decreased expression (Kasumi-3, MUTZ-3). However, VEGF was strongly correlated with the total protein BSG, as measured by western blot in AML cells. This seems to be in line

with the previously proposed mechanism of VEGF upregulation through membrane-bound BSG and MMPs, and may confirm the importance of BSG in VEGF regulation in AML cells.

A major goal of our study was to determine whether BSG is a potential biomarker in AML. While it works primarily as a membrane-bound protein, various studies have found soluble circulating BSG to be a marker of disease [31–33]. In this study, we showed for the first time that sBSG is more abundant in the serum of AML patients than healthy individuals. Moreover, patients with high sBSG were found to have worse overall survival, more blasts in the bone marrow, higher WBC count, and lower haemoglobin levels. All of this suggests that sBSG may be a marker of worse survival, as a high percentage of blasts and high WBC count are major hallmarks of AML. Additionally, patients often have decreased haemoglobin due to anaemia resulting from impaired bone marrow function. The mode of action of sBSG is not fully understood, but it is believed that it can dimerize with membrane-bound BSG to exert its influence [34,44]. Interestingly, we did not observe increased sBSG in AML cell culture supernatants, even though the cells exhibited increased levels of BSG mRNA. Actual protein production may not necessarily correspond to mRNA levels, and our results show that not all of the cell lines produced total BSG in levels corresponding to their BSG mRNA expression. However, this does not seem to explain the decreased sBSG secretion in almost all of the investigated cell lines. Furthermore, an earlier study suggests that total protein production of BSG does correlate with mRNA expression in AML cells [27]. A significant factor influencing the observed levels of sBSG may be the way in which sBSG is secreted. BSG is primarily expressed on the cell surface, and only some of its molecules are released, either by MMPs/ADAM12-mediated cleavage, or in microvesicles [28–30]. This means that sBSG levels may be regulated by more factors and do not only reflect mRNA and protein BSG expression. Furthermore, sBSG may be released by other cells from the bone marrow microenvironment, following stimulation by AML cells. A study on gingival fibroblasts and U937 cells found that their co-culture released more sBSG than the respective cultures did separately [45]. The same study also confirmed that sBSG participated in a self-regulatory mechanism with its membrane-bound form [45]. An earlier study also suggested that sBSG internalization induced secretion of more sBSG [34], acting in a positive feedback loop. All this evidence suggests that sBSG is likely not causative in the associations with AML described here, but it can still be considered a potentially useful diagnostic and prognostic biomarker.

Another aspect of this study was to analyse SNPs in the gene coding for BSG and MCT1 in the context of AML. We previously showed that SNPs can act as biomarkers in AML [46,47], and we identified some BSG and MCT1 variants that were associated with survival in multiple myeloma patients; most importantly, BSG allele rs4919859 C was associated with worse MM progression-free survival [25]. In the current study, allele rs4919859 C was likewise associated with adverse overall survival in AML patients. However, no association was observed in AML patients for BSG allele rs8637 G, which conferred worse survival on MM patients [25]. The rs4919859 BSG SNP is predicted to lie in a transcription factor binding site [35], and therefore is expected to exert its influence by affecting BSG transcription rates. Indeed, we observed that AML cell lines with the highest BSG RNA expression (Kasumi-1 and HT-93) were homozygous for BSG rs4919859 C. Nevertheless, this SNP remains relatively under-studied; other than the current study and our previous work on MM patients [25], only one study has analysed it in the context of coronary heart disease [48]. Another SNP found to be associated with AML survival in the current study is rs4682, which is predicted to be located in an exonic splicing enhancer/silencer (ESE/ESS) [36].

We also analysed two MCT1 SNPs, one of which, rs1049434 (allele T), was associated with better overall survival. This SNP represents a missense Asp (A) to Glu (T) mutation, and the Glu (T) isoform is thought to be better at transporting lactate [49]. The effect of this SNP seems to be inconsistent between different diseases and conditions [25,50–52]. This may be due to MCT1's being quite elastic in its role as a transporter, as it can transport monocarboxylates bidirectionally [13]. In the case of AML, the effect of rs1049434 on

survival may be influenced by the fact that MCT1 was recently found to be more important in lactate uptake, rather than efflux, in differentiating AML cells [42]. This is due to the fact that AML cells, unlike most cancer cells, often employ oxidative phosphorylation for energy production [53]. The dual nature of MCT1 may be the reason behind the varying effects of the rs1049434 SNP between our work and previous works, although this remains to be confirmed in a separate study.

5. Conclusions

In conclusion, our study shows that BSG and MCT1 are overexpressed in most AML cells compared to normal primary bone marrow CD34+ cells. Soluble serum BSG is increased in AML patients compared to healthy individuals and its high level is associated with lower overall survival and worse clinical parameters. BSG and MCT1 genetic variants are associated with overall survival and other clinical parameters. This study confirms the role of BSG and MCT1 in AML and shows that soluble serum BSG and BSG/MCT1 genetic variants may act as markers of AML survival.

Author Contributions: Conceptualization, P.Ł. and K.B.-K.; methodology, P.Ł, E.T., M.S.-S., J.W. and K.B.-K.; formal analysis, P.Ł. and K.B.-K.; investigation, P.Ł. and K.B.-K.; resources, P.Ł., A.B., E.T., J.W., G.M. and K.B.-K.; data curation, P.Ł., A.B. and E.T.; writing—original draft preparation, P.Ł. and K.B.-K.; writing—review and editing, P.Ł., A.B., E.T., M.S.-S., J.W., G.M. and K.B.-K.; visualization, P.Ł.; supervision, J.W., G.M. and K.B.-K.; project administration, P.Ł. and K.B.-K.; funding acquisition, P.Ł. and K.B.-K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Wroclaw Medical University (protocol code: 368/2019, 25 April 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Conflicts of Interest: The authors declare no conflict of interest.

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Article

Soluble CD147 (BSG) as a Prognostic Marker in Multiple Myeloma

Piotr Łacina [1,*], Aleksandra Butrym [2], Diana Frontkiewicz [3], Grzegorz Mazur [4] and Katarzyna Bogunia-Kubik [1]

1 Laboratory of Clinical Immunogenetics and Pharmacogenetics, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wrocław, Poland; katarzyna.bogunia-kubik@hirszfeld.pl
2 Department of Cancer Prevention and Therapy, Wroclaw Medical University, 50-556 Wrocław, Poland; aleksandra.butrym@umw.edu.pl
3 Department of Haematology, Sokołowski Specialist Hospital, 58-309 Wałbrzych, Poland; d.frontkiewicz@gmail.com
4 Department of Internal, Occupational Diseases, Hypertension and Clinical Oncology, Wroclaw Medical University, 50-556 Wrocław, Poland; grzegorz.mazur@umw.edu.pl
* Correspondence: piotr.lacina@hirszfeld.pl; Tel.: +48-713-709-960 (ext. 236)

Abstract: CD147 (basigin, BSG) is a membrane-bound glycoprotein involved in energy metabolism that plays a role in cancer cell survival. Its soluble form is a promising marker of some diseases, but it is otherwise poorly studied. CD147 is overexpressed in multiple myeloma (MM) and is known to affect MM progression, while its genetic variants are associated with MM survival. In the present study, we aimed to assess serum soluble CD147 (sCD147) expression as a potential marker in MM. We found that sCD147 level was higher in MM patients compared to healthy individuals. It was also higher in patients with more advanced disease (ISS III) compared to both patients with less advanced MM and healthy individuals, while its level was observed to drop after positive response to treatment. Patients with high sCD147 were characterized by worse progression-free survival. sCD147 level did not directly correlate with bone marrow CD147 mRNA expression. In conclusion, this study suggests that serum sCD147 may be a prognostic marker in MM.

Keywords: BSG; basigin; CD147; MM; multiple myeloma; survival

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1. Introduction

Multiple myeloma (MM) is an incurable bone marrow malignancy associated with the presence of atypical plasma cells and with occurrence of end organ damage. It is the second most common haematological malignancy and accounts for 2% of all cancer cases [1,2]. MM is proceeded by monoclonal gammopathy of undetermined significance (MGUS), which has a 1% chance of progressing to malignant MM [3]. While novel therapeutic options, such as autologous stem cell transplantation and immunomodulatory drugs, led in the last two decades to a significant increase in overall survival, mean survival of MM patients is still relatively low at approximately 5 years [4,5].

CD147, also known as basigin (BSG) and extracellular matrix metalloproteinase inducer (EMMPRIN), is a heavily glycosylated member of the Ig superfamily. It is encoded by the *BSG* gene located on chromosome 19p13.3 and is ubiquitously expressed on various types of cells [6,7]. It is primarily described as a transmembrane protein. Its main isoform, basigin-2, is composed of a longer extracellular domain including a signal sequence and two Ig domains, as well as of shorter transmembrane and cytoplasmic domains [8,9]. CD147 can also be found in body fluids in the form of soluble CD147 (sCD147). sCD147 can be secreted by cells as a full-length protein released with microvesicles [10]. Alternatively, the extracellular domain can be cleaved and released through one of two pathways, one involving matrix metalloproteinases (MMPs), and one involving ADAM12 [11,12]. CD147

is a multifunctional protein involved in many cellular pathways [9,13]. Additionally, recent reports indicate that it may function as an alternative entry receptor for the SARS-CoV-2 virus associated with the COVID-19 pandemic [14,15].

CD147 is overexpressed in many cancers and is known to promote cancer progression [16,17]. It was found to be a marker of risk, poor prognosis, overall and progression-free survival, as well as chemotherapy resistance [18,19]. Although many CD147-associated pathways may be responsible for this, it was shown that interaction between CD147 and monocarboxylate transporters (MCTs) contributes most to this pro-tumour effect [20]. MCTs are membrane-bound transporters of monocarboxylates such as lactic acid [21]. They are important components of energy metabolism, as lactic acid is a by-product of glycolysis which needs to be removed from the cell to avoid dangerous decrease in cytosolic pH [22]. Most cancer cells rely primarily on glycolysis for energy production, a phenomenon known as the Warburg effect, making proper functioning of MCTs crucial for them [23].

CD147 functions as a chaperone of MCT1 (also known as SLC16A1) and its downregulation is known to be detrimental to proper lactate transport and tumour survival [24–26]. CD147 is also known to be involved with expression of other proteins such as MMPs, and vascular endothelial growth factor (VEGF), while its own expression can be controlled by various other factors, e.g., receptor activator for nuclear Factor κ B ligand (RANKL) [27–29]. Its ability to induce VEGF expression also contributes to cancer development and progression, as VEGF is an important pro-angiogenic factor [29].

Like in other cancers, CD147 was shown to be overexpressed in MM and to be associated with MM progression [30]. Likewise, MCT1 and MCT4 were also overexpressed in MM patients, although only MCT1 was indispensable for continued MM cell proliferation [24]. Genetic variants of both CD147 and MCT1 were found to influence survival in MM patients [31], and CD147 is known to be involved in response to MM treatment [32,33]. Myeloma cells were observed to exhibit increased lactate transport, whereas CD147 gene expression was found to correlate with key regulators of glycolysis and the Warburg effect, further substantiating the pro-myeloma effect of CD147 [24,34].

Soluble CD147 is thought to support cancer proliferation by interacting with membrane-bound CD147 [35]. It was shown to be an easily detectable biomarker in some diseases [36–38], although its role in haematological malignancies is poorly studied. We recently showed thatsCD147 is overexpressed in acute myeloid leukaemia (AML) patients compared to healthy individuals and that high CD147 is associated with worse overall survival [39].

In the present study, we aimed to determine whether serum sCD147 could be used as a potential prognostic marker in MM. Furthermore, we wanted to establish if sCD147 level correlated with the proangiogenic factor VEGF and mRNA expression of CD147 in the bone marrow.

2. Materials and Methods

2.1. Patients and Controls

The study included 62 newly diagnosed MM patients and 25 healthy blood donors serving as the control group. Both groups were nearly equally divided into men and women (the ratio of females was 30/59 and 12/25, respectively). The study was approved by the Wroclaw Medical University Bioethical Committee (ethical approval code: 369/2019). According to International Staging System (ISS) stratification, 21.4% of patients were in stage I, 33.9% were in stage II, and 44.6% were in stage III. Most patients were administered either the bortezomib, melphalan, prednisone (VMP); 35.2%, or the bortezomib, thalidomide, dexamethasone (VTD); 29.6% regimen as first line therapy. Further clinical data of patients analysed in the study are included in Table 1.

Table 1. Characteristics of MM patients included in the study.

Data	Median and Range (n = 59)
Age	70 (43–88)
white blood cell count (G/L)	6.7 (2.4–20.9)
haemoglobin (g/dL)	10.1 (5.6–14.7)
total protein (g/dL)	8.4 (5.1–15.9)
albumin (g/dL)	3.8 (1.7–5.0)
lactate dehydrogenase (U/L)	225 (100–1595)
β2-microglobulin (mg/L)	4.4 (1.3–78.5)
C-reactive protein (mg/L)	10.2 (0.7–102.8)
creatinine (mg/dL)	1.00 (0.36–8.87)
calcium (mg/dL)	9.4 (7.4–14.0)

2.2. ELISA Analysis of Serum Samples

Peripheral blood from 62 MM patients and 25 healthy individuals was collected, allowed to clot, and subsequently centrifuged for 15 min at 1000× *g*. Serum samples were then collected, aliquoted, and kept at −70 °C until further use. Serum samples were used for measurements of sCD147 and VEGF concentrations, which were performed using the Human EMMPRIN/CD147 Quantikine ELISA Kit and Human VEGF Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer's instructions. All samples were run in duplicate. Subsequently, absorbance was measured in a Sunrise microplate reader with Magellan analysis software (Tecan Trading AG, Männedorf, Switzerland).

2.3. RNA Isolation and Gene Expression Analysis

Bone marrow aspirates from a group of 29 MM patients and 3 non-MM patients (working as a PCR control group) were collected, and mononuclear cells were isolated by Lymphodex (inno-train Diagnostik GmbH, Kronberg im Taunus, Germany) density-gradient centrifugation. Total RNA was then extracted using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol. RNA purity and integrity were verified on the DeNovix DS-11 specrophotometer (DeNovix Inc., Wilmington, DE, USA), and by gel electrophoresis. A total of 2000 ng of isolated RNA was used for reverse transcription into cDNA using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Waltham, MA, USA) and RNase Inhibitor (Applied Biosystems, Waltham, MA, USA) was added to the reaction mix. The reaction was performed in a SimpliAmp Thermal Cycler (Applied Biosystems, Waltham, MA, USA) according to manufacturer's instructions. The resulting cDNA was stored at −70 °C.

CD147 (BSG), MCT1 (SLC16A1), MCT4 (SLC16A3), and VEGF gene expression were measured using quantitative real-time PCR, and the raw expression data were normalized to β-actin (ACTB), which was used as a reference gene. TaqMan Gene Expression assays specific to each gene of interest, as well as TaqMan Gene Expression Master Mix (Applied Biosystems, Waltham, MA, USA) were used for the experiment. TaqMan Gene Expression probes (Applied Biosystems, Waltham, MA, USA) used were: Hs00936295_m1 (CD147), Hs01560299_m1 (MCT1), Hs00358829_m1 (MCT4), Hs00900055_m1 (VEGF), and Hs01060665_g1 (ACTB). Samples were run in duplicate. The reactions were performed on LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland) and according to manufacturer's instructions. Relative expression was then calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Statistical Analysis

The non-parametric Mann–Whitney U test was used for comparison between serum sCD147 and clinical parameters such as white blood cell count, haemoglobin, total protein, albumin, lactate dehydrogenase, β2-microglobulin, creatinine or C-reactive protein. Spearman's coefficient was used to assess correlations with serum sCD147, serum

VEGF, CD147/MCT1/MCT4/VEGF gene expression, and clinical parameters. Overall and progression-free survival were analysed using the Kaplan–Meier curves and Gehan–Breslow–Wilcoxon test, as well as multivariate Cox proportional hazards model, and the non-parametric Wilcoxon signed-rank test was employed to compare sCD147 level before and after response to treatment. These analyses were performed with the Real Statistics Resource Pack for Microsoft Excel 2013 (version 15.0.5023.1000, Microsoft, Redmond, WA, USA), RStudio (RStudio, PBC, Boston, MA, USA), and GraphPad Prism (version 8.0.1, GraphPad Software, San Diego, CA, USA). p-values < 0.05 were considered statistically significant, while those between 0.05 and 0.10 were indicative of a trend.

3. Results

3.1. Serum Soluble CD147 Is Increased in MM Patients

We analysed expression of sCD147 in serum of a group of MM patients (n = 62) and in the control group of healthy individuals (n = 25). The median sCD147 value was 4441.78 pg/mL (interquartile range: 3435.10–5798.96 pg/mL) in patients, and 3894.45 pg/mL (interquartile range: 2903.50–4544.15 pg/mL) in the control group. We found sCD147 to be significantly higher in serum of MM patients compared to the control group (p = 0.016, Figure 1).

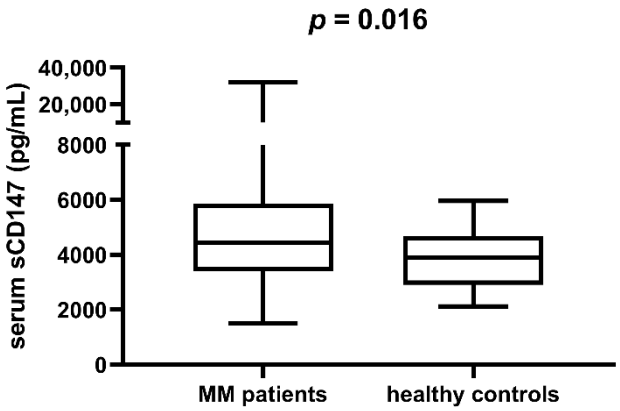

Figure 1. Serum sCD147 in multiple myeloma (MM) patients and the control group of healthy individuals. sCD147 is higher in the former group (p = 0.016).

3.2. Serum Soluble CD147 Is Associated with More Advanced Disease and Worse Survival

We analysed sCD147 expression in the context of some of the clinical parameters of MM. sCD147 was higher in patients in the more advanced stage III (mean: 5202.93 pg/mL, interquartile range: 3909.71–7986.53 pg/mL), compared to patients in stages I-II (mean: 3782.70, interquartile range: 3352.70–5196.10 pg/mL), according to the International Staging System (ISS) criteria (p = 0.012, Figure 2). sCD147 expression in stage III patients was also significantly higher than that of healthy individuals (p = 0.001).

Regarding other clinical parameters, we observed that sCD147 correlated with β2-microglobulin level (R = 0.279, p = 0.033, Figure 3A) and creatinine (R = 0.429, p = 0.001, Figure 3B). However, no associations with either white blood cell count, haemoglobin, total protein, albumin, lactate dehydrogenase, or C-reactive protein were observed. Additionally, we compared serum levels of sCD147 and of the proangiogenic factor VEGF. However, we did not find any correlation between them (R = −0.010, p = 0.941).

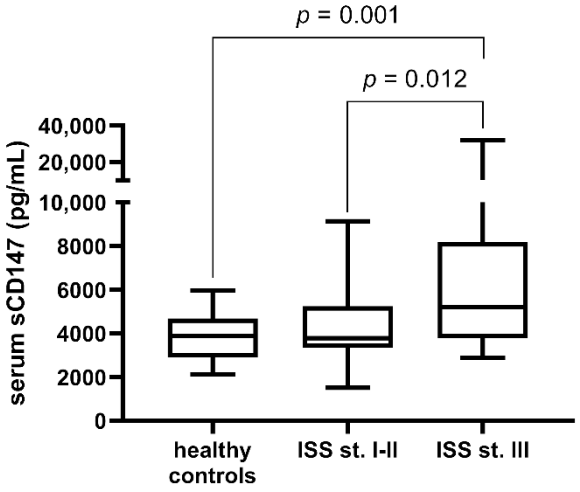

Figure 2. Serum sCD147 in patients in stages I-II and in patients in stage III of the International Staging System, or ISS. Healthy controls are also included. Patients in the more advanced stage III are characterized by higher sCD147 levels than both patients in the stages I-II ($p = 0.014$), and healthy controls ($p = 0.002$). Concurrently, healthy individuals did not significantly differ from patients in stages I-II ($p = 0.279$).

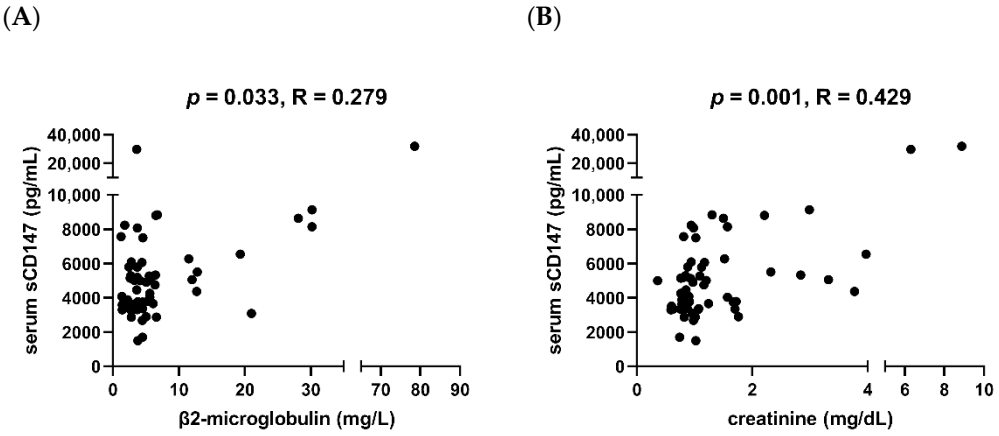

Figure 3. Correlation between serum soluble CD147 and two clinical parameters in MM patients—β2-microglobulin (**A**) and creatinine (**B**). Serum soluble CD147 is characterized by a weak-moderate correlation with these two parameters.

In the next step, we used Kaplan–Meier curves to analyse the difference in overall (OS) and progression-free survival (PFS) between patients with high and low sCD147. High sCD147 was defined in this and further analyses as being above the upper quartile in our study group (5798.96 pg/mL), and low sCD147 was below the upper quartile. While no difference was observed in OS, we observed that patients with high sCD147 were characterized by shorter PFS than patients with low sCD147 ($p = 0.046$, Figure 4).

Additionally, we constructed a Cox proportional hazards model including sCD147 status (low/high) and adjusting for age, β2-microglobulin level, creatinine level, ISS stage, and therapy (use of immunomodulatory drugs). This analysis confirmed high sCD147 to be an independent marker of adverse PFS ($p = 0.038$).

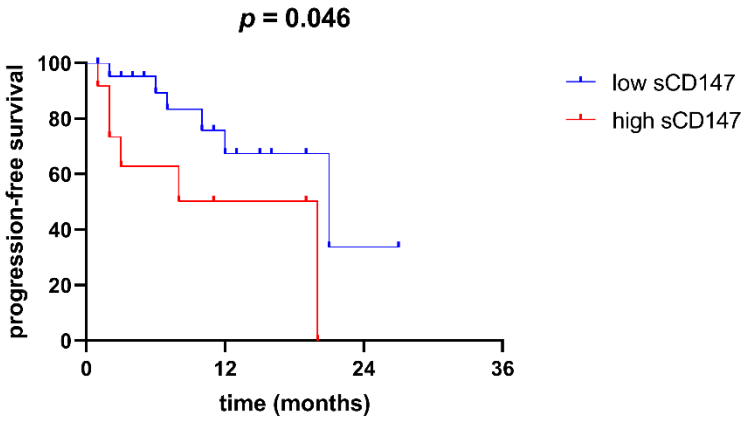

Figure 4. Progression-free survival of patients with high serum sCD147 (above the upper quartile, or 5798.96 pg/mL) and in patients with low serum sCD147 (below the upper quartile). Patients with high sCD147 are characterized by a more adverse PFS (p = 0.046).

3.3. Serum Soluble CD147 Levels Drop in Response to Treatment

In a subsection of MM patients (n = 10), we compared sCD147 levels after positive response to treatment (very good partial response or better) to those at diagnosis. We found sCD147 levels to significantly decrease in response to treatment in most patients (p = 0.025, Figure 5).

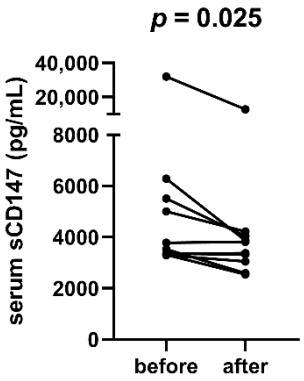

Figure 5. Serum sCD147 levels in multiple myeloma patients before and after positive response to treatment. sCD147 levels are decreased in most patients after remission (p = 0.025).

3.4. Serum Soluble CD147 Does Not Correlate with BSG mRNA Expression Levels

We measured relative mRNA *BSG* expression in bone marrow samples of a subgroup (n = 29) of MM patients. sCD147 level in serum did not correlate with mRNA CD147 expression in those patients (R = 0.027, p = 0.896). In addition to BSG, we also measured mRNA expression of various other genes associated with BSG in MM. We found that, as expected, BSG mRNA expression correlated strongly with expression of VEGF (R = 0.420, p = 0.023), MCT1/SLC16A1 (R = 0.811, $p < 0.001$), but not with MCT4/SLC16A3 (R = 0.061, p = 0.755). While no statistically significant associations between mRNA BSG expression and clinical parameters or survival were observed, we found a trend towards lower BSG expression in patients with positive (very good, partial, or better) response to treatment (p = 0.093).

4. Discussion

A growing body of evidence points to CD147 being a major factor in cancer progression and survival [16–19]. This may be a result of its multifunctional nature and involvement in a multitude of regulatory pathways that include cell migration, proliferation, and angiogenesis. Another pathway associated with tumour progression and promoted by

CD147 is epithelial–mesenchymal transition (EMT) [40,41]. Furthermore, recent research suggests that control of lactate transport through MCTs is a major function of CD147 in the context of cancer [20]. As CD147 was recently shown to function as an auxiliary receptor for SARS-CoV-2 infection [14,15], a good knowledge of its expression patterns may be of importance in the context of the COVID-19 pandemic.

Various studies suggests that CD147 is involved in development, progression, and response to treatment of MM [24,30,42]. CD147 is also implicated in response to treatment, particularly treatment involving immunomodulatory drugs [32,33]. In our previous studies, we showed that some genetic variants of CD147 and MCT1 affect survival of MM patients [31]. Soluble CD147 is a form of CD147 found in all body fluids and an easily measured potential biomarker, which we recently showed to be associated with survival in AML patients [39]. However, little is known about sCD147 in MM patients.

The main goal of the present study was to establish if sCD147 could be a prognostic marker in MM. Earlier study showed that serum/plasma sCD147 is elevated in many diseases, including some cancers [36–38,43,44] and our earlier studies show that it is elevated in AML [39]. Here, we observed that sCD147 level is significantly higher in MM patients compared to healthy individuals. Furthermore, sCD147 is higher in patients with more advanced disease than in both patients with less advanced MM, and healthy individuals. Additionally, we showed that sCD147 dropped in most analysed patients as a result of achieving remission, regardless of treatment regimen. High sCD147 also predicted shorter PFS, independently of variables such as age, ISS stage, or treatment regimen (whether immunomodulatory drugs were used or not).

All this evidence suggests that sCD147 is a potential prognostic marker associated with MM in general, and more particularly with MM progression and survival. Our results resemble those found in a study on breast cancer, which showed higher sCD147 in patients with primary breast cancer compared to benign diseases, as well as in patients with advanced cancer compared to early stage disease [37]. Similarly, sCD147 was shown to be elevated in patients with hepatocellular carcinoma (HCC), and to correlate with HCC tumour size and worse survival [36,45].

In our previous study, we likewise demonstrated that sCD147 is higher in AML patients and correlates with various AML clinical parameters and survival [39]. However, data on sCD147 expression in other cancers are scarce. Given that sCD147 is abundant in serum and plasma, but also in body fluids such as saliva and urine, it can be easily measured by protein detection methods such as ELISA. Therefore, it appears to be an interesting candidate for a potential biomarker not just in MM, but in cancer in general.

The role of sCD147 in multiple myeloma, and in cancer in general, is not very clear. CD147-containing microvesicles were shown to be internalized by myeloma cells and to increase their proliferation [42]. sCD147 is known to dimerize with membrane-bound CD147 in the tumour microenvironment, to stimulate production of more CD147 as well as various other proteins enhancing tumour survival [35,46]. However, we did not observe sCD147 to correlate with levels of the pro-angiogenic factor VEGF, even though there was a correlation between VEGF and CD147 expression on the mRNA level. This suggests that sCD147 might not be involved in BSG-dependent induction of VEGF expression and angiogenesis [29]. However, it is worth noting that VEGF regulation is very complex and involves many factors and pathways [47]. This includes post-transcriptional regulation processes, which means that its mRNA expression may not necessarily correspond to actual protein expression [48]. Additionally, we did not find the sCD147 level to correlate with CD147 mRNA expression. However, mRNA expression of both VEGF and MCT1 (SLC16A1) did correlate with CD147 mRNA level, as expected [29,33]. This may be due to the fact that CD147 is primarily expressed on the cell surface. Only a part of it is secreted, and this secretion is dependent on three specialised secretion pathways (one involving microvesicles, and two involving shedding of the CD147 extracellular domain) [10–12]. These results suggest that sCD147 secretion may not be the main way for CD147 to exert its

pro-tumour effect, although sCD147 still appears to be an interesting biomarker in multiple myeloma.

In conclusion, we showed that sCD147 may be a prognostic marker in MM, although our results may require confirmation on a larger cohort of patients. sCD147 appears to be a promising marker of cancer.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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CONCLUSIONS

1. BSG and MCT1, factors associated with energy metabolism, are confirmed to be overexpressed in acute myeloid leukaemia on mRNA level (Łacina et al, 2022a).
2. BSG and MCT1 genetic variants, especially the BSG polymorphism rs4919859, are associated with survival in multiple myeloma (Łacina et al, 2018) and acute myeloid leukaemia (Łacina et al, 2022a).
3. Soluble form of BSG (sBSG) measured in serum is elevated in multiple myeloma and acute myeloid leukaemia patients as compared to healthy individuals. Its high level is associated with shorter survival and correlates with clinical parameters, such as white blood cell count and beta-2-microglobulin level in blood (Łacina et al, 2022a; Łacina et al, 2022b). Furthermore, sBSG level was shown to decrease during remission in multiple myeloma patients (Łacina et al, 2022b).
4. Results of these studies suggest that BSG an MCT1 genetic variants as well as sBSG could be used as potential biomarkers in haematological malignancies.