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Die prognostische Bedeutung von β-Arrestin-2 und Alpha-Enolase beim Ovarialkarzinom

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Abkürzungsverzeichnis

BMI	-	body mass index
BRCA1/2	-	Brustkrebsgen 1/2
CA125	-	cancer antigen 125
CCC	-	clear cell carcinoma
cDNA	-	complementary desoxyribonucleic acid
CXCR2	-	CXC-Motiv-Chemokinrezeptor 2
EC	-	endometrioid carcinoma
EMT	-	epithelial-mesenchymale Transition
ENO-1	-	alpha-Enolase
EOC	-	epithelial ovarian cancer
ET_AR	-	Endothelin A Rezeptor
ERK	-	extracellular-signal regulated kinase
FSHR	-	follicle stimulating hormone receptor
FIGO	-	Fédération Internationale de Gynécologie et d'Obstétrique
GPCR	-	G protein coupled receptor
GPER	-	G protein coupled estrogen receptor
GRK	-	G-Proteingekoppelte-Rezeptor-Kinase
HBOC	-	hereditary ovarian cancer syndrome
HGSC	-	high Grade seous cancer
HNPCC	-	lynch syndrome
HRD	-	homologe Rekombinations-Defizienz
IκBα	-	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
IRS	-	Immunreaktiver Score
LGSC	-	low grade serous cancer
LHR	-	lunteinizing hormone receptor
MAPK	-	mitogen-activated-proteinkinase
MBP-1	-	c-myc Promoter-Bindeprotein
MC	-	mucinous carcinoma
NF-κB	-	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
OC	-	Ovarialkarzinom
mRNA	-	messenger ribonucleic acid
siRNA	-	small interfering ribonucleic acid
SC	-	serous carcinoma

TP-β	-	Thromboxan-β-Rezeptor
TXA_2	-	Thromboxan Rezeptor
VEGF-A	-	vascular endothelial growth factor
WHO	-	World Health Organisation
Wnt	-	wingless

Publikationsliste

Die vorliegende Dissertation wurde als kumulative Arbeit eingereicht. Grundlage dieser Arbeit sind die folgenden Publikationen:

1. Bastian Czogalla[#], **Alexandra Partenheimer**[#], Udo Jeschke, Viktoria von Schönfeldt, Doris Mayr, Sven Mahner, Alexander Burges, Manuela Simoni, Beatrice Melli, Riccardo Benevelli, Sara Bertini, Livio Casarini[##] and Fabian Trillsch[##]

   β-arrestin 2 is a prognostic factor for survival of ovarian cancer patients upregulating cell proliferation. *Frontiers in Endocrinology 11:554733. doi: 10.3389/fendo.2020.554733*

   # geteilte Erstautorenschaft
   ## geteilte Letztautorenschaft

2. Bastian Czogalla, **Alexandra Partenheimer**, Elisa Schmoeckel, Doris Mayr, Thomas Kolben, Susanne Beyer, Anna Hester, Alexander Burges, Sven Mahner, Udo Jeschke and Fabian Trillsch

   Nuclear Enolase-1/ MBP-1 expression and its association with the Wnt signaling in epithelial ovarian cancer. *Translational Oncology doi.org/10.1016/j.tranon.2020.100910*

Autorenbeitrag

Für die Publikation “β-arrestin 2 is a prognostic factor for survival of ovarian cancer patients upregulating cell proliferation“ führte die Autorin Alexandra Partenheimer (A.P.) zusammen mit den gelisteten Co-Autoren die Experimente durch, die von den Co-Autoren Udo Jeschke (U.J.) und Bastian Czogalla (B.C.) konzipiert wurden. A.P, U.J. und B.C. analysierten anschließend die Daten. Die Figuren und Abbildungen hierzu entwarf A.P. Die Publikation, die im Jahr 2020 veröffentlicht wurde, schrieben A.P. sowie B.C. und teilten sich die Erstautorenschaft.

Für die zweite Publikation “Nuclear Enolase-1/ MBP-1 expression and its association with the Wnt signaling in epithelial ovarian cancer” führte A.P. die Auswertung der immunhistochemischen Färbung im Jahr 2018 eigenständig durch, analysierte zusammen mit B.C. und U.J. die Daten und entwarf die Abbildungen. Der Erstautor B.C. schrieb unter Mitwirkung von A.P., welche als Zweitautorin gelistet ist, den Artikel, welcher 2020 veröffentlicht wurde.

1 Einleitung

1.1 Ovarialkarzinom

1.1.1 Epidemiologie und Risikofaktoren

Das Ovarialkarzinom (OC) (ICD-10: C56) stellt in der Gynäkologie den malignen Tumor mit der höchsten Mortalität dar [1]. Laut Robert Koch Institut kam es 2016 in Deutschland zu 7350 Neuerkrankungen und 5486 Sterbefällen mit einer absoluten 5-Jahres-Überlebensrate von 40%. Sowohl die Inzidenz als auch Mortalität sind jedoch seit der Jahrhundertwende konstant regredient [2]. Weltweit waren es 2018 etwa 295400 Neuerkrankungen und 184800 Sterbefälle [3], wobei Europa und Nordamerika eine höhere Inzidenz an OCs aufweisen als Afrika oder Asien und Japan eine mehr als doppelt so hohe wie in China [4-6]. Außerdem steigt die Inzidenz deutlich mit dem Alter an. Der Höchstwert liegt bei 80-84 Jahren mit einem mittlere Erkrankungsalter von 70 Jahren [2].

Neben dem Alter zählen eine frühe Menarche, eine späte Menopause [7], eine lebenslange Zigarettenrauchexposition und eine genetische Neigung zur Endometriose zu möglichen Risikofaktoren [8]. Ebenfalls können der Body Mass Index (BMI) und hormonelle Faktoren das Risiko steigern an einem OC zu erkranken [2]. Hierzu zählen laut Studien auch erhöhte Androgen-Level, wie zum Beispiel bei Testosteron-Therapien [9] und Hormonersatztherapien in der Menopause [2]. Kontrazeptiva hingegen können das Risiko zur Entwicklung eines OCs mindern [9, 10]. Ebenfalls risikosenkend wirken eine hohe Anzahl an Geburten, das Stillen und eine Tubensterilisation [7, 11]. Neben den sporadisch auftretenden OCs weisen auch etwa 25% der OCs eine hereditäre Komponente auf [12]. Dies ist vor allem auf Mutationen der Gene *BRCA1* und *BRCA2* zurückzuführen [13].

1.1.2 Klassifikation: Histologie, Tumorstaging und Grading

Ovarialtumore können anhand der Gewebsdifferenzierung des Ovars in drei Hauptgruppen eingeteilt werden: Epitheliale Tumore, Keimzelltumore und Keimstrangstroma-Tumore. Das weitaus Häufigste sind die epithelialen Tumore/ OCs [14, 15]. Diese werden nach der World Health Organisation (WHO) wiederum histopathologisch in folgende Subtypen unterteilt: high grade seröse Tumore (HGSC), low grade seröse Tumore (LGSC), endometrioide Tumore (EC), muzinöse Tumore (MC), klarzellige Tumore (CCC), Brenner Tumore, gemischte und undifferenzierte Tumore. Diese unterscheiden sich sowohl ätiologisch, als auch molekular und phänotypisch voneinander [16]. Mit einem Anteil von 70% stellen die high grade serösen Tumore die größte Untergruppe der OCs dar [17]. Muzinöse Tumore machen hingegen mit nur 2-3% einen geringen Teil der OCs aus.

Pathogenetisch werden zwei Typen unterschieden. Zu Typ I, welche meist im Anfangsstadium diagnostiziert werden, zählen die low grade serösen-, endometrioiden-, klarzelligen und muzinösen Tumore, welche verschiedene subtypspezifische Mutationen in Genen wie *KRAS*, *BRAF*, *PIK3CA* und *PTEN* aufweisen können. Typ II beinhaltet äußerst aggressive HGSCs, Karzinosarkome und undifferenzierte Tumore, die meist als bereits fortgeschrittene Krankheit vorliegen [18]. Diese Tumore entstehen *de-novo* aus dem Oberflächenepithel der Tube bzw. des Ovars [19, 20]. HGSCs sind außerdem für den Großteil der Ovarialkarzinomsterbefälle verantwortlich und gehen meist mit *TP53* Mutationen und Mutationen in den Genen *BRCA1* und *BRCA2* einher [18, 21].

Das Tumorstaging beim OC dient der Einschätzung des Schweregrades, der Prognose und der Festlegung des therapeutischen Konzepts. [22]. Es erfolgt intraoperativ nach einer Klassifikation der Fédération Internationale de Gynécologie et d'Obstétrique (FIGO). Diese gilt sowohl für ovariale Malignome als auch für Malignome ausgehend von den Tuben sowie dem Peritoneum und ist angelehnt an die aktuelle TNM-Klassifikation [23]. Die TNM-Klassifikation kann nur zum Teil in die FIGO- Klassifikation übertragen werden und gilt neben den OCs auch für nicht-epitheliale Tumore und Borderlinetumore [24].

Tabelle 1: TNM- und FIGO-Klassifikation der Tumore des Ovars, der Tube und des primären peritonealen Karzinoms (WHO- und FIGO- Klassifikation 2014)[24]

TNM	FIGO	Definition
TX		Primärtumor nicht bekannt, keine Angaben möglich
T0		Kein Anhalt für einen Tumor
T1	**I**	Tumor auf die Ovarien oder Tuben beschränkt

T1a	**IA**	Auf ein Ovar (Kapsel intakt) oder eine Tube (Serosa intakt), Ovar- oder Tubenoberfläche tumorfrei, negative Spülzytologie
T1b	**IB**	Befall beider Ovarien (Kapsel intakt) oder beider Tuben (Serosa intakt), Ovar- oder Tubenoberfläche tumorfrei, negative Spülzytologie
T1c	**IC**	Tumor befällt ein oder beide Ovarien oder Tuben mit Nachweis einer der folgenden Punkte:
T1c1	**IC1**	Iatrogene Kapsel- (Serosa-)ruptur
T1c2	**IC2**	Präoperative Kapsel- (Serosa-)ruptur oder Tumor auf der Ovar- oder Tubenoberfläche
T1c3	**IC3**	Maligne Zellen im Aszites oder in der Spülzytologie nachweisbar
T2	**II**	Tumor befällt ein oder beide Ovarien oder Tuben mit zytologisch oder histologisch nachgewiesener Ausbreitung in das kleine Becken oder primäres Peritonealkarzinom
T2a	**IIA**	Ausbreitung und/oder Tumorimplantate auf Uterus und/oder Tuben und/oder Ovarien
T2b	**IIB**	Ausbreitung auf weitere intraperitoneale Strukturen im Bereich des kleinen Beckens
T3 und/oder N1	**III**	Tumor befällt ein oder beide Ovarien oder Tuben oder primäres Peritonealkarzinom mit zytologisch oder histologisch nachgewiesener Ausbreitung außerhalb des kleinen Beckens und/oder retroperitoneale Lymphknotenmetastasen
T3		Nur retroperitoneale Lymphknotenmetastasen
N1a	**IIIA1i**	Metastasen ≤ 10mm
N1b	**IIIAii**	Metastasen > 10mm
T3a jedes N	**IIIA2**	Mikroskopische extrapelvine Ausbreitung auf das Peritoneum außerhalb des kleinen Beckens mit oder ohne retroperitoneale Lymphknotenmetastasen
T3b jedes N	**IIIB**	Makroskopische extrapelvine Ausbreitung auf das Peritoneum außerhalb des kleinen Beckens ≤ 2cm mit oder ohne retroperitoneale Lymphknotenmetastasen
T3c jedes N	**IIIC**	Makroskopische extrapelvine Ausbreitung auf das Peritoneum außerhalb des kleinen Beckens > 2cm mit oder ohne retroperitoneale Lymphknotenmetastasen; schließt eine Ausbreitung auf die Leberkapsel und/oder die Milz ein
M1	**IV**	Fernmetastasen mit Ausnahme peritonealer Metastasen
M1a	**IVA**	Pleuraerguss mit positiver Zytologie
M1b	**IVB**	Parenchymale Metastasen der Leber und/oder der Milz, Metastasen in außerhalb des Abdomens gelegenen Organen (einschließlich inguinaler Lymphknotenmetastasen und/oder anderer außerhalb des Abdomens gelegener Lymphknotenmetastasen)

1.1.3 Diagnostik, Therapie und Prognose

Das OC verläuft zu Beginn oftmals asymptomatisch [25]. Unklare Schmerzen im Bereich des Abdomens, Verdauungsstörungen und Unregelmäßigkeiten in der Menstruation, die erst seit ein paar Wochen bestehen, können mögliche Symptome sein und schleichend auftreten [26]. Auch Völlegefühl und Zunahme der Miktionsfrequenz, vor allem bei Frauen nach der Menopause, zählen zu möglichen Symptomen [24]. Eine Symptomatik ist zumeist erst in fortgeschrittenen Erkrankungsstadien vorhanden. Dadurch werden etwa zwei Drittel der OCs oftmals erst im Stadium III oder Stadium IV diagnostiziert [26].
Tumormarker werden in der Verlaufsbeurteilung von OCs eingesetzt. Zu den derzeit wichtigsten und meist genutzten Markern gehört das cancer antigen 125 *(CA125)*. Allerdings wird ein Screening mit dem Marker *CA125* aktuell nicht routinemäßig eingesetzt, da ein positiver Einfluss auf die Senkung der Mortalität bislang nicht nachgewiesen werden konnte [24, 27]. Somit besteht weiterhin Bedarf an neuen nützlichen Tumormarkern, welche für eine frühzeitigere Diagnostik verwendet werden können [27].

Die operative Therapie des OCs mit dem Ziel der kompletten makroskopischen Tumorresektion stellt über alle Erkrankungsstadien die Standardtherapie dar [28]. Bei Aussicht auf eine erfolgreiche Komplettresektion können zusätzlich multiviszerale Resektionen vorgenommen werden [24].

Während in frühen FIGO-Stadien eine adjuvante Platin-haltige Monochemotherapie eingesetzt werden kann, wird bei fortgeschrittenen Stadien eine Platin-/Taxan-haltige Polychemotherapie empfohlen [28]. Bei fortgeschrittenen FIGO-Stadien kann zusätzlich zur Polychemotherapie Bevacizumab, ein monoklonaler Antikörper gegen den Gefäßendothel-Wachstumsfaktor VEGF-A, mit anschließender Erhaltungstherapie für insgesamt 15 Monate eingesetzt werden [29-31].

Der Einsatz von Poly-ADP-Ribose-Polymerase (PARP)-Inhibitoren hat die Therapie des OCs in den vergangenen Jahren maßgeblich verändert. Bei fortgeschrittenen OCs ermöglichen PARP-Inhibitoren eine langfristige Krankheitskontrolle nach einem Ansprechen auf eine Platin-haltige Chemotherapie [24]. Basierend auf den Ergebnissen mehrerer Phase III – Studien können PARP-Inhibitoren im Rahmen der Zulassung aktuell sowohl in der Erstlinientherapie als auch in der Rezidivsituation eingesetzt werden. Besonders Patientinnen mit BRCA1/BRCA2-Mutation und/oder homologer Rekombinations-Defizienz (HRD) profitieren von einer PARP-Inhibitor Therapie [32, 33]. Auch eine kombinierte Erhaltungstherapie von Olaparib und Bevacizumab ist aktuell in der Erstlinientherapie bei Patientinnen mit BRCA1/2-Mutation oder HRD im Rahmen eines validierten Testverfahrens zugelassen [34].

Einer initial erfolgreichen Primärtherapie bei Patienten mit einem OC folgt häufig eine Rezidiverkrankung, welche sich wiederum in der niedrigen 5-Jahres-Überlebensrate wiederspiegelt [35]. Häufig handelt es sich hierbei im Verlauf um ein platin-resistentes Rezidiv, welches mit nicht-platinhaltigen Monotherapien, wie pegyliertem liposomalen Doxorubicin, Topotecan oder Gemcitabin behandelt wird [24].

Bei Nachweis von *BRCA1/BRCA2* Mutationen kann auch prophylaktisch eine bilaterale Salpingo-Oophorektomie zur Senkung des Erkrankungsrisikos und der Mortalität vorgenommen werden. Dabei sollte das Resektat der Tube vollständig pathologisch untersucht werden [24].

Am Beispiel der 5-Jahres-Überlebensrate ist auch deutlich zu erkennen, dass die FIGO- Klassifikation einen entscheidenden Parameter in der Prognose für OCs darstellt. Die 5-Jahres-Überlebensrate eines FIGO Stadium I Tumors liegt bei fast 90% [36], wohingegen sie bei FIGO Stadien III und IV nur bei 41% liegt [2]. Die schlechte Prognose der meisten OCs ist mitunter zurückzuführen auf unzureichende Screening Methoden, späte Diagnosestellung in fortgeschrittenen Stadien (III/IV) und die gehäufte Entstehung von Resistenzen auf Chemotherapeutika im Laufe der Therapie [37, 38]. Werden OCs jedoch früh diagnostiziert, ist nach erfolgreicher Operation von einer guten Prognose auszugehen [39].

Allerdings ist der wichtigste Prognosefaktor das Vorhandensein von Residualtumor nach erfolgter operativer Therapie. Daneben spielen weitere Faktoren wie Tumorgrading und histologische Subtypen eine entscheidende Rolle [28]. Als prognostisch gut gelten hierbei die endometrioiden Karzinome, welche ca. 10% der OCs umfassen [40]. Zudem bringt der pathogenetische Typ II Tumor eine günstigere Prognose mit sich als der fortgeschrittene Typ I Tumor [36].

1.2 β-Arrestin-2

1.2.1 Definition und Klassifikation

Arrestine sind eine Gruppe von Proteinen, bestehend aus vier Mitgliedern, welche ähnliche Strukturen aufweisen. Hierzu gehören:
Das visuelle Arrestin (Arrestin-1), hauptsächlich exprimiert in den Stäbchen und Zapfen des Auges. Die beiden nicht-visuellen Arrestine, β-Arrestin-1 (Arrestin-2) und β-Arrestin-2 (Arrestin-3), welche ubiquitär verbreitet und an der Regulierung der meisten G-Protein gekoppelten Rezeptoren beteiligt sind. Und zuletzt das X-Arrestin (Arrestin-4), welches ebenfalls nur von den Zapfen der Netzhaut exprimiert wird [41-43].

1.2.2 Physiologische Funktion

β-Arrestine sind ubiquitär verbreitete Proteine und zusammen mit G-Protein-gekoppelten Rezeptor-Kinasen (GRK) an der Regulierung der meisten G-Protein-gekoppelten Rezeptoren (GPCR) beteiligt [42]. Hierzu zählen unter anderem FSHR und LHCGR [44]. Nach der Phosphorylierung eines GPCRs durch GRKs binden β-Arrestine daran und vermitteln somit eine Desensibilisierung der GPCRs [42, 45]. Zusätzlich fördern β-Arrestine eine Rezeptorinternalisierung. Hierbei fungieren sie als multifunktionelle Adapter. Dies geschieht über rezeptorvermittelte Endozytose an Vertiefungen der Membran, die mit Clathrin umgeben sind, [41, 46]. Ob eine β-Arrestin Rekrutierung auch ohne G-Proteine möglich ist, ist derzeit noch unklar [47-49]
Des Weiteren sollen β-Arrestine als Gerüstproteine für verschiedene intrazelluläre Signalkaskaden dienen. Hierzu zählen unter anderem die Mitogen-aktivierten Proteinkinase (MAPK) -Kinasen und Serin/Threonin-Kinasen [50].

1.2.3 Pathophysiologie

β -Arrestine rücken auch im Zusammenhang mit der Entstehung von Tumoren zunehmend in den Fokus der Wissenschaft [51-53]. Sie sind an vielen verschiedenen Signalwegen beteiligt, die für Metastasierungen und Tumorüberleben zuständig sind. Hierzu gehören laut aktuellen Studien: GPCRs, Signalwege wie Hedgehog, Wingless (Wnt) und Notch sowie MAPKs. Dies wurde bereits für verschiedenste Krebsarten, wie Lungen-, Blasen-, Darm- und Brustkrebs beschrieben [54-58].

Bei Blasenkrebs wurde unter anderem eine erhöhte β-Arrestin-2 Expression entdeckt, die im Zusammenhang mit dem Thromboxan- β-Rezeptor (TP-β) eine Rolle spielt. TP-β fördert als Isoform des Thromboxan (TXA_2)-Rezeptors das Zellwachstum und die Zellmigration von Blasenkrebszellen und ist sowohl *in vitro* als auch *in vivo* an einer malignen Transformation beteiligt. [55, 59].
Ähnliche Forschungsergebnisse konnten auch für Brustkrebs nachgewiesen werden. Dort fand man ebenfalls sowohl eine gesteigerte Ral-Guanosintriphosphat-bindende Proteinase als auch β-Arrestin-2 Expression. Ral-Proteine fördern die Brustkrebszellproliferation und konnten durch einen β-Arrestin Knockdown gehemmt werden [57]. Ebenso soll β-Arrestin-2 den Morphin-induzierten Zelltod von Brustkrebszellen via Akt- und Caspase-8 Signalwegen hemmen [60].

Auch für die β2 Adrenorezeptor vermittelte Prostatakrebsentwicklung und die damit verbundene Aktivierung von extrazellulär regulierten Kinasen (ERK) spielt β-Arrestin-2 eine entscheidende Rolle, indem sogenannte β-Arrestin-2/c-Src Komplexe gebildet werden [61]. Ihre Funktion in der Tumorentstehung ist somit breit gefächert [62-66].
Jedoch wurden bereits auch gegensätzliche Studienergebnisse veröffentlicht. Für Lungenkrebs wurde hierzu herausgefunden, dass das Tumorwachstum und die Metastasierung bei β-Arrestin-2 knockout-Mäusen deutlich stärker ist als bei β-Arrestin-2 Wildtyp-Mäusen, indem es die Aktivierung von CXC-Motiv-Chemokinrezeptor2 (CXCR2), als auch von nuclear factor 'kappa-light-chain-enhancer' of active B-cells (NF-κB) anpasst [67]. Außerdem kann β-Arrestin-2 das Wachstum von Nierenkrebszellen hemmen und hierfür als Tumorsuppressor dienen, indem es die Phosphorylierung von nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) unterdrückt [68].

1.2.4 Pathophysiologie beim Ovarialkarzinom

Beim OC spielen β-Arrestine unter anderem eine Rolle im Zusammenhang mit dem Endothelin A Rezeptor (ET_AR). ET_AR ist ein GPCR und steuert invasive Signalwege, die zur Krebsentstehung beitragen und eine Metastasierung begünstigen. Es ist angewiesen auf β-Arrestine, die die β-Catenin-Signalisierungen aktivieren [53, 58].
Im Gegensatz hierzu konnte anhand eines klinischen Datensatzes von 820 Ovarialkarzinompatientinnen auch gezeigt werden, dass sich eine Angiotensin-vermittelte β-Arrestin-2 Expression positiv auswirkt [69].

Ebenso positive Effekte zeigen Studien für das OC, wobei β-Arrestin-2 daran beteiligt ist *Cdc42* zu aktivieren. Dies geschieht über den TGF-β-TypIII-Rezeptor, welcher als möglicher Tumorsuppressor bei Eierstockkrebs dienen könnte [70].

1.3 Alpha-Enolase (ENO-1)

1.3.1 Definition und Klassifikation

Enolase wird in drei Subtypen unterteilt: Alpha-Enolase (ENO-1), beta-Enolase (ENO-3) und gamma-Enolase (ENO-2). ENO-1 ist ubiquitär und somit in fast allen reifen Geweben aufzufinden [71]. Es weist ein Molekulargewicht von 48 kDa auf [72]. ENO-2 kommt meist in neuronalem und neuroendokrinem Gewebe vor, wohingegen ENO-3 hauptsächlich in Muskelgewebe vorhanden ist. Im Gegensatz zu ENO-1 sind die beiden anderen Subtypen gewebsspezifisch.
Erst kürzlich wurde ein weiterer Subtyp von Enolase entdeckt, der sich von den vorherigen unterscheidet. Das sogenannte Enolase-4 (ENO-4) kommt hauptsächlich in Spermien vor und steht funktionell in Verbindung mit der Spermienmotilität [73].

1.3.2 Physiologische Funktion

ENO-1 ist ein multifunktionelles Schlüsselenzym der Glykolyse, welches in den meisten Geweben, wie auch im Ovar exprimiert wird und an vielen verschiedenen physiologischen sowie pathophysiologischen Vorgängen beteiligt ist. Hauptsächlich zeigt sich die ENO-1 Expression im Zytoplasma. Sie ist allerdings auch an der Zellmembran sowie im Nukleus möglich [74-76]. Im Zytoplasma dient ENO-1 nicht nur als Katalysator in der Glykolyse zur Umwandlung von 2-Phosphoglycerinsäure zu Phosphoenolbrenztraubensäure sondern auch der Regulierung von Zellwachstum und Immunantwort [75, 77]. Abhängig von der verfügbaren Substratkonzentration kann die enzymatische Reaktion im Rahmen der Glykolyse auch reversibel ablaufen [76]. Zusätzlich weisen verschiedene Studien weitere biologische Funktionen von ENO-1, wie den Zusammenhang mit der Entstehung von Stressreaktionen auf. Hier zeigen Ergebnisse, dass ENO-1 vor und nach Stressexposition verschiedene Expressionsgrade aufweist und somit essentiell für das Überleben von Zellen ist [78]. Des Weiteren spielt ENO-1 eine Rolle in Bakterien und Mechanismen, die dem Auftreten von Pilzerkrankungen zu Grunde liegen [74, 79].

1.3.3 Pathophysiologie

Es gibt Anzeichen dafür, dass ENO-1 als Mediator an verschiedenen Prozessen wie Entzündungen, Infektionen, Autoimmunität und Metastasierung beteiligt ist [80-87]. Somit rückt ENO-1 im Zusammenhang mit malignen Tumoren ebenso wie β-Arrestine zunehmend in den Vordergrund und spielt je nach Lokalisation eine entscheidende Rolle in der Entstehung und dem Auftreten von Tumoren [88]. In den meisten Tumoren scheint ENO-1 an der Zelloberfläche als Plasminogen-Rezeptor zu fungieren und dadurch die Aktivierung von Plasmin zu fördern, was zur Degradierung extrazellulärer Matrix führt und somit eine Zellinvasion und Metastasierung begünstigt [89, 90]. In Tumorzellen sind unter anderem glykolytische Enzyme und Glucose Transporter dafür zuständig, dass ENO1 hochreguliert und aktiviert wird und somit zur Aufrechterhaltung des Warburg Effekts beiträgt. Dieser besagt, dass Tumorzellen, trotz genug vorhandenen Sauerstoffs für die Atmungskette ausschließlich Glucose verwenden, um Milchsäure aus Pyruvat zu gewinnen. Somit haben sie einen höheren Glucose Verbrauch als normale Zellen. [91]. Dieser Zusammenhang mit dem Warburg Effekt stellt bereits für verschiedene Krebsarten, wie Gliome, Lymphome, Lungenkrebs, Darmkrebs, Magenkrebs, Brustkrebs sowie für hepatozelluläre Karzinome eine relevante Grundlage dar [72, 91-97]. Die ENO-1 Überexpression dieser Krebsarten hat einen Einfluss auf die Prognose der Erkrankung [77, 90].

Im Nukleus hingegen findet man ENO-1 meist als alternativ translatierte Form wieder, die als c-myc Promoter-Bindeprotein (MBP-1) bezeichnet wird. Mit einem Molekulargewicht von 37kDa [91] fungiert es als Tumorsuppressor, indem es an c-myc Protoonkogene bindet und diese hemmt. Protoonkogene sind Phosphoproteine, welche sich an DNA binden und somit das Zellwachstum fördern [98]. Den inhibitorischen Effekt eines Tumorsuppressors auf verschiedene Krebsarten, bei denen ENO-1 als MBP-1 fungiert, zeigen bisher nur wenige Studien. Dies wurde derzeit sowohl für Brustkrebs als auch für Neuroblastome und nicht-kleinzelligen Lungenkrebs beschrieben [99, 100]. Für Brustkrebs wurde nicht nur herausgefunden, dass die MBP-1 Expression mit einem verbesserten progressionsfreien Überleben einhergeht, sondern verglichen mit gesundem Brustgewebe auch stark herabreguliert ist [101, 102]. Zusätzlich scheint MBP-1 auch an der Regulierung der epithelial-mesenchymalen Transition (EMT) beteiligt zu sein, welches den Übergang von Epithelzellen zu mesenchymalen Stammzellen beschreibt und einen wichtigen Schritt in der Krebsentstehung und Metastasierung darstellt [103-105].

Auch der Zusammenhang der ENO-1-Expression auf das Gesamtüberleben wurde bereits für mehrere Krebsarten, wie Brustkrebs, Gliome, verschiedene Kopf- und Halstumore, Chordome, klarzelligen Nierenkrebs, hepatozelluläres Karzinom und Lungenkrebs gezeigt [91, 92, 106-110].

Die genauen, an der ENO-1 Regulation beteiligten Mechanismen sind bis heute noch nicht ganz nachzuvollziehen [111]. Bisherige Untersuchungen zeigen, dass eine ENO-1 Translation hauptsächlich durch Hypoxie und eine MBP-1 Translation durch endoplasmatischen Retikulum-Stress ausgelöst wird, aber auch durch die Glukosekonzentration beeinflusst werden kann. Eine hohe Glukosekonzentration zeigte in Brustkrebszellen einen höheren MBP-1-Wert und vice versa. [112].

1.3.4 Pathophysiologie beim Ovarialkarzinom

Ein möglicher Zusammenhang von ENO-1 und dem OC ist derzeit noch nicht ausreichend erforscht. Nur wenige *in vitro* Studien liegen hierzu bereits vor. Zum einen soll ENO-1 die mRNA Expression des FSHRs und des LHRs anregen, indem es die Granulosazellen des Ovarialfollikels beeinflusst [113], zum anderen zeigen Auswertungen, dass ein Zusammenhang zwischen einer hohen ENO-1 Expression und Chemotherapie-Resistenzen in Ovarialkarzinomzellen besteht [35, 114, 115]. Außerdem fand man heraus, dass ENO-1, bezogen auf das Endometrium- und Ovarialkarzinom, bei einer Metastasierung stärker exprimiert wird als in der nicht metastasierten Erkrankungssituation [116].

1.4 Fragestellung

Da weiterhin ein großer Bedarf für geeignete Prognosefaktoren oder Tumormarker als auch Targets für eine adjuvante Anti-Krebstherapie des OCs besteht, war das Hauptziel der vorliegenden Studien hier weitere relevante Erkenntnisse zu gewinnen.

Sowohl β-Arrestin-2 als auch ENO-1 rücken im Zusammenhang mit Tumorgenese zunehmend in den Fokus der Wissenschaft. Demnach dienen neben dem β-Arrestin-2 gerade auch die duale Funktion des zytoplasmatischen ENO-1 sowie des nuklearen MBP-1 als interessanter Ansatzpunkt, um die Biologie des OCs in Zukunft besser zu verstehen.

Somit untersuchten wir die Korrelation zwischen dem Ausprägungsgrad der Expression sowohl von β-Arrestin-2 als auch von ENO-1 und klinisch pathologischen Daten von Patientinnen mit einem OC genauer. Eine Korrelation mit weiteren putativen Signalwegpartnern, wie unter anderem den GPCRs wurde ebenfalls analysiert, da dieser Zusammenhang in Verbindung mit Tumoren des Ovars bisher nicht genauer untersucht wurde. Außerdem wurde eine Überlebenszeitanalyse in Abhängigkeit der Expression von sowohl ENO-1 als auch von β-Arrestin-2 durchgeführt, um einen neuen potenziellen Prognosefaktor aufzudecken. Zusätzlich untersuchten wir anhand von *in vitro*-Versuchen funktionelle Zusammenhänge in transfizierten, unausgereiften Nierenzellen (HEK293), humanen Granulosazellen (hGL5) und Ovarialkarzinomzellen (A2780) unter Induktion von β-Arrestin-2 cDNA und dem Einsatz von Anti-β-Arrestin-2 siRNA.

2 Zusammenfassung

Das OC gilt in der Gynäkologie als der maligne Tumor mit der höchsten Mortalität. Die 5-Jahres Überlebensrate beträgt lediglich 40%, was unter anderem auf unzureichende Screening-Methoden und somit eine späte Diagnosestellung in fortgeschrittenen Stadien zurück zu führen ist. Zusätzlich trägt eine vermehrt aufkommende Resistenz gegenüber Chemotherapeutika zu solch schlechter Prognose bei. Die primäre chirurgische Resektion wird derzeit als Standardtherapie für das OC angesehen. Je früher der Tumor diagnostiziert wird, desto besser ist der Behandlungserfolg. Deshalb ist es von besonderer Bedeutung sowohl die Frühdiagnostik voran zu treiben als auch aussagekräftige Prognosefaktoren für das OC sowie passende Targets für eine effektive Systemtherapie zu finden. Zu den aktuell am Häufigsten verwendeten Tumormarkern für ein OC zählt *CA125*, dessen Bestimmung zur Verlaufsbeurteilung insbesondere im Erkrankungsrezidiv eingesetzt werden kann.
Sowohl β-Arrestin-2 als auch ENO-1 wurden bereits in Studien genauer untersucht und mit der Tumorentstehung verschiedener Entitäten in Verbindung gebracht. Hierbei wird sowohl β-Arrestin-2 als auch ENO-1 in vorangegangenen Studien eine konträre Funktion zugesprochen. In Blasen-, Brust- sowie Darmkrebs scheint β-Arrestin-2 eine negative Rolle zu spielen, indem es an Signalwegen beteiligt ist, die zur Tumorentwicklung und Metastasierung beitragen. Hierzu zählen unter anderem Hedgehog, Wnt, Notch und MAPKs. Als ubiquitär verbreitetes Protein ist β-Arrestin-2 vor allem dafür bekannt an der Regulierung G-Protein gekoppelter Rezeptoren beteiligt zu sein, so auch von ET_AR, welches einen Signalweg steuert, der an der Eierstockkrebs-Entwicklung beteiligt ist.
Hingegen konnte β-Arrestin-2 bei Lungen-, Nieren- und auch Eierstockkrebs als möglicher Tumorsuppressor identifiziert werden.
Ebenso wird ENO-1 hauptsächlich beschrieben als Plasminogen-Rezeptor zu fungieren und somit eine Zellinvasion und Metastasierung zu begünstigen. Darüber hinaus zeigten Studien, dass ENO-1 bei einigen Krebsarten, wie Brustkrebs, Neuroblastom oder nicht-kleinzelligem Lungenkrebs als MBP-1 fungiert und somit das Tumorwachstum hemmen kann. Die genauen Vorgänge, die zur Regulierung von ENO-1 beitragen sind derzeit noch nicht ausreichend nachzuvollziehen.

In unserer Studie über β-Arrestin-2 untersuchten wir somit insbesondere dessen Nutzen in Bezug auf die Prognosebeurteilung für das OC. In die Studie eingeschlossen wurden 156 Gewebeproben von Patientinnen, die zwischen 1990 und 2002 an unserer Klinik aufgrund eines OCs behandelt wurden. In klarzelligen Krebszellen war im Vergleich zu den verschiedenen histologischen Subtypen ein signifikant höherer medianer immunreaktiver Score (IRS) zu erkennen ($p = 0,1$). In Bezug auf das Tumorgrading erzielten wir ein signifikantes Ergebnis in high grade serösen Karzinomen ($p = 0,041$; $Cc = 0,169$).

Andere klinisch-pathologische Daten, wie Alter, FIGO-Stadium, Tumorgröße und Lymphknoten wiesen keine signifikanten Resultate im Zusammenhang mit der β-Arrestin-2-Expression auf. Zusätzlich zeigte sich anhand der Kaplan-Meier-Schätzung eine signifikante Korrelation zwischen dem Gesamtüberleben der Patientinnen und der β-Arrestin-2-Expression. Patientinnen, die wenig (IRS: 0-3) β-Arrestin-2 exprimierten, wiesen ein deutlich besseres Gesamtüberleben (50,64 vs. 29,88 Monate, median; $p = 0,025$) auf, als diejenigen mit hoher β-Arrestin-2 Expression (IRS: 4-12). Zusätzlich wurde *in vitro* untersucht, wie sich die Funktionsfähigkeit in transfizierten, unausgereiften Nierenzellen (HEK293), in humanen Granulosazellen (hGL5) und Ovarialkarzinomzellen (A2780) unter Induktion von β-Arrestin-2 cDNA und Einsatz von Anti-β-Arrestin-2 siRNA verhält. β-Arrestin-2 cDNA führte zu einer gesteigerten Proliferationsrate der hGL5 Zellen, wohingegen Anti-β-Arrestin-2 siRNA zum Zelltod von HEK293 Zellen führte, nicht aber in den hGL5 Zellen, was vermutlich auf den Einbau von exogenen Onkogenen, wie E6 und E7, in die hGL5 Zelllinie zurückzuführen ist [117]. Diese Ergebnisse unterstreichen auch, dass β-Arrestine als Gerüstproteine auch der Regulierung proliferativer Signalkaskaden dienen, indem sie an verschiedenen Signalwegen mit GPCRs und ERK1/2 beteiligt sind [118].

Da GPCRs, wie der luteinisierende Hormon/Choriogonadotropin-Rezeptor (LHCGR) und der Follikel-stimulierende Hormonrezeptor (FSHR), gehäuft bei OCs exprimiert werden und bereits mit β-Arrestinen in Verbindung gebracht wurden, führten wir ebenso eine bivariate Korrelationsanalyse nach Spearman mit diesen beiden sowie weiteren putativen Signalwegpartnern durch. Dies war uns aufgrund vorliegender Ergebnisse vorangegangener Studien unserer Forschungsgruppe möglich. Signifikante Ergebnisse erzielten wir für LHCGR ($p = 0,001$; $Cc = 0,284$), FSHR ($p = 0,029$; $Cc = 0,183$), das humane Choriongonadotropin β (hCGβ) ($p < 0,001$; $Cc = 0,36$) sowie für den G-Protein-gekoppelten-Östrogen-Rezeptor (GPER) ($p = 0,009$; $Cc = 0,215$). GPERs gehören zur Gruppe der G-Protein-gekoppelten-Rezeptoren und werden durch Östrogene induziert. Sie konnten bereits sowohl in gesundem als auch in neoplastischem Gewebe nachgewiesen werden, wo sie als Mediatoren für nicht-genomische Signalkaskaden fungieren [119, 120]. Da der GPER außerdem bereits mit einer verbesserten Gesamtüberlebensrate bei Ovarialkarzinompatientinnen in Verbindung gebracht wurde [121-123], vermuten wir, dass β-Arrestin-2 Einfluss auf die physiologische Funktion des Östrogen-Rezeptors nimmt. Allerdings sollte dies genauer untersucht werden, da bisher davon ausgegangen wurde, dass β-Arrestine keine Auswirkung auf die Regenerierung von GPER und dessen intrazellulären Austausch haben [124].

Anschließend wurde eine Multivariaten-Analyse durchgeführt, um unabhängige Prognosefaktoren für das Gesamtüberleben zu identifizieren. Hierbei konnten wir signifikante Ergebnisse für das Alter ($p = 0,014$) und FIGO-Stadium ($p < 0,001$) nachweisen.

In der zweiten Studie untersuchten wir die prognostische Bedeutung von ENO-1 für das OC. Auch in dieser Studie wurden 156 Gewebeproben von Patientinnen eingeschlossen, die zwischen 1990 und 2002 an unserer Klinik aufgrund eines OCs behandelt wurden. Von 156 erfolgreich gefärbten Schnitten wiesen 142 eine positive ENO-1-Expression auf. Die Auswertung erfolgte für das Zytoplasma und den Zellkern separat. Signifikante Ergebnisse konnten wir lediglich im Kern erzielen (nukleares ENO-1/MBP-1). In Bezug auf Tumorgrading erzielten wir hierbei ein signifikantes Ergebnis für low grade seröse Karzinome ($p = 0,01$; $Cc=0,205$) und umgekehrt eine negative Korrelation mit high grade serösen Karzinomen ($p < 0,001$; $Cc = -0,341$). Ein zusätzlicher Zusammenhang zwischen der ENO-1- sowie MBP-1-Expression und weiteren klinisch pathologischen Daten konnte nicht dargestellt werden. Jedoch konnte ein signifikantes Ergebnis bezüglich des Gesamtüberlebens der Patientinnen gezeigt werden. Patientinnen mit einer hohen ENO-1-Expression (IRS: 3-12) im Kern (MBP-1 Expression) wiesen ein deutlich besseres Überleben auf als diejenigen, die wenig ENO-1 (IRS: 0-2) im Kern (MBP-1) exprimierten (88,6 vs 33,1 Monate, median; $p = 0,013$).
Um unabhängige Prognosefaktoren für das Gesamtüberleben zu identifizieren, wurde zusätzlich eine Multivariaten-Analyse durchgeführt. Diese zeigte sowohl für das Patientenalter ($p = 0,031$) als auch für das FIGO-Stadium ($p = 0,001$) ein signifikantes Ergebnis. Wie auch für β-Arrestin-2 konnten wir für ENO-1 aufgrund von Ergebnissen vorangegangener Studien unserer Forschungsgruppe eine Korrelationsanalyse nach Spearman durchführen. Hierbei wurde die ENO-1-Expression im Kern/MBP-1 Expression mit anderen putativen Signalweg-Partnern verglichen. Als positiv signifikant erwiesen sich die Wnt-Signalmarker beta-Catenin ($p = 0,007$; $Cc = 0,235$) und Glykogensynthase-Kinase-3-beta (GSK-3β) ($p < 0,001$; $Cc = 0,341$). Eine negative Korrelation ergab sich mit den Trankriptionsfaktoren Snail/Slug ($p = 0,004$; $Cc = -0,257$). Der Wnt Signalweg ist von großer Bedeutung für die Embryogenese und soll an der epithelial-mesenchymalen Transition (EMT) beteiligt sein. Somit spielt dieser ebenso eine entscheidende Rolle in der Kanzerogenese eines OCs [125, 126]. In vorangegangenen Studien wurde bereits festgestellt, dass MBP-1 an der Regulierung der EMT beteiligt ist, in dem es diese reduziert und somit einen hemmenden Einfluss auf die Tumorentstehung hat [104, 105]. Die positive Korrelation der Wnt Signalmarker beta-Catenin und GSK-3β mit MBP-1 in unserer Analyse lassen auf einen inaktivierten Wnt Signalweg und somit auch EMT schließen. Hingegen weist die negative Korrelation mit Snail/Slug auf einen aktivierten Signalweg hin. Demnach

stellt MBP-1 einen vielversprechenden Angriffspunkt für die Therapie des OCs dar, weil es basierend auf unseren Ergebnissen in den Wnt Signalweg und die EMT involviert ist.
In dieser Studie konnten wir erstmalig eine nukleare ENO-1/MBP-1 Expression für das OC nachweisen und deren begünstigenden Effekt auf das Gesamtüberleben darlegen. Diese Ergebnisse bestätigen die Auswertungen zuvor erwähnter Studien für verschiedene Krebsarten, in denen ENO-1 eine Tumor-hemmende Funktion zugesprochen wurde [99-102]. Unterstützend wirkt ebenso die Tatsache, dass wir in high grade serösen Karzinomen eine geringere nukleare ENO-1/MBP-1-Expression zeigen konnten.

Zusammenfassend konnte im Rahmen der hier vorliegenden Dissertation die prognostische und funktionelle Bedeutung von β-Arrestin-2 und ENO-1 hinsichtlich der Entstehung des OCs beleuchtet werden. In zukünftigen Studien sollte der Zusammenhang von β-Arrestin-2 und ENO-1 im OC verifiziert werden und deren genauer Wirkmechanismus weiter untersucht werden.

3 Summary

In gynecology epithelial ovarian cancer (EOC) is considered the most lethal malignancy. 5-year-survival accounts for only 40% and is based on insufficient screening methods and therefore late stage detection. Furthermore, an increased occurrence of chemoresistance contributes to the poor prognosis. At present primary resection of tumor tissue is considered standard therapy for EOC. The sooner a tumor is diagnosed the better the treatment success. Therefore, it is of increased importance to promote an early diagnosis and find decent prognostic factors for EOC, as well as suitable targets for an endocrine cancer therapy. Currently, the mostly used tumor marker for EOC is *CA125,* which determination can be used to evaluate the course of the disease, especially in the event of a relapse.

Both β-arrestin-2 and ENO-1 have been analyzed closely and linked to tumor development of different kinds of entities. Here β-arrestin-2, just as ENO-1 shows a contrary function. In bladder-, breast- and colonic cancer β-arrestin-2 seems to play a negative role, while being involved in signaling pathways that lead to tumor development and metastasis. Among others these include Hedgehog, Wnt, Notch and MAPK.

As ubiquitous spread protein β-arrestin-2 is mostly known to be involved in regulating G-protein coupled receptors, just like the ET_AR, which regulates a pathway that is involved in EOC development.

Whereas in lung-, renal- and ovarian cancer β-arrestin-2 might serve as a tumor suppressor.

Also, ENO-1 is mainly described to act as a plasminogen receptor and therefore promotes cell invasion and metastasis. In addition, studies verify that ENO-1 inhibits tumor growth while functioning as MBP-1 in several kinds of cancers including breast cancer, neuroblastoma and non-small-cell lung cancer. The exact mechanism that contributes to the regulation of ENO-1 is not yet sufficiently understood.

In our study about β-arrestin-2, we especially examined its usefulness regarding prognosis evaluation for EOC. We included 156 histologic cancer samples of female patients that have been treated for EOC in our clinic between 1990 and 2002. Clear cell cancer cells showed a significant higher median immunoreactive score (IRS) compared to the different histological subtypes ($p = 0.01$).

Regarding tumor grading we only received a significant outcome in high grade serous carcinomas ($p = 0.041$; $Cc = 0.169$). Other clinicopathological data, like age, FIGO stage, tumor size and lymph nodes did not show any significant association to the β-arrestin-2 expression. Additionally, a significant correlation between patients' overall survival and β-arrestin-2 expression was found based on Kaplan-Meier estimates. Patients expressing a low (IRS: 0-3) amount of β-arrestin-2 showed a notable better survival rate than the ones with a high (IRS: 4-12) expression (50.64 vs. 29.88 months, median; $p = 0.025$). Additionally,

we investigated the viability in transfected embryonic kidney cells (HEK293), in human granulosa cells (hGL5) and EOC cells (A2780) with induction of β-arrestin-2 cDNA and application of anti-β-arrestin-2 siRNA *in vitro*. β-Arrestin-2 cDNA led to an increased proliferation rate of the hGL5 cells, whereas anti-β-Arrestin-2 siRNA resulted in cell death within the HEK293 cells but not the hGL5 cells, which is probably due to the integration of exogenous oncogenes such as E6 and E7 in the hGL5 cell line [117]. These results also underline that β-arrestins, as scaffold proteins are also involved in regulation of proliferative signaling cascades by being part in various signaling pathways with GPCRs and ERK1 / 2 [118].

Since GPCRs such as the luteinizing hormone/choriogonadotropin receptor (LHCGR) and the follicle-stimulating hormone receptor (FSHR) are frequently expressed in ovarian carcinoma and have already been associated with β-arrestins in the past we also performed a bivariate correlation analysis according to Spearman including those and other putative signaling pathway partners. We were able to do this based on the results of previous studies by our research group. We achieved significant results for LHCGR ($p = 0.001$; $Cc = 0.284$), FSHR ($p = 0.029$; $Cc = 0.183$), the pregnancy hormone chorionic gonadotropin-β (hCGβ) ($p < 0.001$; $Cc = 0.36$) as well as the G-coupled estrogen receptor (GPER) ($p = 0.009$; $Cc = 0.215$). GPERs belong to the GPCR group and are estrogen induced. They have already been detected in both healthy and neoplastic tissue where they act as mediators for non-genomic signal cascades [119, 120]. Since the GPER has already been associated with an improved overall survival rate in EOC patients [121-123], we assume that β-arrestin-2 influences the physiological function of the estrogen receptor. However, this should be investigated further, since previously it has been presumed that β-arrestins do not effect GPER regeneration and its intracellular exchange [124].

Afterwards, a multivariate analysis was performed to determine any appearance of independent prognostic factors for overall survival. Here significant outcomes were detected for patient's age ($p = 0.014$) and FIGO stage ($p < 0.001$).

In a further study, we additionally examined the prognostic usefulness of ENO-1 in EOC. In this study, we also included 156 histologic cancer samples of female patients that have been treated for EOC in our clinic between 1990 and 2002. 142 out of 156 successfully stained samples showed a positive ENO-1 expression. The evaluation was performed separately for cytoplasm and nucleus. Significant outcomes were only detected for the nucleus (nuclear ENO-1/MBP-1). Here regarding tumor grading, we achieved a significant result for low grade serous carcinomas ($p = 0.01$; $Cc=0.205$) and vice versa a negative correlation with high grade serous carcinomas ($p < 0.001$; $Cc = -0.341$). An additional coherence between ENO-1 expression as well as MBP-1 expression and further clinicopathological data could not be described. However, a significant outcome regarding patients' overall survival was detected.

Patients with higher nuclear ENO-1 (MBP-1) expression (IRS: 3-12) showed a clearly better overall survival than patients expressing little ENO-1 (IRS: 0-2) in the nucleus (MBP-1) (88.6 vs 33.1 months, median; $p = 0.013$).

To detect independent prognostic factors for overall survival we also conducted a multivariate analysis which showed a significant result for patient's age ($p = 0.031$) and FIGO stage ($p = 0.001$). As for β-arrestin-2, we could perform a spearman analysis for ENO-1 due to previous studies of our research group. Here we compared the nuclear ENO-1/MBP-1 expression to other putative signaling pathway partners. Wnt signaling markers like beta-catenin ($p = 0.007$; $Cc = 0.235$), glycogen synthase kinase 3 beta (GSK-3β) ($p < 0.001$; $Cc = 0.341$) proved positive significant, whereas we obtained a negative correlation to the transcription factor snail/slug ($p = 0.004$; $Cc = -0.257$).

The Wnt signaling pathway is of great importance for embryogenesis and is supposed to be involved in epithelial-mesenchymal transition (EMT). Therefore, it also plays a decisive role in the carcinogenesis of EOC [125, 126]. In previous studies it has already been established that MBP-1 is involved in the regulation of EMT by reducing it and as a consequence having an inhibiting influence on tumor development [104, 105]. The positive correlation of Wnt signaling markers beta-catenin and GSK-3β with MBP-1 in our analysis suggests an inactivated Wnt signaling pathway and also EMT. In contrast, the negative correlation with snail / slug indicates an activated signaling pathway. According to this, MBP-1 represents a promising target for EOC as, based on our results, it is involved in the Wnt signaling pathway and EMT.

In this study, we were also able to show a nuclear ENO-1/MBP-1 expression for EOC for the first time and demonstrate its beneficial effect on overall survival. These results confirm the evaluations of the previously mentioned studies for various types of cancer in which ENO-1 played a tumor-inhibiting role [99-102]. The fact that we were able to show lower nuclear ENO-1 / MBP-1 expression in high grade serous cells also has a supportive impact.

In summary, the prognostic and functional importance of β-arrestin-2 and ENO-1 regarding EOC development could be examined in the context of the present dissertation. In future studies, the connection between β-arrestin-2 and ENO-1 in EOC cells should therefore be verified and their exact mechanism of action further investigated.

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β-arrestin 2 Is a Prognostic Factor for Survival of Ovarian Cancer Patients Upregulating Cell Proliferation

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Establishing reliable prognostic factors as well as specific targets for new therapeutic approaches is an urgent requirement in advanced ovarian cancer. For several tumor entities, the ubiquitously spread scaffold protein β-arrestin 2, a multifunctional scaffold protein regulating signal transduction and internalization of activated G protein-coupled receptors (GPCRs), has been considered with rising interest for carcinogenesis. Therefore, we aimed to elucidate the prognostic impact of β-arrestin 2 and its functional role in ovarian cancer. β-arrestin 2 expression was analyzed in a subset of 156 samples of ovarian cancer patients by immunohistochemistry. Cytoplasmic expression levels were correlated with clinical as well as pathological characteristics and with prognosis. The biologic impact of β-arrestin 2 on cell proliferation and survival was evaluated, *in vitro*. Following transient transfection by increasing concentrations of plasmid encoding β-arrestin 2, different cell lines were evaluated in cell viability and death. β-arrestin 2 was detected in all histological ovarian cancer subtypes with highest intensity in clear cell histology. High β-arrestin 2 expression levels correlated with high-grade serous histology and the expression of the gonadotropin receptors FSHR and LHCGR, as well as the membrane estrogen receptor GPER and hCGβ. Higher cytoplasmic β-arrestin 2 expression was associated with a significantly impaired prognosis (median 29.88 vs. 50.64 months; $P = 0.025$). Clinical data were confirmed in transfected HEK293 cells, human immortalized granulosa cell line (hGL5) and the ovarian cancer cell line A2780 *in vitro*, where the induction of β-arrestin 2 cDNA expression enhanced cell viability, while the depletion of the molecule by siRNA resulted in cell death. Reflecting the role of β-arrestin 2 in modulating GPCR-induced proliferative and anti-apoptotic signals, we propose β-arrestin 2 as an important prognostic factor and also as a promising target for new therapeutic approaches in advanced ovarian cancer.

Keywords: β-arrestin 2, ovarian cancer, immunohistochemistry, survival analysis, G protein-coupled receptor, *in vitro* analyses

INTRODUCTION

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy and is considered the fifth leading cause of death among women (1), mainly due to late diagnosis in advanced tumor stage leading to overall poor prognosis. Lack of inadequate screening methods and rising resistances toward chemotherapy over the clinical course further contribute to the relatively low 5-years survival at around 45% (1, 2). Standard treatment for advanced EOC consists of primary cytoreductive surgery, followed by platinum-based combination chemotherapy followed by targeted therapies like the anti-angiogenic antibody bevacizumab or PARP inhibitors. To date, the most reliable prognostic factors include volume of post-operative residual disease, tumor stage diagnosed according to the International Federation of Gynecology and Obstetrics (FIGO) staging system, patient's age and histology (2–7). EOCs are classified into four histological subtypes: serous, mucinous, endometrioid, and clear-cell, which vary in terms of phenotype, etiology and molecular background (8). Taking the heterogeneity of ovarian cancer into account appears crucial for developing new prognostic and therapeutic strategies.

β-arrestin 2 is a multifunctional scaffold protein regulating signal transduction and internalization of activated G protein-coupled receptors (GPCRs) like follicle-stimulating hormone receptor (FSHR) and luteinizing hormone/choriogonadotropin receptor (LHCGR) by promoting clathrin-dependent endocytosis (9). The β-arrestin family is a known activator of signaling pathways involved in tumorgenesis (10–12) and proliferation of certain tumor entities (13–17), including ovarian cancer (18). Functional association between FSHR/LHCGR and β-arrestin in granulosa cells could be demonstrated after gonadotropin treatment *in vitro* (19, 20). In immortalized ovarian cell lines, β-arrestin 2 triggers the activation of FSH-induced mitogen-activated protein (MAP) kinase signaling cascades linked to anti-apoptotic and proliferative events (21).

In this study, we aimed to evaluate the possible prognostic impact of β-arrestin 2 on clinical course of ovarian cancer. The role of β-arrestin 2 in modulating cell viability and survival was elucidated by functional analyses *in vitro*, suggesting new therapeutic approaches in ovarian cancer.

TABLE 1 | Patient characteristics.

Parameters	*N*	Percentage (%)
HISTOLOGY		
Serous	110	70.5
Clear cell	12	7.7
Endometrioid	21	13.5
Mucinous	13	8.3
LYMPH NODE		
pNX	61	39.1
pN0	43	27.6
pN1	52	33.3
DISTANT METASTASIS		
pM0/X	150	96.2
pM1	6	3.8
GRADING SEROUS		
Low	24	23.0
High	80	77.0
ENDOMETRIOID		
G1	6	31.6
G2	5	26.3
G3	8	42.1
MUCINOUS		
G1	6	50.0
G2	6	50.0
G3	0	0
CLEAR CELL		
G3	9	100
FIGO		
I	35	23.1
II	10	6.6
III	103	68.2
IV	3	2.0
AGE		
≤60 years	83	53.2
>60 years	73	46.8

MATERIALS AND METHODS

Patients and Specimens

156 ovarian cancer samples of patients that underwent surgery between 1990 and 2002 at the Department of Obstetrics and Gynecology, Ludwig Maximilian University in Munich, Germany, were analyzed (**Table 1**). The clinical data were received from the patient's charts, whereas the follow-up data from the Munich Cancer Registry (MCR). Only patients with a clear diagnosis of ovarian cancer and were included in this study, while benign tumors, just as borderline tumors were excluded. These patients had no history of previous or secondary cancers. Also, none of the patients had neoadjuvant chemotherapy. After the samples had been formalin-fixed and paraffin-embedded (FFPE), they were evaluated by a specialized pathologist at the department of Pathology, Ludwig Maximilian University, who classified them into histological subtypes [serous ($n = 110$), endometrioid ($n = 21$), mucinous ($n = 13$), clear-cell ($n = 12$)] and rated the tumor grading. The serous ovarian cancer samples were divided into low and high grading. Endometrioid ovarian cancer was graded according to G1–G3. For the mucinous carcinoma, there is no WHO classification; however, the subtype is often classified into G1–G3. The clear cell cancer was always categorized as G3. Staging was performed using the FIGO (WHO) and TNM classification. I ($n = 35$), II ($n = 10$,) III ($n = 103$), and IV ($n = 3$). Data on primary tumor extension was available in 155 cases: T1 ($n = 40$), T2 ($n = 18$), T3 ($n = 93$), T4 ($n = 4$), and on lymph node involvement in 95 cases: N0 ($n = 43$), N1 ($n = 52$). In nine cases available was the data on distant metastasis: M0 ($n = 3$), M1 ($n = 6$).

Ethical Approval

This study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 227-09 and 18-392). All tissue samples used for this study were obtained from leftover material from the archives of LMU Munich, Department Gynecology and Obstetrics, Ludwig-Maximilians-University, Munich, Germany, initially used for pathological diagnostics. The diagnostic procedures were completed before the current study was performed. During all experimental and statistical analysis, the observers were fully blinded to patient's data. All experiments were performed according to the standards of the Declaration of Helsinki (1975). As per declaration of our ethics committee no written informed consent of the participants or permission to publish is needed given the circumstances described above.

Immunohistochemistry

The formalin-fixed and paraffin-embedded ovarian cancer tissue samples were dewaxed in xylene for 20 min, before adding 100% ethanol to completely remove the Xylol. Unspecific color responses were avoided by blocking the endogenous peroxidase with 3% H_2O_2 in methanol (3 ml 30% H_2O_2 + 97 ml methanol), followed by rehydrating it in 70%, then 50% ethanol. Afterwards the slides were placed in a pressure cooker for 10 min, using sodium citrate buffer (pH = 6), made from 0.1 M citric acid and 0.1 M sodium citrate). After cooling down, this was followed by washing the samples first in distilled water, then in phosphate buffered saline (PBS) twice. After preprocessing, the slides were incubated in a blocking solution (ZytoChem Plus HRP Polymer System, Berlin, Germany, POLHRP-100) for 5 min to prevent an unspecific staining reaction. This was followed by a 16-h incubation overnight at 4°C with the primary antibodies: anti-β-arrestin 2, rabbit IgG, polyclonal, Abcam, ab151774 at a 1:1,500 dilution; anti-FSHR, rabbit IgG, polyclonal, Novus Biologicals, NLS2231 at a 1:100 dilution, anti-LHCGR, rabbit IgG, polyclonal, Acris, SP4594P at a 1:800 dilution; anti-GPER, rabbit IgG, polyclonal, Lifespan Biosciences, LS92467 at a 1:600 dilution and anti-hCGβ, rabbit IgG, polyclonal, Dako, A-0231 at a 1:300 dilution—then washed in PBS twice. Next step was the application of reagent 2 (ZytoChem Plus HRP Polymer System, Berlin, Germany, POLHRP-100), consisting of a corresponding biotinylated secondary anti-rabbit IgG antibody and the associated avidin-biotin-peroxidase complex, for 20 min. For the visualization reaction 3,3 Diaminobenzidine (DAB) and a substrate buffer (Liquid DAB and Substrate Chromogen System, Dako, Munich, Germany, K3468) was used for 30 min, followed by distilled water, to stop the reaction. After counterstaining the slides with Mayer's acidic hematoxylin (Waldeck-Chroma, Münster, Germany, catalog-number 2E-038) for 2 min, they were dehydrated in an ascending series of alcohol (70, 96, 100%), brightened by adding xylol and then covered. Negative and positive controls were used to assess the specificity of the immunoreactions. Negative controls (colored in blue) were performed in kidney, placental and uterine tissue by replacement of the primary antibodies by species-specific (rabbit) isotype control antibodies (Dako, Glostrup, Denmark). For positive controls, placental and kidney tissues were used.

Staining Evaluation

All EOC specimens were examined with a Leitz photomicroscope (Wetzlar, Germany) and specific β-arrestin 2 immunohistochemically staining reaction was observed in the cytoplasm of the cells. The intensity and distribution pattern of β-arrestin 2 staining was rated using the semi-quantitative immunoreactive score (IR score, Remmele's score). To obtain the IR score result, the optional staining intensity (0 = no, 1 = weak, 2 = moderate, and 3 = strong staining) and the percentage of positive stained cells (0 = no staining, 1 = <10% of the cells, 2 = 11–50% of the cells, 3 = 51–80% of the cells and 4 = >81%) were multiplied. Cut-off points for the IR scores were selected for the β-arrestin 2 staining considering the distribution pattern of IR scores in the collective. β-arrestin 2 staining was regarded as low with IRS 0–3 and as high with IRS $\geq$ 4. Renal biopsies from patients with different *ARRB2* expression levels served as positive and negative control staining (22) (**Figures 1E,F**).

Cell Lines and Transfection Protocol

The immortalized human granulosa (hGL5) cell line of ovarian origin (23), the human embryonic kidney (HEK293) and the ovarian carcinoma (A2780) cell lines were used for *in vitro* experiments. Cells were cultured and handled as previously described (21, 24–26). hGL5 cells culture medium was DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/mL penicillin and 50 μg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and 2% ITS + Premix Universal Culture Supplement (#354352; Corning Incorporated, Corning, NY, USA). The HEK293 cell line was cultured in DMEM additioned of 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin and 50 μg/mL streptomycin. The A2780 cell line served as a model of ovarian cancer and was cultured in RPMI1640 medium (Sigma-Aldrich) additioned of 10% FBS, 1% Hepes (Sigma-Aldrich), 2 mM L-glutamine, 100 IU/mL penicillin and 50 μg/mL streptomycin. Cell lines were maintained in an incubator at 37°C and 5% CO_2.

The transfection protocol was described previously (25–28). Briefly, 3.5×10^4 cells were transiently transfected in a 96-well plate using increasing amount (range: 0–200 ng/well) of β-arrestin 2-encoding pcDNA3.1 plasmid (29) or commercial siRNA probes against β-arrestin 2 (#14387; Life-Technologies, Carlsbad, CA, USA) (21), using an electroporator (Gene Pulser MXcell; Bio-Rad Laboratories Inc., Hercules, CA, USA) in accordance with established protocol and settings (21, 30). Control probes without specific target mRNAs were loaded for achieving equal amount of siRNA among wells.

Western Blot Analysis and Antibodies

Overexpression and knock-down of β-arrestin 2 and pro-caspase 3 were evaluated by Western blotting, as previously described (21, 24). Briefly, transfected cells were seeded in 24-well plates (1×10^5 cells/well) and cultured 48 h before to be lysed using 4°C RIPA buffer. β-arrestin 2 and pro-caspase 3 were evaluated by 12% SDS-page and Western blotting, using specific antibodies (#3857, Cell Signaling Technology Inc., Danvers, MA, USA; #MA1-91637, Thermo-Fisher Scientific,

respectively). β-actin signals served as loading controls and were detected using a horseradish peroxidase (HRP)-conjugated specific antibody (#A3854; Sigma-Aldrich). Signals were revealed by ECL chemiluminescent compound (GE HealthCare), after incubation of the membranes with a secondary anti-rabbit HRP-conjugated antibody (#NA9340V; GE HealthCare), except for β-actin. Western blotting signals were acquired by the Quantity One analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Analysis of Cell Viability and Death

hGLC, HEK293, and A2780 cells were seeded in 96-well plates (1×10^4 cells/well) before viability assessment the by MTT colorimetric assay (31), using a previously validated protocol (21, 30). The absorbance was detected by a Victor3 plate reader (Perkin Elmer Inc., Waltham, MA, USA). A measure of death cells was obtained by incubating samples 30 min with propidium iodide (32). Cells were washed twice by PBS before excitation and light emission detection by a CLARIOstar microplate reader (BMG LabTech, Ortenberg, Germany), following the manufacturer's protocol. Data were represented as box and whiskers plots in a graph, as a measure of cell viability, and control of protein amount per well was performed by Bradford assay.

Statistical Analysis

IBM SPSS Statistics, version 25.0 (IBM Corporation, Armonk, NY, USA) was used for collecting and analyzing all the data for statistical significance. Overall-survival was compared with Kaplan-Meier curves and log-rank testing was used to detect differences in patients' overall survival times. The Spearman correlation coefficient (cc) was used for detecting correlations between immunohistochemical staining outcomes. For multivariate analyses, the cox regression was performed. Results obtained from cell lines were analyzed by D'Agostino and Pearson normality test before applying the Kruskal-Wallis test together with Dunn's correction for multiple comparisons, using the GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). For all analysis a P-value of <0.05 was considered to be statistically significant.

RESULTS

β-arrestin 2 Expression and the Correlation to Clinical and Pathological Characteristics

The clinicopathologic characteristics of the analyzed ovarian cancer patients are listed in **Table 1**. 104 (67%) out of 156 ovarian cancer specimens showed positive cytoplasmic β-arrestin 2 expression. The median immunoreactive score (IRS) of cytoplasm staining was 2 (with a range of 0–12). Clear cell histology exhibited the strongest β-arrestin 2 staining (Kruskal-Wallis test; $p = 0.01$) when compared with different histological subtypes (**Figures 1A–D**).

Representative staining of benign ovarian and fallopian tube tissue show positive results in epithelial cells, granulosa cells and theca cells. The ovarian and fallopian tube stroma did not show a β-arrestin 2 staining (**Figures 2A,B**).

We analyzed the correlation between β-arrestin 2 and clinicopathological data, such as histology, grading, and FIGO classification (**Table 2**). A positive correlation was observed between high β-arrestin 2 expression and high-grade serous histology (Spearman's correlation analysis; $p = 0.041$; $cc = 0.169$). Moreover, no other correlations were observed.

β-arrestin 2 Correlates With Gonadotropin Receptors Expression

We then analyzed the correlation between β-arrestin 2 expression and other parameters previously examined (33–37) in the same ovarian cancer cohort (**Table 3**). A positive correlation was detected to the expression of the gonadotropin receptors FSHR ($p = 0.029$; $cc = 0.183$) and LHCGR ($p = 0.001$; $cc = 0.284$), the G protein-coupled membrane estrogen receptor GPER ($p = 0.0009$; $cc = 0.215$) as well as with the choriogonadotropin beta subunit (hCGβ) ($p < 0.001$; $cc = 0.36$). Representative stainings of the correlated parameters are shown in **Supplementary Figure 1**.

High β-arrestin 2 Expression Is Associated With Impaired Overall Survival

Age of the patient cohort was 58.7 ± 31.4 years (median ± standard deviation, SD; $n = 156$) with a range of 31–88 years. The overall survival of the EOC patients was 34.4 ± 57.8 months (median ± SD; $n = 156$). High β-arrestin 2 expression (IRS 4–12) is associated with shorter overall survival. As depicted on the Kaplan-Meier curve, higher cytoplasmic β-arrestin 2 expression correlates with a significantly impaired prognosis (**Figure 3**; 50.64 vs. 29.88 months, median; $p = 0.025$).

Clinicopathological Parameters as Independent Prognostic Factors

A multivariate cox-regression analysis was performed to detect which parameters were independent prognostic factors for overall survival in the present cohort. In this analysis, patients' age ($p = 0.014$) and FIGO stage ($p < 0.001$) were independent factors for overall survival. High β-arrestin 2 ($p = 0.152$), however, was not confirmed as an independent factor of prognostic independence (**Table 4**).

Effects of β-arrestin 2 Overexpression and Depletion on Cell Viability

Since β-arrestin 2 expression levels were found to correlate with overall survival of ovarian cancer patients, the impact of the molecule on cell proliferation and survival was evaluated, *in vitro*. Cell viability and death are represented by box and whiskers plots (**Figure 4**).

Western blot analysis demonstrated that β-arrestin 2 expression levels increase together with the amount of cDNA encoding the molecule, in the HEK293, hGL5 and A2780 cell lines (**Figure 4A**). We found increased viability of all cell models overexpressing β-arrestin 2, compared to mock-transfected cells used as controls (**Figures 4B–D**; Kruskal-Wallis test; $p < 0.01$; $n = 8$). Moreover, highest levels of cell viability corresponded to the highest amount of plasmid transfected. These results are corroborated by data from propidium iodide-stained HEK293

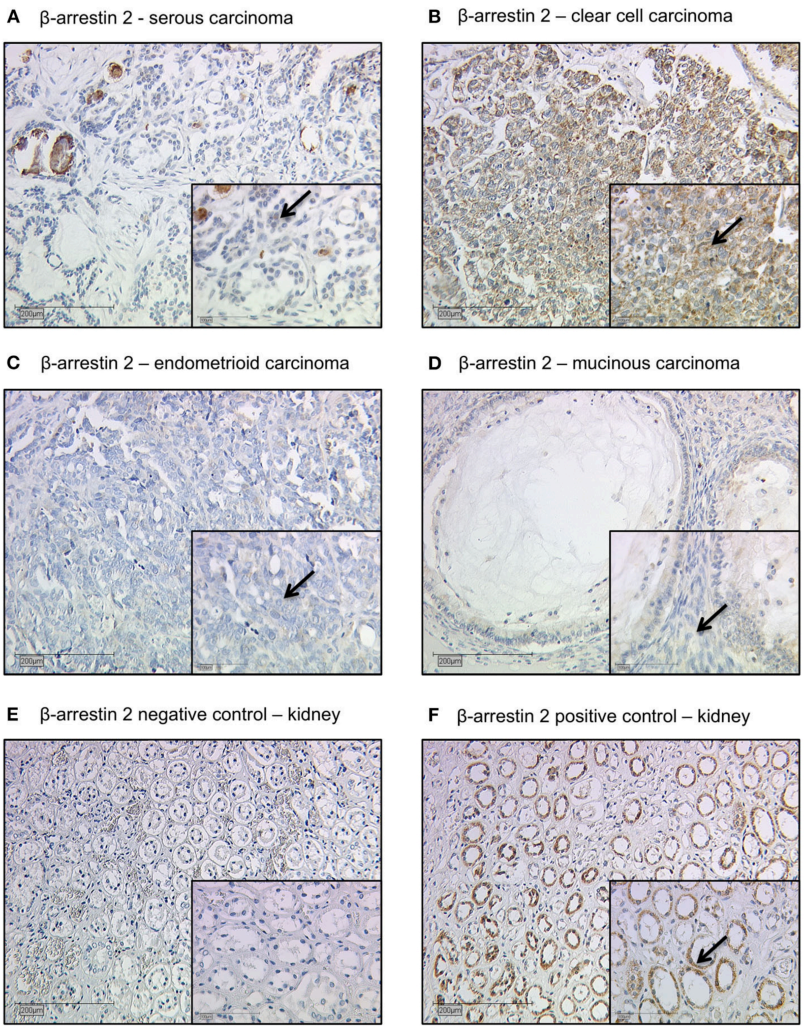

FIGURE 1 | Detection of β-arrestin 2 with immunohistochemistry. **(A)** Cytoplasmic β-arrestin 2 staining in ovarian cancer with serous, **(B)** clear cell, **(C)** endometrioid, and **(D)** mucinous histology. **(E)** β-arrestin 2 negative control **(F)** and positive control in human kidney tissue. Clear cell histology exhibited the strongest cytoplasmic β-arrestin 2 expression (Kruskal-Wallis test; $p = 0.01$) when compared with different histological subtypes. Arrows demonstrate representatively β-arrestin 2 stained cells. Images representative of three independent experiments.

and A2780 cells overexpressing β-arrestin 2, indicating that the least cell death occurs with the highest amounts of β-arrestin 2-encoding plasmid transfected (**Figures 4E–G**; Kruskal-Wallis test; $p < 0.01$; $n = 8$). However, data from transfected hGL5 cells did not reveal any association between β-arrestin 2 expression and cell death (**Figure 4F**; Kruskal-Wallis test; $p \geq 0.05$; $n = 8$).

Since a positive correlation between β-arrestin 2 expression and cell viability was found (**Figure 4**), control experiments were performed by evaluating the cell viability and death of cells maintained under β-arrestin 2 depletion, *in vitro*. Therefore, HEK293, hGL5, and A2780 cells were treated by increasing concentrations of siRNA probes silencing the endogenous molecule, then MTT assay and propidium iodide staining were performed (**Figure 5**).

The viability of all the cell lines is significantly reduced upon β-arrestin 2 depletion (**Figures 5A–C**; Kruskal-Wallis test; $p < 0.05$; $n = 8$). These data are reflected by increased propidium iodide signals occurring together with increasing amounts of siRNAs, in HEK293 cells (**Figure 5D**; Kruskal-Wallis test; $p < 0.05$; $n = 8$), while similar signals were found between siRNA-

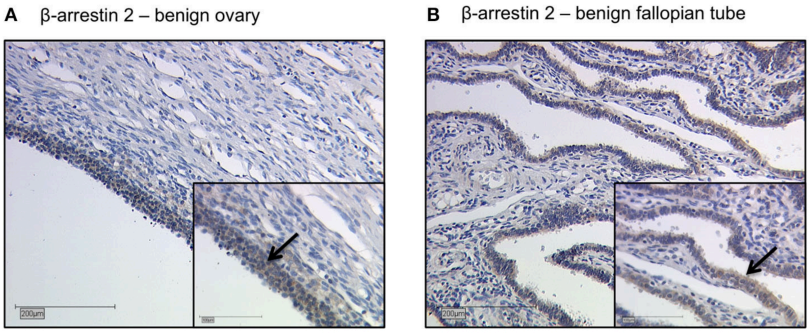

FIGURE 2 | Detection of β-arrestin 2 with immunohistochemistry in benign ovary and fallopian tube. Detection of β-arrestin 2 with immunohistochemistry in benign ovary **(A)** and fallopian tube **(B)**. Positive β-arrestin 2 staining was observed in epithelial cells, granulosa cells and theca cells. The ovarian and fallopian tube stroma did not show a β-arrestin 2 staining. Arrows demonstrate representatively β-arrestin 2 stained cells. Images representative of three independent experiments.

TABLE 2 | Correlation analysis between β-arrestin 2 expression and clinicopathological data.

Variable	*P*	Correlation coefficient
Histology	0.075	−0.083
FIGO stage	0.673	0.086
Age	0.206	0.105
GRADING		
Clear cell, endometrioid, and mucinous (G1–G3)	0.520	−0.088
Serous—low grading	0.052	−0.160
Serous—high grading	0.041*	0.169

*Clinicopathologic data and β-arrestin 2 expresssion were correlated to each other using Spearman's correlation analysis. Significant correlations are indicated by asterisks (*p < 0.05). p, two-tailed significance.*

TABLE 3 | Correlation analysis of β-arrestin 2.

Staining	β-arrestin 2	FSHR	LHCGR	GPER	hCGβ
β-ARRESTIN 2					
cc	1.000	0.183	0.284	0.215	0.360
p	–	0.029	0.001	0.009	0.000
n	148	143	146	148	137
FSHR					
cc	0.183	1.000	0.164	0.214	0.082
p	0.029	–	0.046	0.009	0.340
n	143	151	149	148	137
LHCGR					
cc	0.284	0.164	1.000	0.170	0.200
p	0.001	0.046	–	0.037	0.018
n	146	149	154	151	140
GPER					
cc	0.215	0.214	0.170	1.000	0.114
p	0.009	0.009	0.037	–	0.179
n	148	148	151	153	141
hCGβ					
cc	0.360	0.082	0.200	0.114	1.000
p	0.000	0.340	0.018	0.179	–
n	137	137	140	141	142

cc, correlation coefficient; p, two-tailed significance; n, number of patients.

and mock-treated hGL5 and A2780 cells (**Figures 5E,F**; Kruskal-Wallis test; $p \geq 0.05$; $n = 8$). Western blotting analysis confirmed the siRNA-dependence of β-arrestin 2 silencing, which is linked to pro-caspase 3 cleavage in all the cell lines (**Figure 5G**).

DISCUSSION

Our study revealed for the first time that β-arrestin 2 expression significantly correlates with impaired overall survival of ovarian cancer patients and these results are consistent with the proliferative role of β-arrestin 2 demonstrated *in vitro*. β-arrestins are known to act as scaffold proteins controlling multiple cellular functions, such as MAP kinase signaling, GPCR trafficking and transcriptional modulations (38, 39). Several studies demonstrated that β-arrestins are involved in carcinogenesis of the ovary (11, 18) and other types of cancer (10, 12–17). The relevance of the GPCR/β-arrestin pathway in ovarian cancer cells is demonstrated by the finding that endothelin-1 receptor/β-arrestin complex activates beta-catenin signaling pathways with impact on carcinogenesis and metastatic progression (11). Nevertheless, conflicting results have been reported with a positive impact of angiotensin-mediated β-arrestin expression in a clinical-genomic dataset from 820 ovarian cancer patients (40). Indicative results may be provided by the analysis of gene expression data stored in public cancer repositories. For instance, The Human Protein Atlas database (https://www.proteinatlas.org; last accession: 03 March 2020) revealed that β-arrestin 2-encoding gene (*ARRB2*) expression is an unfavorable prognostic marker of prostate cancer, although the association is not replicated for ovarian cancer. These data corroborate, at least in part, our finding although they should be considered cautiously, since gene expression levels

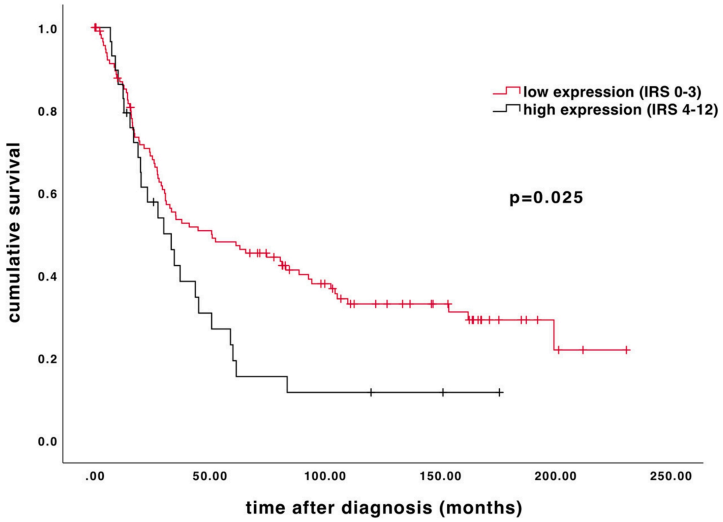

FIGURE 3 | Kaplan–Meier estimate of β-arrestin 2. β-arrestin 2 expression (IRS 4–12) was associated with impaired overall survival (50.64 vs. 29.88 months, median; $p = 0.025$). Censoring events have been marked in the graphs.

TABLE 4 | Multivariate analysis of the analyzed ovarian cancer patients ($n = 156$).

Covariate	Coefficient (b_i)	[HR Exp (b_i)]	95% CI		*P*-value
			Lower	Upper	
Patient's Age (≤60 vs. >60)	0.625	1.869	1.133	3.084	0.014
FIGO (I, II vs. III, IV)	0.813	2.255	1.499	3.393	<0.001
GRADING					
Clear cell, endometrioid, and mucinous (G1–G3)	0.142	1.153	0.749	1.774	0.517
Serous—low grading	−0.695	0.499	0.160	1.562	0.232
Serous—high grading	0.654	1.924	0.765	4.838	0.164
High β-arrestin 2	−0.097	0.907	0.795	1.036	0.152

are not necessarily corresponding to protein levels and the database does not allow to analyze all the specific histological tumor subtypes.

The link between GPCR/β-arrestins complexes and cancer is an established concept (41) and seems to be result of sustained intracellular signaling occurring upon dysregulation of intracellular β-arrestin protein levels (42). These molecules were associated with drug resistance of breast (43) and lung cancer cells (44), cancer cell migration and invasion (45), tumor progression (46) and metastasis (47), as well as a number of other tumors (48). Thus, β-arrestins were proposed as a target for therapeutic strategies (49). Our data *in vitro* confirm the proliferative role of β-arrestin 2 also in an ovarian cancer cell line, as well as in hGL5 and HEK293 cells. There are converging evidences that β-arrestin 2 depletion leads to decreasing cell viability and/or activating a caspase 3-mediated cell death, at least in HEK293 cells and confirmed in the ovarian cancer cell line A2780. Interestingly, in hGL5 cells, increased β-arrestin 2 expression is linked with upregulation of cell viability, while the depletion of this molecule by siRNA did not result in cell death. This is likely due to the integration of exogenous *E6* and *E7* oncogenes in the hGL5 cell line genome (23), which served for immortalizing them and, likely, results in increased resistance to caspase 3 activation. On the other hand, these oncogenes encode proteins inhibiting tumor protein 53 (p53) and retinoblastoma (Rb) molecules and interfering with apoptosis and cell cycle blockade. hGL5 have characteristics reflecting epithelial tumor cells, such as fibroblast-like spindle shape and relatively high proliferation rate, a feature depending on β-arrestins functioning (21), as well as in other ovarian cancer cell lines (40). However, these results underline that β-arrestins are modulators of proliferative signals exerted through a molecular signaling module with GPCRs and the extracellular-regulated MAP kinases 1 and

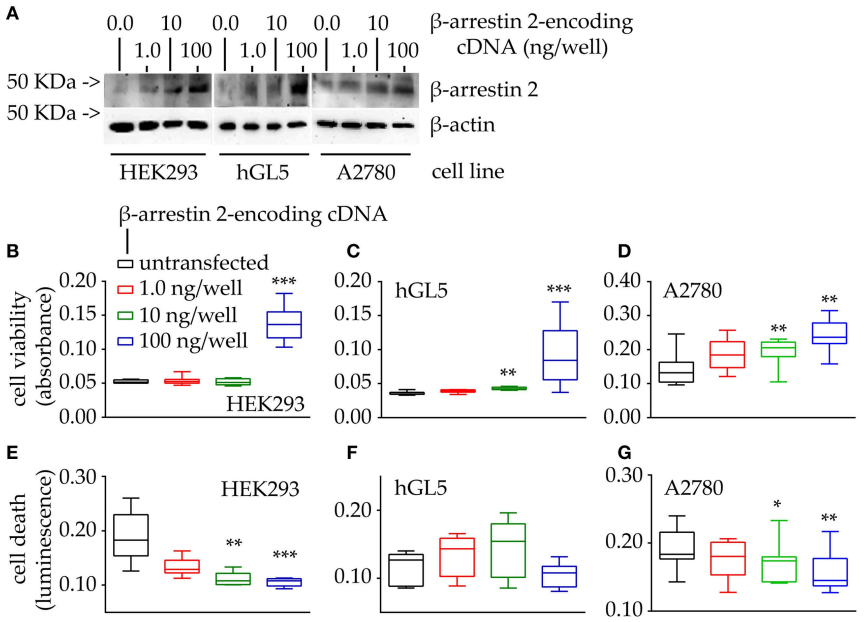

FIGURE 4 | Evaluation of cell viability and death under 48-h β-arrestin 2 overexpression. HEK293, hGL5, and A2780 cells were transfected by increasing amount of plasmid encoding β-arrestin 2 and cell viability and death were assessed by MTT and propidium iodide, respectively. Total protein content from 0.5×10^4 70% confluent log phase cells were loaded. **(A)** Representative image of β-arrestin 2 detected in cell lysates by Western blotting using a specific rabbit antibody using β-actin as a normalizer. **(B)** Box and Whiskers plot of MTT data, collected from transfected HEK293 cells overexpressing increasing levels of β-arrestin 2, representing cell viability. **(C)** hGL5 cell viability evaluated by MTT. **(D)** β-arrestin 2-dependent cell viability of the A2780 ovarian cancer cell line. **(E)** Data from the β-arrestin 2-overexpressing HEK293, representing cell death, in propidium iodide-stained samples. **(F)** β-arrestin 2-overexpressing hGL5 cell death. **(G)** β-arrestin 2-overexpressing A2780 ovarian cancer cell death. Brightness/contrast of Western blotting pictures were adjusted uniformly in all panels. Asterisks indicate significantly different data distribution than mock-transfected (untransfected) cells (Kruskal-Wallis test and Dunn's correction for multiple comparisons; $^*P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$; $n = 8$).

2 (ERK1/2), in a number of cells (50), including ovarian cells (21). Gonadotropin receptors are expressed in ovarian cells and targeted by β-arrestin 2 (51). It is well-known that gonadotropin receptor expression is dysregulated in ovarian cancer (34, 36, 52) as well adrenal tumor (53), although the specific influence of these receptor on carcinogenesis is still not determined (54). One reason for this, may be the bi-directional regulation of pro- and anti-apoptotic signals which depend on FSHR and LHCGR expression levels (55). Interestingly, the depletion of β-arrestin 2 may be linked to increased tumor cell growth and angiogenesis, at least in certain cases, such as lung cancer (56). In this case, the absence of β-arrestin 2 would lead to uncontrolled activation of proliferative signals induced by other GPCRs, e.g., the interleukin 8 receptor beta (CXCR2). Taken together, we could speculate that β-arrestins may positively or negatively impact proliferative events, modulating the activity of various GPCRs specifically expressed in the tumor cells.

In correlation analyses, a link between β-arrestin 2 and the G-protein coupled estrogen receptor (GPER) was identified. This receptor is known for rapidly mediating non-genomic signals induced by estrogens and was found in both healthy and neoplastic human tissue (57, 58). Indeed, previous studies revealed that GPER is related to better overall survival in ovarian cancer patients (34, 59, 60). Since GPER belongs to the GPCRs family, we may suppose that β-arrestin 2 might interact with this membrane estrogen receptor and influence its physiological function. However, this issue must be further investigated because GPER recycling and intracellular trafficking seems to be regulated by mechanisms not involving β-arrestins (61).

Interestingly, we also found an association between β-arrestin 2 and one of the two LHCGR ligands, hCGβ. This hormone was shown to be associated with an increased 5-years survival in LHCGR positive/FSHR negative ovarian cancer cases (33). Although this study had limitations related to low specificity of the anti-gonadotropin receptor antibodies employed (62), it suggests that hCGβ and its receptor might be linked to survival in these patients. These data would be in line with the relatively high potency of hCGβ in inducing LHCGR-mediated intracellular cAMP increase (27), as an effect linked to the steroidogenic potential of the hormone and to pro-apoptotic effects (24). On the other hand, these data would

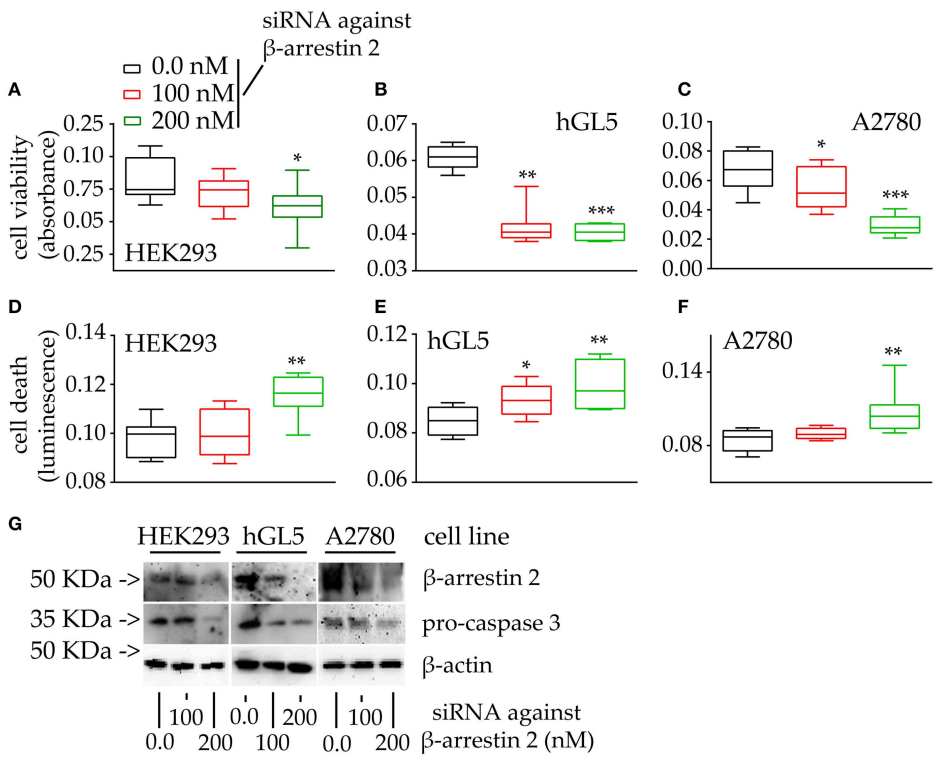

FIGURE 5 | Viability and death of 48-h β-arrestin 2-silenced HEK293, hGL5, and A2780 cells. Samples were transfected by increasing amount of siRNA probes targeting the β-arrestin 2 mRNA and 48-h cell viability and death were assessed by MTT and propidium iodide. Total protein content from 3×10^4 70% confluent log phase cells were loaded. **(A)** Box and Whiskers plot representing the HEK293 cell viability under β-arrestin 2 depletion. **(B)** β-arrestin 2-silenced hGL5 cell viability. **(C)** β-arrestin 2-silenced A2780 ovarian cancer cell viability. **(D)** Data from the β-arrestin 2-silenced HEK293, representing cell death evaluated using propidium iodide staining. **(E)** hGL5 cell death evaluated by propidium iodide. **(F)** Cell death in β-arrestin 2-silenced A2780 evaluated by propidium iodide. **(G)** Representative Western blotting images demonstrating β-arrestin 2 depletion and pro-caspase 3 cleavage in HEK293, hGL5, and A2780 cell lysates. β-actin was the normalizer. Brightness/contrast of Western blotting pictures were adjusted uniformly in all panels. Significantly different data distribution than mock-transfected (untransfected) cells were indicated by asterisks. Kruskal-Wallis test and Dunn's correction for multiple comparisons; $^*P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$; $n = 8$).

be in contrast with the fact that hCGβ has been used as a marker of certain tumors, such as the choriocarcinoma (63), which reflects the involvement of β-arrestin 2 in the hCGβ-mediated intracellular signaling (29) and suggests that the connection between the two molecules in cancer merits further investigations.

β-arrestin 2 is associated with a significantly impaired overall survival of ovarian cancer patients. This finding is supported by *in vitro* data demonstrating that β-arrestin 2 upregulates the viability of an ovarian cancer cell line, and transfected HEK293 and hGL5 cells. Reflecting the role of β-arrestins as scaffold proteins linked to the GPCRs activity, correlations between the β-arrestin 2 and FSHR and LHCGR expression was identified ovarian cancer tissue. Taken together, we indicate that β-arrestin 2 may be a promising new target in ovarian cancer so that clinical implications should be further addressed in future research.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BC, UJ, MS, LC, and FT: conceptualization. DM, SM, and AB: validation. BC, AP, UJ, and LC: formal analysis. AP, BM, RB,

and SB: investigation. BC, AP, LC, and FT: writing-original draft preparation. UJ, VS, DM, SM, AB, MS, BM, RB, and SB: writing—review and editing. AP, BC, and LC: visualization. UJ, SM, and MS: supervision. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.554733/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Nuclear Enolase-1/ MBP-1 expression and its association with the Wnt signaling in epithelial ovarian cancer

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ABSTRACT

Background:: Enolase-1, primarily known for its role in glucose metabolism, is overexpressed in various cancer entities. In contrast its alternative spliced nuclear isoform MBP-1 acts as a tumor suppressor. The aim of this study is to analyze the prognostic impact of Enolase-1/ MBP-1 and its functional significance in epithelial ovarian cancer (EOC).

Methods:: By immunohistochemistry, Enolase-1 staining was examined in 156 EOC samples. Evaluation of Enolase-1 staining was conducted in the nucleus and the cytoplasm using the semi-quantitative immunoreactive score. Expression levels were correlated with clinical and pathological parameters as well as with overall survival to assess for prognostic impact.

Results:: Cytoplasmic and nuclear Enolase-1 expression did not show a significant difference between the histological subtypes ($p = 0.1$). High nuclear Enolase-1/ MBP-1 staining negativly correlated with the tumor grading ($p<0.001$; Cc= −0.318). Cytoplasmic Enolase-1 did not correlate with clinicopathological data. Higher nuclear Enolase-1/ MBP-1 staining was detected in low-grade serous cancer cases compared to high-grade ones (median IRS 3 (range 0–8) vs. median IRS 2 (range 0–4), $p<0.001$). Nuclear Enolase-1/ MBP-1 expression correlated with the Wnt signaling markers membranous beta-catenin ($p = 0.007$; Cc=0.235), serine residue 9-phosphorylated glycogen synthase kinase 3 beta ($p<0.001$; Cc=0.341) and snail/slug ($p = 0.004$; Cc= −0.257). High nuclear Enolase-1/ MBP-1 expression was associated with improved overall survival (88.6 vs. 33.1 months, median; $p = 0.013$).

Conclusion:: Additional knowledge of Enolase-1/ MBP-1 as a biomarker and its interactions within the Wnt signaling pathway and epithelial–mesenchymal transition potentially improve the prognosis of therapeutic approaches in EOC.

Introduction

Epithelial ovarian cancer (EOC) is one of the most lethal tumor entities [47]. Lack of adequate screening methods and rising resistances towards chemotherapy over the clinical course further contribute to the relatively low 5-year survival at around 45% [4,47]. Histologically EOC is classified into five main subtypes: high-grade serous, low-grade serous, mucinous, endometrioid, and clear-cell histology [43]. Standard treatment for advanced EOC consists of primary cytoreductive surgery, followed by platinum-based combination chemotherapy followed by targeted therapies like the anti-angiogenic antibody bevacizumab or poly-ADP-ribose-polymerase inhibitors [11]. To date, most reliable prognostic factors include the presence of residual disease after initial debulking surgery, the International Federation of Gynecology and Obstetrics (FIGO) –stage, ascites volume, patient age, and histological subtype [14,55,1,17]. However, widely accepted prognostic biomarkers are missing. Taking the heterogeneity of ovarian cancer into account appears crucial for developing new prognostic and therapeutic strategies.

Enolase, a glycolytic metalloenzyme, catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate and therefore plays a pivotal role in glycolysis and gluconeogenesis. Enolase-1/ alpha-Enolase (ENO-1) is one of three different isoforms and ubiquitously expressed in the cytoplasm of most human tissue including the ovaries [42,16]. Beyond its cellular housekeeping function in glucose metabolism, increasing evidence suggests that ENO-1 is an important mediator in pathological

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Table 1
Clinicopathologic characteristics of the ovarian cancer patients.

Clinicopathologic parameters	n	Percentage (%)
Histology		
Serous	110	70.5
Clear cell	12	7.7
Endometrioid	21	13.5
Mucinous	13	8.3
Primary tumor expansion		
TX	1	0.6
T1	40	25.6
T2	18	11.5
T3	97	62.3
Nodal status		
pNX	61	39.1
pN0	43	27.6
pN1	52	33.3
Distant Metastasis		
pMX	147	94.2
pM0	3	1.9
pM1	6	3.8
Grading Serous		
Low	24	21.8
High	80	72.7
Grading Endometrioid		
G1	6	28.6
G2	5	23.8
G3	8	38.1
Grading Mucinous		
G1	6	46.2
G2	6	46.2
G3	0	0
Grading Clear cell		
G3	12	100.0
FIGO		
I	35	22.4
II	10	6.4
III	103	66.0
IV	3	1.9
Age		
≤ 60 years	83	53.2
> 60 years	73	46.8

conditions like infection, inflammation, autoimmunity and malignancy [2,29,39,35,5,34,31,32,21]. Hence, ENO-1 is overexpressed in a subset of cancers – a fact that is partially described by the Warburg effect, an adaptive response of anaerobic glycolysis in tumor cells to hypoxia [54,8].

In contrast, myc promoter-binding protein 1 (MBP-1) as alternatively spliced nuclear isoform of the *ENO1* gene binds and suppresses the activity of c-myc transcription factor, a master regulator of cellular functions, and therefore acts as a tumor suppressor [50,13]. Thus, the dual function of cytoplasmic ENO-1 and nuclear MBP-1 is of great interest for a better understanding of ovarian cancer biology.

The expression analysis of ENO-1/ MBP-1 in different histological subtypes of EOC and its correlation with survival was the primary aim of the current study which is the first of its kind.

Material and methods

Patients and specimens

156 EOC samples of patients that underwent surgery between 1990 and 2002 at the Department of Obstetrics and Gynecology, Ludwig Maximilian University in Munich, Germany, were analyzed (Table 1). The clinical data were received from the patient's charts, whereas the follow-up data derived from the Munich Cancer Registry (MCR). Only patients with pathologically confirmed EOC were included, while benign tumors, just as borderline tumors were excluded. Also, none of the patients had neoadjuvant chemotherapy. Previously, other pathological parameters were investigated in the same patients' collective, which enables correlation analysis. After the samples had been formalin-fixed and paraffin-embedded (FFPE), they were evaluated by a specialized pathologist at the department of Pathology, Ludwig Maximilian University, who classified them into histological subtypes (serous ($n = 110$), endometrioid ($n = 21$), mucinous ($n = 13$), clear-cell ($n = 12$)) and rated the tumor grading. Serous ovarian cancer samples were divided into low and high grading. Endometrioid ovarian cancer was graded according to G1 to G3. For mucinous carcinoma, there is no WHO classification; however, the subtype is often classified into G1 to G3. Clear cell cancer was always categorized as G3. Staging was performed using the FIGO (2014) and TNM classification. I ($n = 35$), II ($n = 10$,) III ($n = 103$), and IV ($n = 3$). Data on primary tumor extension was available in 155 cases: T1 ($n = 40$), T2 ($n = 18$), T3 ($n = 93$), T4 ($n = 4$) and on lymph node involvement in 95 cases: N0 ($n = 43$), N1 ($n = 52$). In nine cases the data on distant metastasis was available: M0 ($n = 3$), M1 ($n = 6$). Information to FIGO stage and grading are missing in 5 and 9 cases respectively.

Ethical approval

This study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 227-09, 18-392 and 19-972). All tissue samples used for this study were obtained from leftover material from the archives of the Department of Gynecology and Obstetrics, Ludwig-Maximilians-University, Munich, Germany, initially used for pathological diagnostics. The diagnostic procedures were completed before the current study was performed. During all experimental and statistical analysis, the observers were fully blinded to patient's data. All experiments were performed according to the standards of the Declaration of Helsinki (1975). As per declaration of our ethics committee no written informed consent of the participants or permission to publish is needed given the circumstances described above.

Immunohistochemistry

The formalin-fixed and paraffin-embedded ovarian cancer tissue samples were dewaxed in xylene for 20 min, before adding 100% ethanol to completely remove the Xylol. Unspecific color responses were avoided by blocking the endogenous peroxidase with 3% H_2O_2 in methanol, followed by rehydrating it in 70%, then 50% ethanol. Afterwards the slides were placed in a pressure cooker for 10 min, using sodium citrate buffer (pH=6; 0.1 M citric acid and 0.1 M sodium citrate). After cooling down, this was followed by washing the samples first in distilled water, then in phosphate buffered saline (PBS) twice. After preprocessing, the slides were incubated in a blocking solution (ZytoChem Plus HRP Polymer System, Berlin, Germany, POLHRP-100) for 5 min to prevent an unspecific staining reaction. This was followed by a 16-hour incubation overnight at 4 °C with the primary antibodies: anti-ENO-1, mouse IgG, monoclonal, Abcam, ab190365 at a 1:3000 dilution; anti-beta-catenin, rabbit IgG, polyclonal, Novus Biologicals, NLS2231 at a 1:100 dilution, anti- GSK-3β[pS9], rabbit IgG, polyclonal, Acris, SP4594P at a 1:800 dilution and anti-snail/slug, rabbit IgG, polyclonal, Lifespan Biosciences, LS92467 at a 1:600 dilution – then washed in PBS twice. Next step was the application of reagent 2 (ZytoChem Plus HRP Polymer System, Berlin, Germany, POLHRP-100), consisting of a corresponding biotinylated secondary anti-mouse/rabbit IgG antibody and the associated avidin-biotin-peroxidase complex, for 20 min. For the visualization reaction 3,3 Diaminobenzidine (DAB) and a substrate buffer (Liquid DAB and Substrate Chromogen System, Dako, Munich, Germany, K3468) was used for 30 min, followed by distilled water, to stop the reaction. After counterstaining the slides with Mayer's acidic hematoxylin (Waldeck-Chroma, Münster, Germany, catalog-number 2E-038) for two minutes, they were dehydrated in an ascending series of alcohol (70%, 96%, 100%), brightened by adding xylol and covered. Negative and positive controls were used to assess the specificity of the immunoreactions. Negative controls were performed in kidney and placental tissue by replacement of the primary antibodies by species-specific

(mouse/rabbit) isotype control antibodies (Dako, Glostrup, Denmark). For positive controls, placental, fallopian tube, vulva and colon tissues were used. (Supplementary Fig. 1)

Staining evaluation

All EOC specimens were examined with a Leitz photomicroscope (Wetzlar, Germany) and specific ENO-1 immunohistochemically staining reaction was observed in the cytoplasm and nucleus of the cells. The intensity and distribution pattern of ENO-1 staining was rated using the semi-quantitative immunoreactive score (IRS) [45]. To obtain the IRS result, the staining intensity (0=no, 1=weak, 2=moderate, and 3=strong staining) and the percentage of positive stained cells (0=no staining, 1=<10% of the cells, 2 = 11–50% of the cells, 3 = 51–80% of the cells and 4=>81%) were multiplied. Cut-off points for the IRS were selected for the ENO-1 staining considering the distribution pattern of IRS in the collective. Nuclear ENO-1 staining was regarded as low with IRS 0–2 and as high with IRS≥3. Staining evaluation of the Wnt signaling markers was equally performed.

Statistical analysis

IBM SPSS Statistics, version 25.0 (IBM Corporation, Armonk, NY, USA) was used for collecting and analysing all data. To compare the distribution of more than two independent samples Kruskal–Wallis H-test was used [30]. Bivariate correlations between clinical and pathological data have been calculated with Pearson's chi-squared test and Spearman's analysis [49]. Overall-survival was compared with Kaplan–Meier curves and log-rank testing was used to detect differences in patients' overall survival times. To identify an appropriate cut-off, the ROC curve was drawn. It is considered as one of the most reliable methods for cut-off point selection. In this context, the ROC curve is a plot representing sensitivity on the y-axis and (1-specificity) on the x-axis [37]. Consecutively Youden index, defined as the maximum (sensitivity+specificity-1) [57], was used to find the optimal cut-off maximizing the sum of sensitivity and specificity [19,41]. For multivariate analyses, the cox regression was performed. For all analysis a *P*-value of less than 0.05 was considered to be statistically significant.

Results

Nuclear ENO-1/ MBP-1 expression correlates with clinicopathologic characteristics

The clinicopathologic characteristics of the analyzed ovarian cancer patients are listed in Table 1. Out of 156 successfully stained ovarian cancer specimens, 142 (91%) showed positive cytoplasmic and nuclear ENO-1/ MBP-1 expression. Median (range) immunoreactivity scores (IRS) for ENO-1/ MBP-1 in cytoplasm and nucleus were 6 (0,8) and 2 (0,8), respectively.

Cytoplasmic and nuclear ENO-1/ MBP-1 expression did not show a significant difference between the histological subtypes ($p = 0.1$) (Fig. 1).

ENO-1/ MBP-1 expression displayed correlations to clinicopathologic characteristics (Table 2). A negative correlation was observed between high nuclear ENO-1/ MBP-1 staining and grading ($p<0.001$; Cc= −0.318). In the cytoplasm, ENO-1 does not correlate with clinicopathological data. Nuclear and cytoplamic expression correllated to each other ($p = 0.001$; Cc= 0.278).

Higher nuclear ENO-1/ MBP-1 staining was detected in low-grade serous cancer cases compared to high-grade ones (median IRS 3 (range 0–8) vs. median IRS 2 (range 0–4), $p<0.001$) (Fig. 2). The distribution pattern of nuclear ENO-1/ MBP-1is shown in Supplementary Table 1.

Table 2
Correlation between cytoplasmic/ nuclear ENO-1/ MBP-1 expression and clinicopathological data.

	Cytoplasmic ENO-1 expression		Nuclear ENO-1/ MBP-1 expression	
Variables	*p*	Correlation coefficient	*p*	Correlation coefficient
Age	0.429	0.067	0.393	−0.072
Histology	0.379	0.074	0.819	0.019
FIGO	0.925	−0.008	0.482	−0.061
Grading	0.444	0.067	<0.001	−0.318**

Clinicopathologic data and ENO-1 expresssion were correlated to each other using Pearson's chi-squared test. Significant correlations are indicated by asterisks (*: $p<0.05$; **: $p<0.01$).
p: two-tailed significance.

Nuclear ENO-1/ MBP-1 expression correlates with Wnt signaling markers

We then analyzed the correlation between nuclear ENO-1/ MBP-1 expression and Wnt signaling markers in the same ovarian cancer cohort (Table 3). A positive correlation was detected to the expression of membranous beta-catenin ($p = 0.007$; Cc=0.235) and serine residue 9-phosphorylated glycogen synthase kinase 3 beta (GSK-3β[*p*S9]) ($p<0.001$; Cc=0.341), a negative correlation to the transcription factor snail/slug ($p = 0.004$; Cc=−0.257).

High nuclear ENO-1/ MBP-1 expression is associated with improved overall survival

The median age of the patients was 58.7 (standard deviation [SD] 31.4) years, with a range of 31–88 years. The median follow-up OS of the EOC patients was 34.4 (SD 57.8) months. Nuclear ENO-1/ MBP-1 expression was significantly associated with a longer OS in the whole cohort (Fig. 3, median 88.6 vs. 33.1 months; $p = 0.013$). Association of high MBP-1 expression with OS in the different EOC subtypes was not statistically significant. (Supplementary Fig. 2)

Clinicopathological parameters as independent prognostic factors

A multivariate cox-regression analysis was performed to detect which parameters were independent prognostic factors for overall survival in the present cohort. In this analysis, patients' age ($p<0.001$), FIGO stage ($p = 0.002$) and serous grading ($p<0.001$) were independent factors for overall survival. High nuclear ENO-1/ MBP-1 expression ($p = 0.676$), however, was not confirmed as an independent prognostic factor (Table 4).

Discussion

This present study focused on ENO-1 expression in the cytoplasm and nucleus of different histologic subtypes of EOC and its correlation with clinicopathological parameters and different Wnt signaling markers. Whereas cytoplasmic ENO-1 expression has no impact in our EOC cohort, nuclear ENO-1/ MBP-1 was significantly higher expressed in patients with low-grade serous histology and lower expressed in high-grade serous histology, respectively.

Nuclear ENO-1/ MBP-1 expression showed correlations to the Wnt signaling markers membranous beta-catenin, GSK-3β[pS9] and the transcriptionfactor snail/slug. Moreover patients with an increased nuclear ENO-1/MBP-1 expression confirmed to have a significantly improved OS. Our results suggest a functional role of MBP-1 in ovarian cancerogenesis, which merits further investigations.

In recent years, increasing evidence suggests that ENO-1's molecular function differs depending on its cellular localization [7,15,16]. The

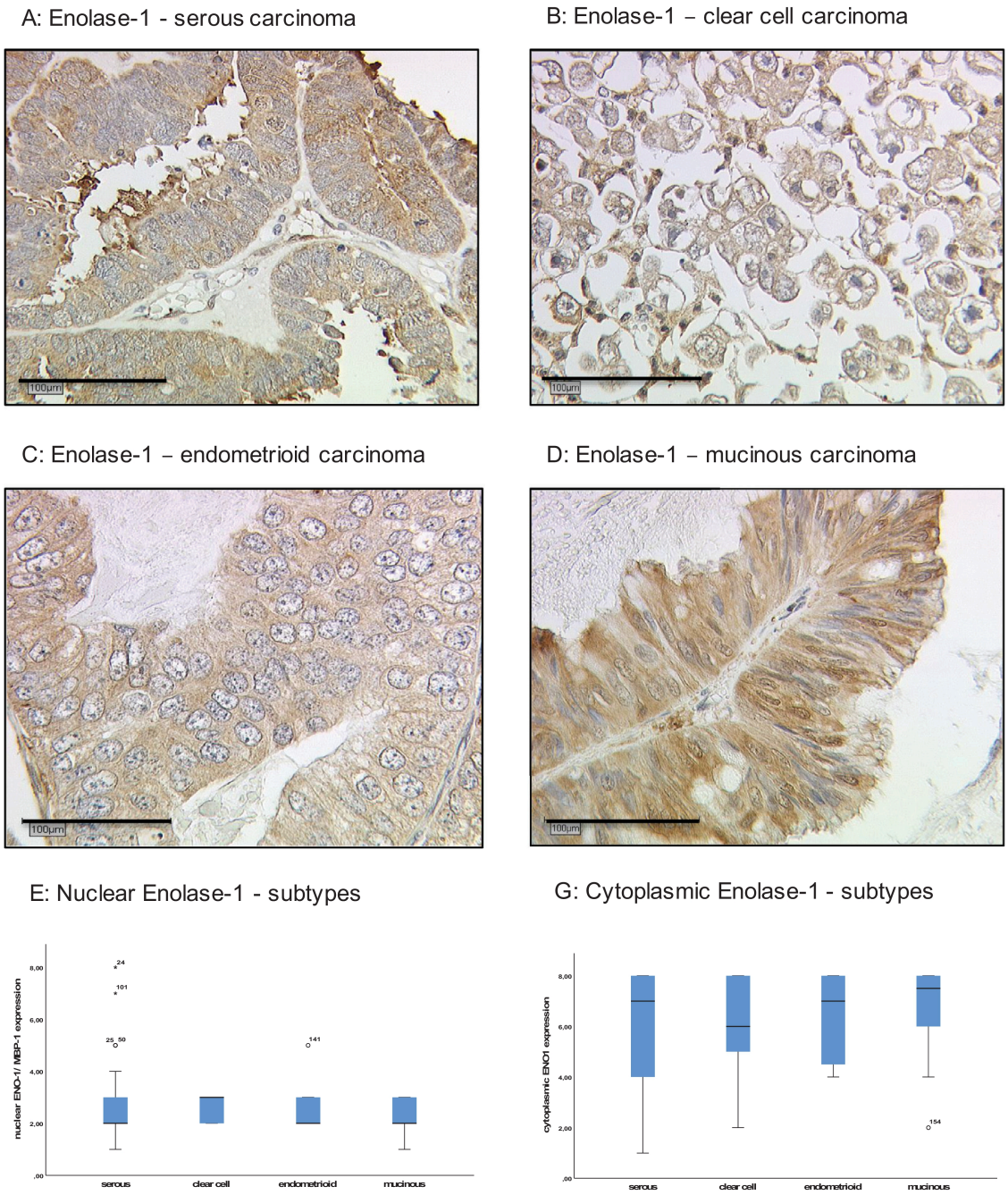

Fig. 1. Detection of Enolase-1 with immunohistochemistry. Notes: (A) Enolase-1 staining in ovarian cancer with serous, (B) clear cell, (C) endometrioid, (D) and mucinous histology. (E) Nuclear and (G) cytoplasmic Enolase-1 expression in histological subtypes. Cytoplasmic and nuclear Enolase-1 expression did not show a significant difference between the histological subtypes ($p = 0.1$). Images representative of 3 independent experiments. scale bar = 100 μm.

primary catalytic function of the cytoplasmic glycolytic enzyme ENO-1 is essential for glucose metabolism [40]. Dysregulation of this physiological process in context with the Warburg effect is a relevant condition in different kinds of cancer including glioma, lymphoma, thyroid carcinoma, lung cancer, colorectal cancer, gastric cancer, pancreatic cancer, hepatocellular carcinoma, endometrial cancer and breast cancer [51,53,22,9,48,20,60,59,44,58]. Overexpression of ENO-1 in these malignant tumors promotes cell proliferation, migration and invasion through FAK-mediated PI3K/AKT pathway and influencing plasminogen signaling [2,36,20,44].

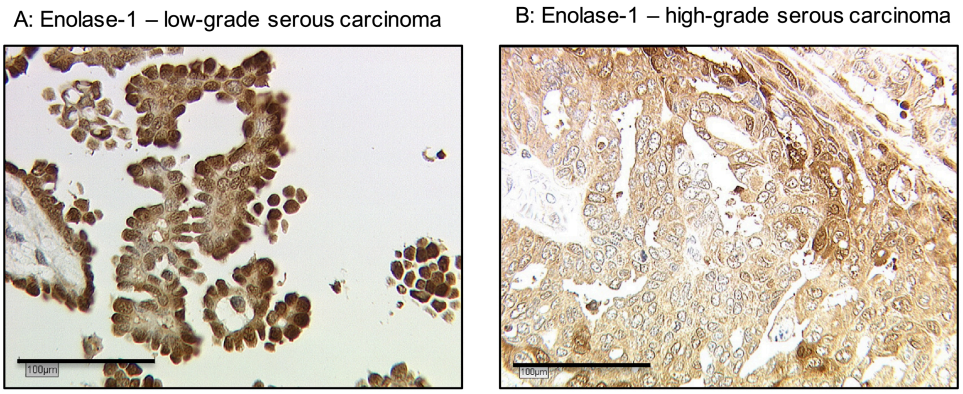

Fig. 2. Detection of nuclear Enolase-1/ MBP-1 in low/high-grade serous carcinoma. Notes: Higher nuclear ENO-1/ MBP-1 staining was detected in (A) low-grade serous carcinoma compared to (B) high-grade serous carcinoma (median IRS 3 (range 0–8) vs. median IRS 2 (range 0–4), $p<0.001$).

Table 3
Correlation analysis between nuclear ENO-1/ MBP-1 expression and different Wnt signaling markers.

Staining	Nuclear ENO-1/ MBP-1	Membranous beta-catenin	GSK-3β[pS9]	Snail/slug
Nuclear ENO-1/ MBP-1				
Cc	1.000	0.235	0.341	−0.257
p	–	0.007*	<0.001*	0.004*
n	142	129	112	126
Membranous beta-catenin				
Cc	0.235	1.000	0.393	−0.390
p	0.007*	–	<0.001*	<0.001*
n	129	147	126	134
GSK-3β[pS9]				
Cc	0.341	0.393	1.000	−0.076
p	<0.001*	<0.001*	–	0.419
n	112	126	140	116
snail/slug				
Cc	−0.257	−0.390	−0.076	1.000
p	0.004*	<0.001*	0.419	–
n	126	134	116	145

IRS of nuclear ENO-1/ MBP-1 was correlated to different Wnt signaling markers using Spearman's correlation analysis. Significant correlations are indicated by asterisks (*: $p<0.05$).
Cc: correlation coefficient, p: two-tailed significance, n: number of patients.

Table 4
Multivariate analysis of the analyzed ovarian cancer patients ($n=156$).

Covariate	Hazard ratio (95% CI)	*p*-value
Patient's Age	1.009* (1.004–1.014)	<0.001
FIGO (I, II vs. III, IV)	2.723* (1.434–5.170)	0.002
Grading serous (low vs. high)	2.845* (1.590–5.089)	<0.001
nuclear ENO-1/ MBP-1 (high vs. low)	0.885 (0.500–1.569)	0.676

A multivariate Cox regression model was established to investigate independency of prognostic factors.
Significant independent factors are indicated by asterisks (*: $p<0.05$). CI: confidence interval.

Alternative splicing of the *ENO1* gene results in a 36 kDa nuclear isoform, called myc promoter-binding protein 1 (MBP-1) [50]. This shorter gene product binds to the c-myc promoter and prevents the formation of an active transcription initiaton complex [50,18]. Thus the proto-oncogene c-myc, a key transcriptional factor influencing cell proliferation, migration, differentiation and apoptosis, is negatively regulated in the context of a tumor suppressor [13].

Indeed studies in gastric, prostate and breast cancer revealed that elevated levels of MBP-1 reduce proliferation, migration and invasion in respective cancer cells [24,26,28]. In addition MBP-1 seems to be a regulator in epithelial–mesenchymal transition (EMT) [26]. Immunohistochemical analyses in breast cancer showed that MBP-1 was highly downregulated compared to benign breast tissue and correlated with better progression free survival [33,6].

The regulating mechanism of ENO-1/ MBP-1 ratio is not well understood so far. Present studies revealed that ENO-1 translation is mostly triggered by hypoxia whereas MBP-1 translation by endoplasmic reticulum stress. In addition cellular glucose concentration may influence ENO-1/ MBP-1 ratio: whereas breast cancer cells under normal or higher concentrations show lower MBP-1 levels, the contrary effect is observed under lower glucose concentrations [46].

Our present study distinguished the expression profile of ENO-1/ MBP-1 in ovarian cancer for the first time. Up to date only few *in-vitro* studies in ovarian cancer exist. Former analyses showed an association of higher ENO-1 expression and chemotherapy resistance in ovarian cancer cells and suggested ENO-1 as putative target to overcome these drug resistance [23,52,12]. In addition ENO-1 expression was elevated in brain metastases of ovarian cancer compared with the primary tumor tissue [56]. No previous studies examined nuclear ENO-1/ MBP-1 in ovarian cancer before. Our expression analysis of nuclear ENO-1/ MBP-1 with its beneficial survival impact is in line with the above described results in other cancer entities. The putative positive impact of nuclear ENO-1/ MBP-1 on ovarian cancer biology is supported by the fact that

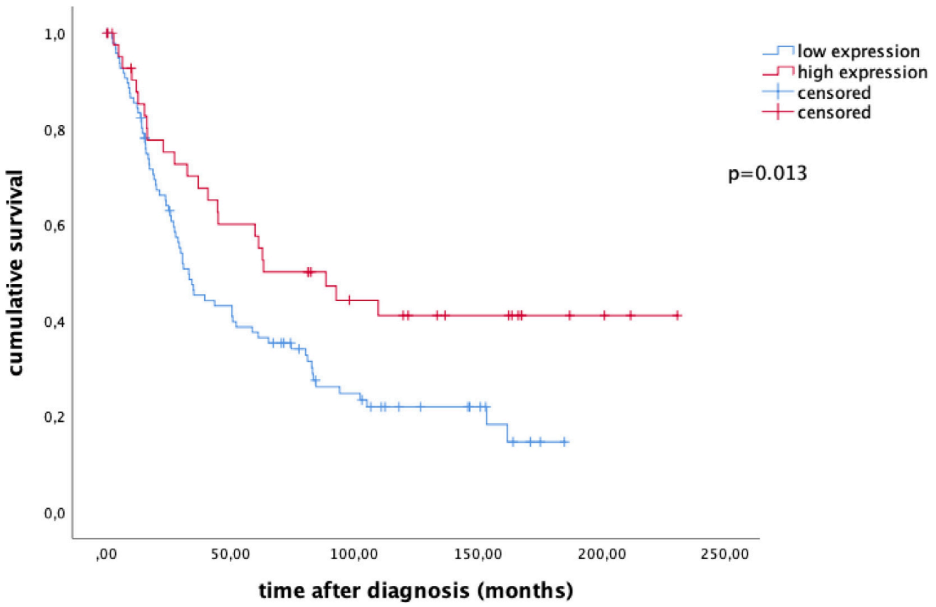

Fig. 3. Kaplan–Meier estimate of nuclear ENO-1/ MBP-1. Notes: High ENO-1/ MBP-1 expression (IRS≥3) was associated with improved overall survival (88.6 vs. 33.1 months, median; $p = 0.013$). Censoring events have been marked in the graphs.

nuclear ENO-1/ MBP-1 expression is reduced in high-grade serous ovarian cancer compared to the low-grade cases.

As mentioned before MBP-1 reduces EMT, an essential process in the initation of metastasis in cancer progression [25]. The Wnt signaling pathway, which is activated in EOC and therefore plays a pivotal role in cancerogenesis, is one of the major signaling pathways thought to be involved in EMT [3,38]. The correlation analysis between nuclear ENO-1/ MBP-1 expression and the Wnt signaling markers membranous beta-catenin, GSK-3β[pS9] and snail/slug revealed a putative association of nuclear ENO-1/ MBP-1 with an inactivated Wnt signaling and EMT. Nuclear ENO-1/ MBP-1 positively correlated to membranous beta-catenin and inactivated GSK-3β[pS9], which represent an inactivated Wnt signaling. In contrast nuclear ENO-1/ MBP-1 negatively correlated to snail/slug, an important transcription factor in the activated Wnt signaling. Thus our findings support nuclear ENO-1/ MBP-1 as possible new target involved in the Wnt signaling and EMT. In vitro studies showed that ENOblock, a unique small molecule inhibitor of the non-glycolytic functions of enolase, caused higher expression of MBP-1 linked to c-myc repression and negativly influenced cancer cell migration and invasion [27,10]. Further studies are needed to verify our findings and prove ENOblock as putative new therapeutical approach in EOC.

Taken together, we indicate that nuclear ENO-1/ MBP-1 may be a promising new target in EOC so that clinical implications should be further addressed in future research.

Declaration of Competing Interest

Thomas Kolben holds stock of Roche AG and his relative is employed at Roche AG. Anna Hester has received a research grant from the “Walter Schulz” foundation and advisory board, speech honoraria and travel expenses from Roche and Pfizer. Alexander Burges has received advisory board and honoraria from AstraZeneca, Clovis, Roche and Tesaro. Research support, advisory board, honoraria, and travel expenses from AstraZeneca, Clovis, Medac, MSD, Novartis, PharmaMar, Roche, Sensor Kinesis, Tesaro, Teva have been received by Sven Mahner and from AstraZeneca, Medac, PharmaMar, Roche, Tesaro by Fabian Trillsch. All other authors declare no conflict of interest.

CRediT authorship contribution statement

Bastian Czogalla: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Project administration, Software, Visualization, Writing - original draft. **Alexandra Partenheimer:** Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing - review & editing. **Susann Badmann:** Formal analysis, Writing - review & editing. **Elisa Schmoeckel:** Formal analysis, Validation, Writing - review & editing. **Doris Mayr:** Formal analysis, Supervision, Validation, Writing - review & editing. **Thomas Kolben:** Writing - review & editing. **Susanne Beyer:** Writing - review & editing. **Anna Hester:** Writing - review & editing. **Alexander Burges:** Writing - review & editing. **Sven Mahner:** Supervision, Writing - review & editing. **Udo Jeschke:** Conceptualization, Methodology, Project administration, Supervision, Writing - review & editing. **Fabian Trillsch:** Conceptualization, Project administration, Supervision, Writing - original draft.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2020.100910.

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