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Untersuchungen zur prognostischen und immunologischen Bedeutung der COX-2- und PPARγ- Expression im Tumorgewebe bei Patientinnen mit Vulvakarzinom

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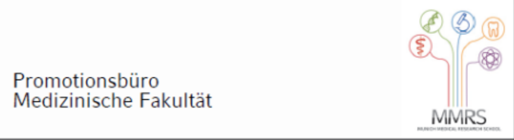

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Abkürzungsverzeichnis

AGO	Arbeitsgemeinschaft Gynäkologische Onkologie
CCL22	C-C motif chemokine ligand 22
CCR4	C-C Chemokin-Rezeptor Typ 4
COX-2	Cyclooxygenase-2
DGGG	Deutsche Gesellschaft für Gynäkologie und Geburtshilfe
dVIN	Differenzierte vulväre intraepitheliale Neoplasie
EGF	Epidermal growth factor
FOXP3	Forkhead-Box-Protein P3
HPV	Humane Papillomviren
HSIL	High-grade squamous intraepithelial lesion
IL	Interleukin
ISSVD	International Society for the Study of Vulvovaginal Disease
iTILs	Intratumoral tumor infiltrating lymphocytes
ITILWG	International TIL Working Group
LSIL	Low-grade squamous intraepithelial lesion
MAPK	Mitogen-activated protein kinase
MPS	Mononukleär-phagozytäres System
NF-κB	Nuclear factor ‘kappa-light-chain-enhancer’ of activated B cells
NK-Zelle	Natürliche Killerzelle
PGE2	Prostaglandin E2
PGJ2	Prostaglandin J2
PPAR	Peroxisom- Proliferator-aktivierter Rezeptor
PPARγ	Peroxisom- Proliferator-aktivierter Rezeptor Gamma
PPRE	PPAR response element
RKI	Robert Koch-Institut
RXR	Retinoid-X- Rezeptor
STIKO	Ständige Impfkommission
sTILs	Stromal tumor infiltrating lymphocytes
TAM	Tumor-assoziierte Makrophagen
TILs	Tumor infiltrating lymphocytes
Tregs	Regulatorische T-Zellen

Publikationsliste

Publikationen, die Bestandteile der kumulativen Dissertation sind:

1. **Ansorge N**, Dannecker C, Jeschke U, Schmoeckel E, Mayr D, Heidegger HH, Vattai A, Burgmann M, Czogalla B, Mahner S, Fuerst S.
   Combined COX-2/PPARγ expression as independent negative prognosticator for vulvar cancer patients.
   Diagnostics (Basel). 2021 Mar 10; 11(3):491.
   doi: 10.3390/diagnostics11030491.
   PMID: 33802010

2. **Ansorge N**, Dannecker C, Jeschke U, Schmoeckel E, Heidegger HH, Vattai A, Burgmann M, Czogalla B, Mahner S, Fuerst S.
   Regulatory T cells with additional COX-2 expression are independent negative prognosticators for vulvar cancer patients.
   International Journal of Molecular Sciences. 2022 Apr 22; 23(9):4662.
   doi: 10.3390/ijms23094662.
   PMID: 35563052

Weitere Publikationen:

3. Garrido F, Wild CM, Mittelberger J, Dobler F, Schneider M, **Ansorge N**, Köpke M, Strieder A, Ditsch N, Jeschke U, Dannecker C.
   The role of chemokines in cervical cancers.
   Medicina (Kaunas). 2021 Oct 21; 57(11):1141.
   doi: 10.3390/medicina57111141
   PMID: 34833360

4. Pochert N, Schneider M, Ansorge N, Strieder A, Sagasser J, Reiger M, Traidl-Hoffmann C, Neumann A, Jeschke U, Dannecker C, Kühn T, Ditsch N.
   Seroma after simple mastectomy in breast cancer- the role of CD4+ T Helper Cells and the evidence as a possible specific immune process.
   International Journal of Molecular Sciences. 2022 Apr 27; 23(9):4848.
   doi: 10.3390/ijms23094848.
   PMID: 35563236

1. Beitrag zu den Publikationen

1.1 Beitrag zu Publikation 1

Der Ethikantrag der Studie war bei Aufnahme meiner Dissertationsarbeit bereits durch meine Betreuerin Frau Dr. Sophie Fürst gestellt und durch die Ethikkommission der LMU München (Ethikvotum 367-16 vom 29.12.2016) im weiteren Verlauf bewilligt. Die Planung der zugehörigen Experimente ist in gemeinsamer Absprache mit Herrn Prof. Dr. Udo Jeschke und Frau Dr. Sophie Fürst erfolgt.

Nach Einführung in die Methodik sind alle Experimente von mir selbstständig und eigenverantwortlich durchgeführt worden. Die Patientinnendaten aus den Archiven der Frauenklinik der LMU München und des Tumorregisters München habe ich selbst recherchiert, gesammelt und daraus die Datenbank des Patientinnenkollektivs neu formiert und maßgeblich aufgebaut. Die statistische Auswertung ist in Rücksprache mit Herrn Prof. Dr. Udo Jeschke erfolgt. Das gesamte Manuskript der Publikation 1 sowie alle darin eingefügten Tabellen und Grafiken habe ich eigenständig verfasst und angefertigt; im Anschluss wurde das Manuskript durch meine Betreuerin Frau Dr. Sophie Fürst und die Co-Autorenschaft evaluiert.

Die Einreichung der Publikation beim Journal wurde vor allem von Herrn Prof. Dr. Udo Jeschke unterstützt.

1.2 Beitrag zu Publikation 2

Bei vorliegendem Ethikvotum und bereits von mir angelegter Datenbank des Patientinnenkollektivs erfolgte die erneute Auswertung der Gewebeschnitte durch mich. Die Idee der Erweiterung meiner wissenschaftlichen Arbeit hinsichtlich der Untersuchung von Immunzellen in der Tumorumgebung entstand durch meine intensive Arbeit an der immunhistochemischen Auswertung für die Daten von Publikation 1. Die Konzeption und Planung dieses Vorhabens wurden von Herrn Prof. Dr. Udo Jeschke unterstützt. Die weiteren Experimente wurden ebenfalls von mir selbstständig und eigenverantwortlich durchgeführt. Die verwendeten Patientinnendaten aus den Archiven der Frauenklinik der LMU München und des Tumorregisters München wurden bereits von mir als Datenbank zusammengestellt und konnten mit den aus dieser Arbeit entstandenen Daten durch mich erneut erweitert werden.

Nach der statistischen Auswertung in Rücksprache mit Herrn Prof. Udo Jeschke habe ich das gesamte Manuskript der Publikation 2 eigenständig erstellt. Alle darin enthaltenen Tabellen und Grafiken wurden von mir konzeptioniert und selbstständig umgesetzt. Nach Evaluation durch meiner Betreuerin Frau Dr. Sophie Fürst und die Co-Autorenschaft erfolgte die anschließende Einreichung der Publikation, die vor allem von Herrn Prof. Udo Jeschke unterstützt wurde.

2. Einleitung

2.1 Das Vulvakarzinom

2.1.1 Epidemiologie

Etwa 3300 Frauen sind im Jahre 2018 in Deutschland an einem Vulvakarzinom erkrankt. Das Zentrum für Krebsregisterdaten im Robert Koch-Institut (RKI) hat 2018 einen Anstieg der Neuerkrankungen in Deutschland verzeichnet [1]. Die Deutsche Gesellschaft für Gynäkologie und Geburtshilfe (DGGG) hat diese epidemiologische Entwicklung zum dringenden Anlass genommen, die Leitlinie zum Vulvakarzinom zu überarbeiten, nachdem die International Society for the Study of Vulvovaginal Disease (ISSVD) die neue Einteilung der Karzinomvorstufen in low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) und differenzierte vulväre intraepitheliale Neoplasie (dVIN) im Jahr 2014 vorgenommen hat [2]. Nicht nur die epidemiologische Entwicklung unterstreicht die wachsende Bedeutung des Vulvakarzinoms, sondern auch der steigende Anteil der jungen Patientinnen. Seit vielen Jahrzehnten wird eine Zunahme des Anteils jüngerer Patientinnen beobachtet [3-6].

2.1.2 Ätiologie und Pathogenese

Etwa 90% aller Vulvakarzinome sind Plattenepithelkarzinome [7]. Vereinfacht lässt sich das Vulvakarzinom in zwei ätiologische Klassen einteilen. Zum einen wird bei Vulvakarzinomen, vorwiegend bei nicht-verhornenden Formen, davon ausgegangen, dass deren Entstehung direkt mit einer Infektion durch Humane Papillomviren (HPV) im Zusammenhang steht [8-10] und aufgrund des erhöhten Expositionsrisikos daher oft jüngere Frauen betroffen sind [11-13]. Der Pathogenese der HPV-abhängigen Karzinome liegt eine Inaktivierung der Tumorsuppressoren wie p53 [14, 15] und des Retinoblastom-Proteins durch die Virusproteine E6 und E7 zugrunde; dies führt zum Proliferationserhalt und Schutz der Tumorzellen vor der Apoptose [16, 17]. Des Weiteren gibt es Vulvakarzinome, die sich unabhängig von einer HPV-Infektion entwickeln und meist bei älteren, postmenopausalen Patientinnen vorkommen. Häufig treten diese Karzinome als stark verhornender Phänotyp auf; sie entstehen zumeist auf dem Boden einer Inflammation wie z.B. im Rahmen einer chronischen Erkrankung wie Lichen sclerosus [18, 19]. Neben diesen pathogenetischen Modellen der Karzinogenese sind auch Faktoren wie Immunsuppression, Rauchen [9, 20], sexuell übertragbare Infektionen wie das Herpes simplex- Virus Typ 2 [9] oder HIV [21] mit einem höheren Risiko der Entstehung eines Vulvakarzinoms verbunden.

2.1.3 Therapie und Prognose

Neben den lokalen oder radikal chirurgischen Therapiemöglichkeiten stehen Maßnahmen wie die systemische Chemotherapie und die Radiatio zur Verfügung [22]. In Studien sind u.a. die Auswirkungen dieser radikal chirurgischen Verfahren hinsichtlich der postoperativen Komplikationen physischer wie auch psychischer Natur untersucht worden. Sexuelle Dysfunktion und schlechtere Lebensqualität sind neben den allgemeinen postoperativen Komplikationen wie Wundheilungsstörung, Miktionsprobleme und Schmerzen [23, 24] schwerwiegende Langzeitkomplikationen, unter welchen Frauen nach Vulvektomien vergleichsmäßig häufig leiden.

Durch den ätiologischen Zusammenhang unverhornter Vulvakarzinome und einer HPV-Infektion wird ein präventiver Effekt der HPV-Vakzine erhofft, welche seit 2007 von der Ständigen Impfkommission (STIKO) in Deutschland für Mädchen und junge Frauen vor der Kohabitarche empfohlen wird [25]. Seit 2018 gilt die Empfehlung der STIKO auch für Jungen und junge Männer ab 9-14 Jahren [26]. Laut Erhebungen des RKI von 2020 haben 51,1% der Mädchen bis 18 Jahre in Deutschland die vollständige HPV-Immunisierung erhalten [27].

Derzeit gibt es keinerlei prognostische Marker, die in der Diagnostik oder Therapie des Vulvakarzinoms eine relevante Rolle spielen, der Bedarf an Prognosemarkern ist daher hoch. Prof. Dr. Hans-Georg Schürch aus Neuss, damaliger Koordinator der Organkommission Vulva/Vagina der Arbeitsgemeinschaft Gynäkologische Onkologie (AGO), hat in einer Pressemitteilung des DGGG im März 2016 einen Ausblick auf die zukünftige epidemiologische Zunahme der Krankheitszahlen gegeben: „[…] [S]chon bald werden mehr Frauen am Vulvakarzinom als am Zervixkarzinom erkranken“ [28].

2.2 Fragestellung der Dissertation und der enthaltenen Publikationen

In beiden Publikationen, die im Rahmen dieser Dissertation angefertigt worden sind, wird als übergeordnete Fragestellung untersucht, ob das Enzym Cyclooxygenase -2 (COX-2) und der Transkriptionsfaktor Peroxisom-Proliferator-aktivierter Rezeptor Gamma (PPARγ) als prognostische Faktoren für das Vulvakarzinom fungieren. Inwiefern diese Faktoren einen möglichen Einfluss auf das Überleben haben und somit als potenzielle Targets in der Therapie des Vulvakarzinoms in Frage kommen, ist die zentrale Frage dieser Arbeit.

Hierzu wurden die Expression von COX-2 und PPARγ im Tumorgewebe (Publikation 1) sowie die COX-2-Expression von stromalen Immunzellen und deren Subtypen in der Tumorumgebung in Geweben von Patientinnen mit Vulvakarzinom (Publikation 2) untersucht, ausgewertet und in einen gemeinsamen Kontext gesetzt.

Beide Publikationen vereinen somit die Betrachtung der Tumorbiologie und -immunologie des Vulvakarzinoms und unterstreichen damit die weiter wachsende Bedeutung der zielgerichteten, onkologischen Therapie der Gegenwart und Zukunft.

2.3 COX-2 und PPARγ (Publikation 1)

2.3.1 COX-2 im physiologischen Kontext und als Therapietarget

Die COX-2 zählt als Enzym zu den pathogen-induzierten Oxygenasen. Im Gegensatz zur COX-1, einem weiteren Subtyp der Cyclooxygenasen, wird die COX-2 in der Regel nicht konstitutiv exprimiert. Ausnahmen stellen die Gewebe des Gehirns sowie die der Macula densa der Nieren, der Hoden und des Trachealepithels dar. [29-31]
Die COX-2 bedarf einer Induktion, beispielsweise durch Zytokine, die im Rahmen einer Läsion oder Inflammation sezerniert werden. Beschriebene spezifische Induktoren der COX-2 sind u.a. Wachstumsfaktoren wie der epidermal growth factor (EGF) [32], Interleukine (IL) [33] und die Retinsäure [34].

COX-2 setzt die Arachidonsäure als Substrat um und es entstehen, neben Thromboxan A2, vorwiegend Prostaglandine als Produkte. Prostaglandine, vor allem das Prostaglandin E2 (PGE2), spielen eine wichtige Rolle in der Vermittlung von Inflammation [35], aber auch in Prozessen der Angiogenese durch Induktion vaskulär endothelialer Wachstumsfaktoren im Rahmen der Karzinogenese [36, 37].

Pharmakologisch ist das Enzym COX-2 bereits seit 1999 als Target für spezifische COX-2- Inhibitoren bekannt: Medikamente wie Rofecoxib sind erstmalig zur Behandlung von Arthritiden eingesetzt worden [38]. Nachdem Rofecoxib aufgrund des erhöhten kardiovaskulären Risikos und dem vermehrten Auftreten von Myokardinfarkten keine Zulassung mehr erhalten hat, existiert aktuell mit Celecoxib ein Medikament, welches seit vielen Jahren als Analgetikum und Antiphlogistikum im klinischen Einsatz ist [39].

2.3.2 COX-2 und Karzinogenese

Aktuelle Forschungsansätze gehen davon aus, dass COX-2 und ihr Produkt PGE2 eine wichtige Rolle in Prozessen der Tumorentstehung wie der Regulation der Angiogenese spielen

[40, 41]. Das Substrat der COX-2, die Arachidonsäure, ist ein Abkömmling der Linolsäure. Die Linolsäure ist eine langkettige, gesättigte Fettsäure und zählt zu denjenigen Fettsäuren, welche im Kontext der Ernährung als Risikofaktoren im Rahmen der Entwicklung von Karzinomen gelten [42, 43]. Ein negativer Einfluss dieser Fette zeigte sich bereits u.a. für die Entstehung kolorektaler Karzinome [44], Pankreaskarzinome [45] und Mammakarzinome [46-48]. Der Haupteinflussfaktor der langkettigen, gesättigten Fettsäuren, welche sich vorwiegend in tierischen Fetten befinden, lässt sich auf die höhere Produktionslast der Prostaglandine, wie PGE2, zurückführen [49].

Die erhöhte Expression von COX-2 konnte in zahlreichen Karzinomen nachgewiesen werden. Untersuchungen am hepatozellulären Karzinom [50], Lungenkarzinom [51], Prostatakarzinom [52], Kolonkarzinom [53], aber auch an Karzinomen gynäkologischer Organe wie dem Endometrium [54], der Mamma [55, 56], dem Ovar [57] und der Zervix [58] zeigten eine hohe COX-2-Expression. Nur vereinzelt existieren Untersuchungen bezüglich der COX-2-Expression in Geweben des Vulvakarzinoms [59, 60].

Einige Studien haben bereits einen Einfluss der COX-2- Expression auf das Überleben von Tumorpatient*innen beobachtet und beschrieben [61-63].

Die COX-Inhibitoren haben bereits in zahlreichen Studien vielversprechende Ergebnisse bezüglich der Chemoprävention bei Kolonkarzinomen [64, 65], aber auch hinsichtlich eines möglichen positiven additiven Effekts bei der Anwendung zur Chemotherapie [66-68] gezeigt.

2.3.3 PPARγ im physiologischen Kontext und als Therapietarget

PPARγ gehört zu der Gruppe der Peroxisom-Proliferator-aktivierten Rezeptoren (PPAR). Es bildet gemeinsam mit PPARα, PPARβ bzw. -δ die einzelnen Subtypen der nukleären Rezeptorfamilie ab. Die Tatsache, dass Peroxisom-Proliferatoren aktivierend auf die Rezeptoren wirken, ist namensgebend. 1990 haben Issemann et al. erstmals erfolgreich die DNA eines PPAR vervielfältigt, 1992 ist dies erstmalig für den Subtyp PPARγ gelungen.[69, 70]

Die PPAR bilden die Gruppe der Liganden-abhängigen Transkriptionsfaktoren, welche an einer spezifischen Dann-Sequenz, dem sog. PPRE (PPAR response element) binden. Nach Heterodimerisierung mit dem Retinoid-X-Rezeptor (RXR) beeinflusst PPARγ in seiner Funktion als Transkriptionsfaktor unter anderem die Adipogenese, die Differenzierung der Adipozyten aber auch die Insulinsensitivität und den Glucosestoffwechsel. Hohe Expressionen von PPARγ werden vor allem in weißen und braunen Fettgeweben nachgewiesen. [71-73]

Wie auch die Namensgebung der Rezeptorfamilie verrät, werden diese Rezeptoren vorwiegend im Kern vorgefunden; jedoch zeigen einige Studien den zytoplasmatischen Nachweis von PPARγ [74-76]. In meiner Studie wird PPARγ im Gewebe des Vulvakarzinoms auch im Zytoplasma immunhistochemisch nachgewiesen (Publikation 1). Der nukleo-zytoplasmatische Transport von Rezeptoren in und aus dem nukleären Wirkungsort wurde bereits in zahlreichen anderen Untersuchungen beobachtet: Steroidrezeptoren wie Glucocorticoidrezeptoren [77, 78], Progesteronrezeptoren [79, 80], Androgenrezeptoren [81] und Thyreoidrezeptoren [82] und ihr sog. nukleo-zytoplasmatisches Shuttling werden zunehmend verstanden. Ein genauer Prozess ist bislang für den PPAR, speziell für PPARγ, noch wenig beschrieben. Jedoch geht man davon aus, dass dieser Transportmechanismus zwischen beiden Zellkompartimenten eine wichtige Rolle hinsichtlich der Funktionsregulation von PPARγ und seiner Transkriptionsaktivität spielt [83-87].

Glitazone sind als spezifische PPARγ-Agonisten zur Therapie des Diabetes mellitus Typ 2 zugelassen und führen zur genetischen Modulation der Insulinsensitivität. Aufgrund schwerer Nebenwirkungen verloren Roseglitazon-haltige Arzneimittel 2010 nach Beschluss der europäischen Arzneimittelagentur die Zulassung [88]. Derzeit ist noch Pioglitazon zugelassen [89]. Wie in einer Studie mit Thiazolidinedionen behandelten Diabetiker*innen gezeigt wurde, ist die Einnahme von PPARγ-Agonisten mit einem geringeren Risiko der Entwicklung eines Lungen-, Prostata- und Kolonkarzinoms vergesellschaftet [90].

2.3.4 PPARγ und Karzinogenese

Der Nachweis von PPARγ in malignem Gewebe wurde bereits mehrfach beschrieben. Sarraf et al. [91] zeigte neben einer hohen Expression von PPARγ eine Wirkung der PPARγ-Agonisten auf die Differenzierung des Kolonkarzinomgewebes. Untersuchungen mit einigen Zellkulturen ergaben eine hemmende Wirkung von PPARγ-Agonisten auf das Wachstum der Karzinomzellen sowie einen aktivierenden Effekt auf deren Apoptose [92, 93]. Es konnte gezeigt werden, dass Prostaglandin J2 (PGJ2) als PPARγ-Aktivator nicht nur einen Einfluss auf die Zelldifferenzierung [72, 73], sondern auch auf die Proliferation und Induktion von Apoptose hat [94, 95].

Die Untersuchungen mit PPARγ-Knock-Out-Mäusen ergaben, dass PPARγ-defiziente Mäuse ein erhöhtes Risiko für die Entwicklung von Neoplasien im Kolon, Ovar und in der Haut besitzen [96]. Im Kolonkarzinomgewebe konnte außerdem eine loss-of-function- Mutation von PPARγ im Großteil der Gewebeproben detektiert werden, was wiederum einen Einfluss des Rezeptors auf die Karzinogenese untermauert [97].

2.3.5 COX-2 und PPARγ: Kontext und Wechselwirkung

Nachdem COX-2 und PPARγ als mögliche prognostische Faktoren im Vulvakarzinom untersucht worden sind (Publikation 1), stellt sich die Frage des Zusammenhangs beider einzelnen Marker zueinander. Das Enzym COX-2 produziert Prostaglandine. PGJ2 ist als natürlicher Agonist für PPARγ bekannt [98]. Somit kann das Enzym COX-2 durch seine entstandenen Produkte die Aktivität des Transkriptionsfaktors PPARγ indirekt beeinflussen. Es sind jedoch nicht nur aktivierende COX-2-Produkte bekannt: das Prostaglandin F2$\propto$ hemmt beispielsweise PPARγ durch Phosphorylierung mittels der Mitogen-activated protein kinase (MAPK) [99].

Andererseits nimmt im Gegenzug PPARγ Einfluss auf die COX-2-Expression. Es ist bekannt, dass PGJ2 als aktivierender Ligand für PPARγ die COX-2-Transkription fördert, da in der Promotorregion von COX-2 das erforderliche PPRE als Bindungsstelle vorhanden ist [100].

Als Beispiel für weitere Verknüpfungspunkte zu COX-2 und PPARγ kann der nuclear factor-'kappa- light- chain- enhancer' of activated B cells (NF-κB) - Reaktionsweg zur näheren Betrachtung herangezogen werden. Er fördert u.a. die COX-2-Expression. Wie bereits in mehreren Studien untersucht wurde, fungiert PPARγ im Zellkern in seiner aktivierten Form als Inhibitor auf den Transkriptionsfaktor NF-κB, was wiederum eine Hemmung der Expression von COX-2 zur Folge hat. [101-103]

So entstehen Regulationskreise und Verbindungen zwischen einzelnen Prozessen, die die Zusammenhänge und Einflüsse von COX-2 und PPARγ auf molekularer Ebene aufdecken, verständlicher machen und so zu potenziellen pharmakologischen Angriffspunkten werden.

2.4 Regulatorische T-Zellen und Makrophagen (Publikation 2)

2.4.1 Tumor-infiltrierende Lymphozyten

Tumor-infiltrierende Lymphozyten (TILs) sind eine spezifische Gruppe von Immunzellen im Tumorgewebe, die laut der International TIL Working Group (ITILWG) in zwei Subgruppen eingeteilt werden: die stromalen TILs (sTILs) und die intratumoralen TILs (iTILs). Die sTILs befinden sich innerhalb des Tumorstromas und bilden keinen direkten Zellkontakt zu den Karzinomzellen aus; hingegen die iTILs binden in Form von Zellnestern durch Zell-Zell-Kontakte an die Karzinomzellen. [104]

Die sTILs gewinnen zunehmend Aufmerksamkeit in der histopathologischen Auswertung maligner Erkrankungen. Das triple-negative Mammakarzinom wird hinsichtlich des

histopathologisch evaluierten Anteils an sTILs untersucht und dies wird als Kriterium in die Therapieplanung einbezogen [105].

2.4.2 Regulatorische T-Zellen

Innerhalb der TILs befinden sich neben verschiedenen Subtypen der T- und B-Zellen u.a. natürliche Killer (NK)-Zellen, dendritischen Zellen, Makrophagen und Plasmazellen. Hierunter wird vor allem den sog. regulatorischen T-Zellen (Tregs) ein tumorförderliches Verhalten zugeschrieben [106].

Dieser T-Zell-Subtyp, der aufgrund seiner immunsupprimierenden Eigenschaften einen großen Einfluss in zahlreichen immunologischen Interaktionen ausübt, ist mit 4-8% als Teil der CD4-positiven (CD4(+)) T-Zellen im peripheren Blut vertreten. Gershon et al. [107, 108] beschrieb diese Zellen zunächst als suppressive T-Zellen. Sakaguchi et al. [109] erläuterte bereits die Funktion der regulatorischen Zellen im Kontext der Immuntoleranz bei Autoimmunerkrankungen anhand eines Mausmodells. Der Transkriptionsfaktors FOXP3 (Forkhead-Box-Protein P3) konnte im Verlauf als spezifischer Marker für Tregs beschrieben werden und ist maßgeblich für deren Entwicklung und Funktion [110, 111]. Neben der Einflussnahme der Tregs auf die Begrenzung chronischer Entzündungsprozesse [112, 113] und auf die Homöostase der Lymphozyten [114] ist ebenso die hemmende Wirkung auf die Immunantwort bei z.B. Virusinfektionen [115] bekannt.

Im Umfeld sowie im Tumorgewebe selbst kommt es zur Ansammlung von Tregs durch drei grundlegende Mechanismen. Diese werden von Stockis et al. [116] detailliert zusammengefasst: die Konversion zu Tregs durch Umwandlung von CD4(+)CD25(-) T-Zellen zu CD4(+)CD25(+)FOXP3(+) Tregs [117], die Chemokin-induzierte Rekrutierung der Tregs aus der Peripherie [118] und die Akkumulation gewebsständiger Tregs.

Ein Bezug zwischen einem erhöhten Vorkommen an infiltrierenden Tregs im Tumorgewebe und einem ungünstigen Prognoseprofil ist in einer Reihe von Untersuchungen an unterschiedlichen Karzinomen festgestellt worden[118-120]. Untersuchungen für Effekte auf das Outcome für Patientinnen mit Neoplasien und Karzinome der Vulva gibt es hierzu bislang nur sehr vereinzelt [121, 122].

2.4.3 Makrophagen und M1- bzw. M2-Polarisation

Die Makrophagen sind Bestandteil des zellulären Immunsystems und des sog. Mononukleär-phagozytären Systems (MPS). Sie entwickeln und entdifferenzieren sich ursprünglich aus Monozyten. Die Phagozytose, Antigenpräsentation und Immunmodulation mittels Sezernierung von Chemokinen sind ihre bedeutenden Funktionen. Die Makrophagen

befinden sich vorwiegend aufgrund von Chemotaxis und Antigenexposition an bestimmten Orten, um dort ihre Funktionen im Sinne der jeweiligen Immunreaktion auszuführen.[123] Man unterscheidet seit dem Jahr 2000 zwischen unterschiedlichen Phänotypen der Makrophagen hinsichtlich ihrer sog. M1- bzw. M2- Polarisation [124]. Je nach Polarisation ergibt sich für die jeweiligen Makrophagen eine andere Funktionalität: Die M1-Polarisation bedingt die Entwicklung eines pro-inflammatorischen Makrophagen-Phänotyps, die M2- Polarisation hingegen bewirkt die Ausbildung eines anti-inflammatorischen Phänotyps. Die M2-Polarisation wird durch Chemokine wie IL-4 induziert und führt im Rahmen der Ausbildung der antiinflammatorisch- wirksamen Makrophagen zu einer erhöhten Phagozytoseaktivität. [125]

Die anti-inflammatorische Wirkung der M2-Makrophagen unter den sog. Tumor-assoziierten Makrophagen (TAM) führt zur Erzeugung eines immunsuppressiven Tumormilieus, welches eine konsekutiven Immunevasion des Karzinoms ermöglicht. Dadurch entsteht eine sog. Immunevasion des Tumors und das höhere Risiko eines prognostisch ungünstigen Wachstumsverhaltens.[126]

2.4.4 Interaktion von Tregs und M2-Makrophagen

M2-Makrophagen besitzen die Funktion und Fähigkeit Tregs im Tumorumfeld zu induzieren. Die Induktion der Tregs verläuft über die initiale Rekrutierung von Lymphozyten durch Zytokine und Chemokine mit anschließender Differenzierung der rekrutierten Lymphozyten in Tregs. Chemokine wie C-C motif chemokine ligand 22 (CCL22) fördern hierdurch das sog. Trafficking der Tregs, welche den C-C Chemokin-Rezeptor Typ 4 (CCR4) exprimieren und damit an CCL2 binden können. [118, 127, 128]

Tregs produzieren Interleukine wie z.B. IL-10 und IL-32. Diese bewirken eine Aktivierung der M2-polarisierten Makrophagen, sodass wiederum die Produktion der Chemokine, die Rekrutierung der Lymphozyten und die Entwicklung weiterer Tregs gefördert wird. [129]

Tregs und Makrophagen bilden auf diese Weise eine Form von Synergismus und verstärken sich über diese gegenseitige Regulation und Wechselwirkung.

Das Zusammenspiel von Tregs und Makrophagen sowie ihre negativen Einflüsse auf die Prognose und das Überleben der Patient*innen mit Karzinomerkrankungen wurden bereits untersucht [130-132].

Der gezielte Angriff von Tregs [133-135] und Makrophagen [136] ist bereits in der experimentellen Erprobung und stellt einen großen Schritt für die Erweiterung des immuntherapeutischen Spektrums in der zielgerichteten, onkologischen Therapie dar.

3. Publikationen der Dissertation

3.1 Publikation 1

Titel:

Combined COX-2/ PPAR*γ* expression as independent negative prognosticator for vulvar cancer patients

Autoren:

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Abstract:

Vulvar cancer incidence numbers have been rising steadily over the past decades. Especially the number of young patients with vulvar cancer increased recently. Therefore, the need to identify new prognostic factors for vulvar carcinoma is more apparent. Cyclooxygenase-2 (COX-2) has long been an object of scientific interest in the context of carcinogenesis. This enzyme is involved in prostaglandin synthesis and the latter binds to nuclear receptors like PPARγ. Therefore, the aim of this study was to investigate COX-2- and PPARγ- expression in tissues of vulvar carcinomas and to analyze their relevance as prognostic factors. The cytoplasmatic expression of COX-2 as well as PPARγ is associated with a significantly reduced survival, whereas nuclear expression of PPARγ results in a better survival. Especially the combined expression of both COX-2 and PPARγ in the cytoplasm is an independent negative prognosticator for vulvar cancer patients.

Article

Combined COX-2/PPARγ Expression as Independent Negative Prognosticator for Vulvar Cancer Patients

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Abstract: Vulvar cancer incidence numbers have been rising steadily over the past decades. Especially the number of young patients with vulvar cancer increased recently. Therefore, the need to identify new prognostic factors for vulvar carcinoma is more apparent. Cyclooxygenase-2 (COX-2) has long been an object of scientific interest in the context of carcinogenesis. This enzyme is involved in prostaglandin synthesis and the latter binds to nuclear receptors like PPARγ. Therefore, the aim of this study was to investigate COX-2- and PPARγ- expression in tissues of vulvar carcinomas and to analyze their relevance as prognostic factors. The cytoplasmatic expression of COX-2 as well as PPARγ is associated with a significantly reduced survival, whereas nuclear expression of PPARγ results in a better survival. Especially the combined expression of both COX-2 and PPARγ in the cytoplasm is an independent negative prognosticator for vulvar cancer patients.

Keywords: COX-2; PPARγ; vulvar cancer; survival

1. Introduction

In 2018, more than 15,000 women worldwide died of vulvar cancer. However, with a worldwide incidence of 44,235 new cases, the number has been rising steadily over the past decades [1]. In addition, there is a continuing increase in new cases in young females [2,3].

A total of 90% of vulvar carcinomas are squamous cell carcinomas (VSCC). Unkeratinized squamous cell carcinomas are often human papilloma virus (HPV)-associated and mainly affect postmenopausal women [4,5]. Keratinized squamous cell carcinomas, on the other hand, are mostly due to a chronic genital disease such as lichen sclerosis [6,7]. In addition to HPV, other risk factors are associated with the development of vulvar carcinoma: immunosuppression, smoking [8] and sexually transmitted diseases such as herpes simplex virus 2 infections [5] are associated with an increased risk.

With regard to therapy, surgical interventions are predominantly used which end often in a vulvectomy if the findings are extensive. The consequences of such a serious and extensive surgery have been rarely studied to this date. Restrictions in sexual behavior, micturition problems or even psychological effects impairing the quality of life are late effects of this radical form of therapy [9,10]. Regarding prevention, the HPV vaccination, for instance, was seen as a great beacon of hope in the fight against HPV-associated tumors such as cervical, anal, and vulvar cancer [11,12]. The EURO vaccination meeting 2016

listed Belgium as a top performer with a vaccination rate of 84%, while in Germany the vaccination rate reached critically 31% in 2015 [13].

Based on the low vaccination rate in Germany it can be assumed that the need for newly found prognostic factors for vulvar carcinoma is even more apparent. In addition, we find an increasing number of new cases, younger patients, radical therapy, and no comprehensive prevention due to the low vaccination rates, at least in this country.

Cyclooxygenase-2 (COX-2) has long been an object of scientific interest in the context of carcinogenesis. In contrast to constitutive housekeeping enzyme COX-1, COX-2 is as a known enzyme of inflammation inductively expressed [14,15]. Exceptions regarding constitutive COX-2 expression are tissues of the brain, kidneys, testes, and tracheal epithelium [16,17]. The induction of the COX-2 enzyme is triggered by cell damage or inflammation through the release of various factors such as growth factors like epidermal growth factor (EGF) [18], prostaglandins, or chemokines like TNF-γ [19]. It is assumed that products of COX-2 like prostaglandin E2 (PGE2) have a decisive influence on the development of tumors, e.g., in angiogenesis [20,21].

The nuclear receptor superfamily for steroids, hormones, vitamin D, and retinoid is formed by isoforms like PPARα, PPARß, and PPARγ (peroxisome proliferator activated receptor gamma). In general, PPARs act as ligand-dependent transcription factors that bind to specific DNA sequences, the PPREs (PPAR response elements). After heterodimerization with retinoid X-receptor (RXR) a regulatory effect on transcription can occur. Ligands altering the conformation of the receptors lead to co-activation or -repression [22–26]. PPARγ has a proven influence on the regulation of insulin sensitivity and glucose metabolism. Based on this knowledge, the PPARγ agonist from the group of thiazolidinediones made its way into the pharmacological therapy of type 2 diabetes mellitus [27]. An interesting observation of PPARγ and its activators like prostaglandin J2 is the effect on cell differentiation [28,29], cell proliferation and apoptosis induction [30,31]. The expression and related antiproliferative property have been demonstrated in some carcinomas [32–35].

This study investigates COX-2- and PPARγ- expression in tissues of vulvar carcinomas and their relevance as prognostic factors.

2. Materials and Methods

2.1. Clinical Data and Tissue Collection

177 patients with vulvar carcinoma primarily diagnosed in the period from 1990 to 2008 were included in this study. The entire patient group was treated at the department of Gynecology and Obstetrics of the Ludwig-Maximilians-University in Munich, Germany. Surgically obtained tissue samples were histopathologically processed and specified. All follow-up and survival data were provided by the tumor register of Munich.

For immunohistochemical staining, 157 of the 177 samples were available. During the evaluation, a further 16 tissue samples were excluded, as the incisions did not contain a tumor, but only precancerous stages of the carcinoma. Therefore, in the end a collective of 141 slides was assessed.

Median age of the investigated collective was 70 years, ranging from 20 to 96 years, with 72 of the 141 patients younger than 70 years (=51.8%% of the collective). All relevant clinic-pathologic parameters are listed in Table 1 below.

2.2. Ethical Approval

All patients' data were completely anonymized, and the study performance was carried out according to the standards set in the Declaration of Helsinki 1975. The examined tissues were residual material that had been collected in first instance for histopathological diagnostic procedures. The actual study was approved in writing by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 367-16, 29 December 2016). Authors were blinded for clinical information during experimental analysis.

Table 1. Clinicopathological Parameters of Vulvar Carcinoma Patients' Collective.

Clinicopathologic Parameters	*n*	Percentage (%)
Histology		
keratinizing	134	95
wartv/basaloid	7	5
Tumor size		
T1	51	36.2
T2	74	52.5
T3	9	6.4
missing	7	5
Nodal status		
N0	60	42.6
N1	31	22
N2	8	5.7
missing	42	29.8
Metastasis		
missing	141	100
FIGO		
I	45	31.9
II	45	31.9
III	36	25.5
IV	9	6.4
missing	6	4.3
Grading		
G1	24	17
G2	87	61.7
G3	29	20.6
missing	1	0.7
p16 status		
positive	34	24.1
negative	57	40.4
missing	50	35.5
Progression status		
positive	61	43.3
negative	79	56
missing	1	0.7
Local recurrence status		
positive	35	24.8
negative	105	74.5
missing	1	0.7

2.3. *Immunohistochemistry*

After formalin-fixing and paraffin-embedding, all samples were cut to 4 µm from paraffin block. They were mounted on SuperFrost Plus microscope slides (Menzel Glaeser, Braunschweig, Germany). For deparaffinizing tissue patterns were processed with xylol for 20 min and washed by 100% ethanol. All slides were prepared with 3% hydrogen peroxide diluted in methanol for 20 min to stop activity of endogenous peroxidase. Afterwards rehydration took place in a descending alcohol series (100%, 70%, 50%) and were washed with distilled water. The samples were heated with citric acid buffer in a pressure cooker to uncover epitopes of antigens. Furthermore, slides were washed two times with phosphate buffered saline (PBS). Zytochem-Plus HRP Polymer-kit (Zytomed, Berlin, Germany) was utilized for blocking and antibody staining. After saturating electrostatic charges in tissue with blocking solution for 5 min, either the polyclonal rabbit IgG anti- COX-2 antibody (Sigma, St. Louis, MO, USA, SAB4502491) or the polyclonal rabbit IgG anti- PPARγ antibody (abcam, Cambridge, Great Britain, ab59256) were applied on tissue specimens.

Anti-COX-2- antibody was diluted at a ratio of 1:400 and anti- PPARγ -antibody at a ratio of 1:100. The incubation time of both antibodies amounts to 16 h at 4 °C in humid chamber. Slides were incubated by post-block reagent for 20 min and thereafter by HRP-Polymer for 30 min at room temperature in a humid chamber. After each application with the antibody, post-block and HRP-Polymer the samples were washed two times with PBS. 3,3′-Diaminobenzidine (Dako, Hamburg, Germany) catalyzed the peroxidase substrate staining so that the color precipitation is detectable with a light microscope. Finally, slides were counterstained with hemalum, again washed by 100% ethanol and covered with glass. As positive control, both antibodies were stained in placenta tissue for validating the staining method.

Under use of the semi quantitative immunoreactive score (IRS) by Remmele and Stegner [36], tissue patterns were evaluated with the light microscope (Leitz, Wetzlar, Germany). For this purpose, the intensity score and the percentage score in the tumor tissue were formed. The intensity score is divided into 0 = no, 1 = weak, 2 = moderate, 3 = strong; the percentage score is also categorized into 0 = no staining, $1 \leq 10\%$, 2 = 11% to 50%, 3 = 51% to 80%, $4 \geq 81\%$. IRS score is formed by product of both scores (intensity score x percentage score). The antibodies showed expressions in cytoplasm and in nucleus, so both expression templates were examined independently by IRS. Patients' data were correlated by IRS and by its two IRS-forming factors of staining intensity and percentage of positively stained cells.

2.4. Statistical Analysis

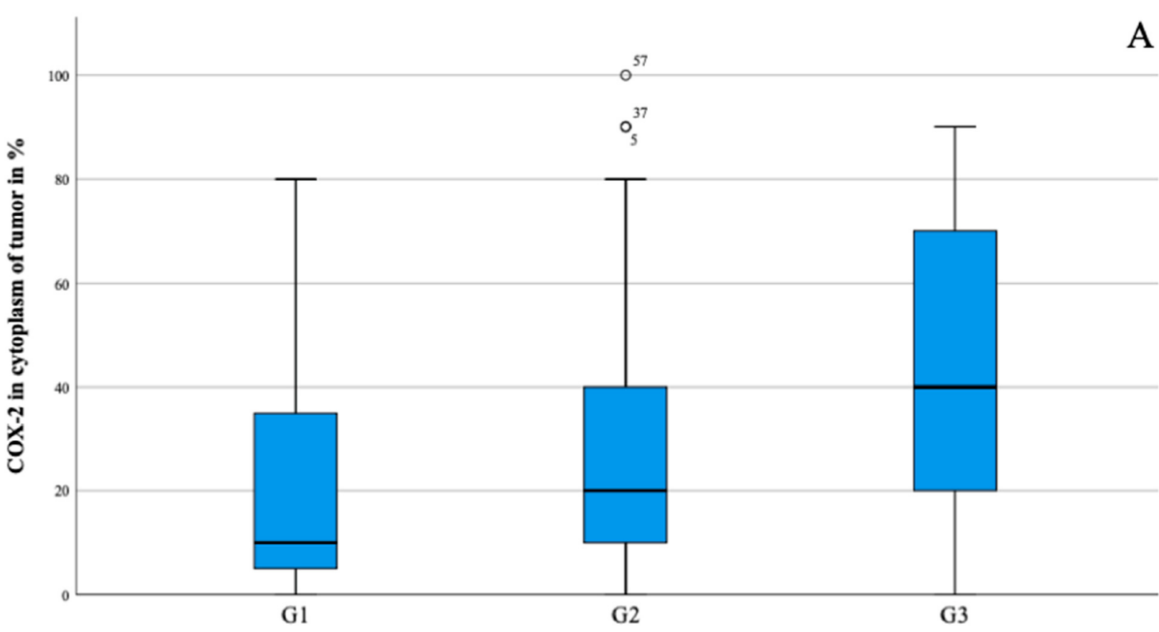
For statistical analysis, the SPSS Statistic version 25 (IBM Corp., Armonk, NY, USA) was used. The non-parametric Kruskal-Wallis test was used to compare between and among groups. Correlation analyses were performed using the Spearman rank correlation coefficient. Kaplan-Meier curves were generated using collected survival data, differences between these curves were tested by the log-rank test. The level of statistical significance was accepted at $p \leq 0.05$ and all test were two-sided.

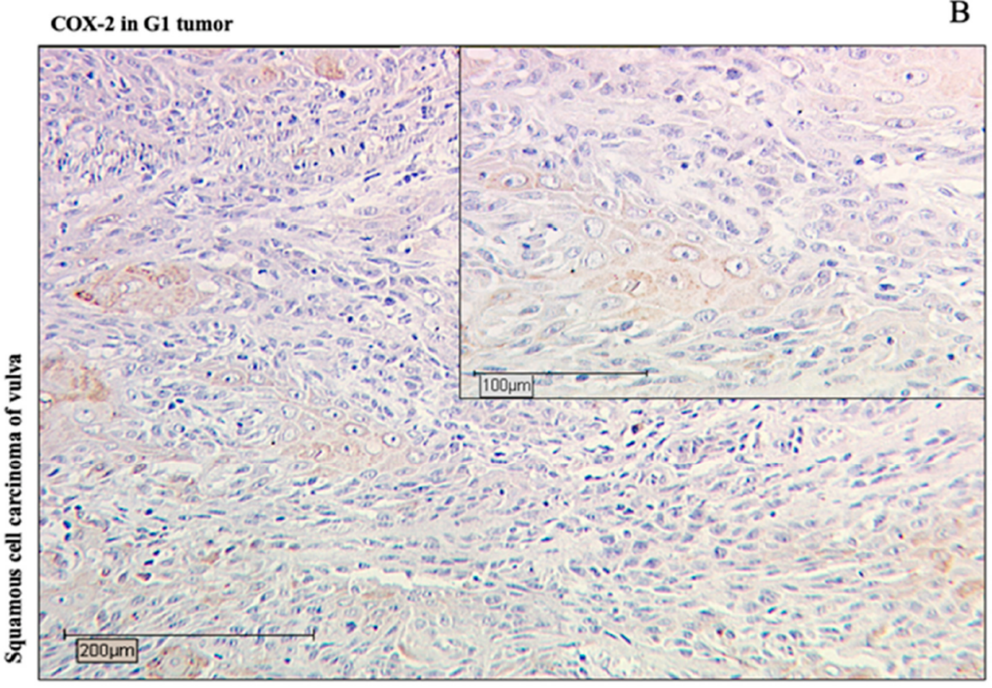
3. Results

3.1. COX-2 as Predictor for Grading and for Overall Survival

In the patient group, 91.3% of the stained samples were positive for COX-2 in the cytoplasm. The immunohistochemical evaluation showed a positive correlation of COX-2 amount in the cytoplasm of the tumor tissue to the respective degree of differentiation (grading) (Spearman-Rho, * $p = 0.003$, Figure 1).

There is a distinct statistical correlation between the increase in COX-2 expression per immunoreactive score (IRS) and the grading (Spearman-Rho, * $p = 0.020$, images B-D in Figure 1). Considering tumor stage T, there is also a significant, concurrent relationship to the proportion of COX-2 expression in tumor tissue (Spearman-Rho, * $p = 0.021$). Furthermore, a reduction in overall survival was demonstrated for the group of patients who had an IRS value > 3 in the cytoplasm in COX-2 staining (* $p = 0.003$, Figure 2).





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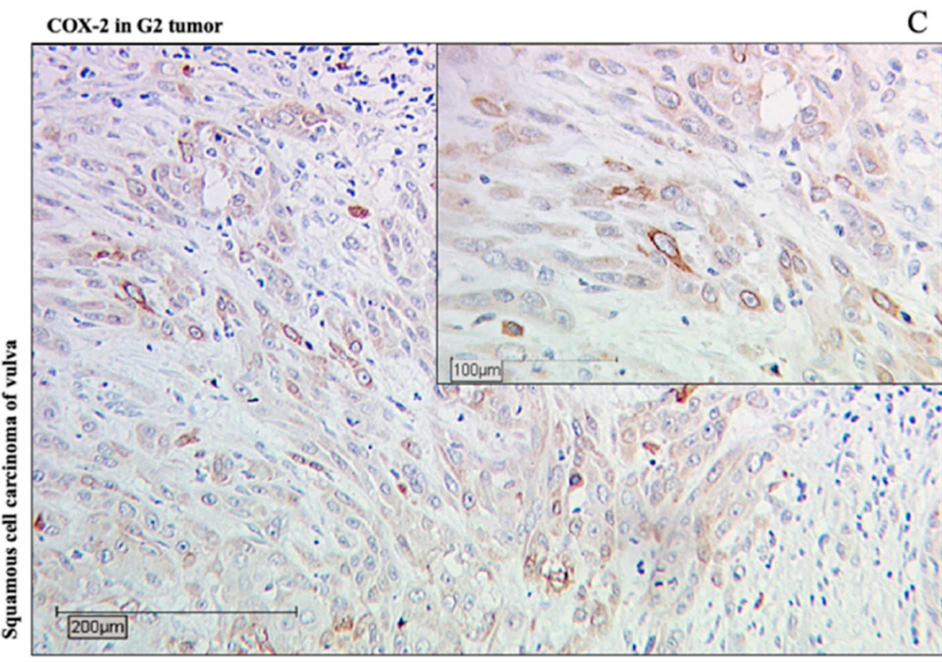


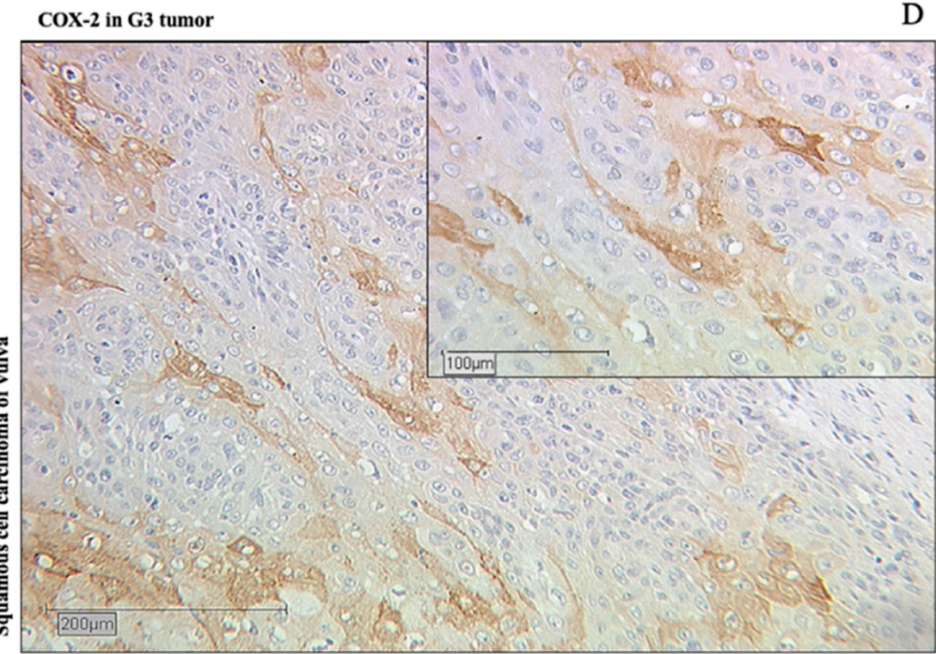
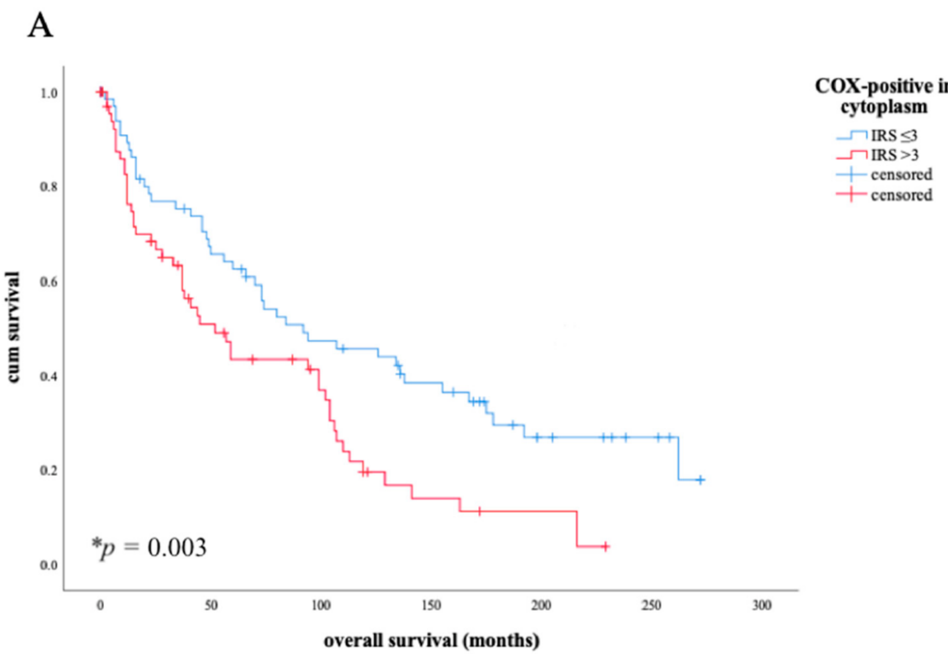


Figure 1. Boxplots (**A**) presenting positive correlation (* $p = 0.001$ in Spearman-Rho) between COX-2 positive tissue amount and the individual degree of tumor grading (G1: well-differentiated for (**B**), G2: moderately-differentiated for (**C**), G3: poorly-differentiated for (**D**)). The boxplots indicate mild outliers, which are marked with circles. These outliers show an interquartile distance to the third quartile of values that is less than three times higher than the third quartile of values. The numbers on the circles denote the cases (case numbers 5, 37, 57) in concern. Immunohistochemistry staining of cytoplasmic COX-2 (10× and 25× magnification) showing correlation to Grading 1–3 with increase of COX-2 intensity in vulvar cancer (**B**–**D**). The medians of the percentage COX-2 expression shown in the boxplots of the individual grading categories are represented in the immunohistochemical images of (**B**–**D**) (amount 10% in (**B**), amount 20% in (**C**), and amount 40% in (**D**)).



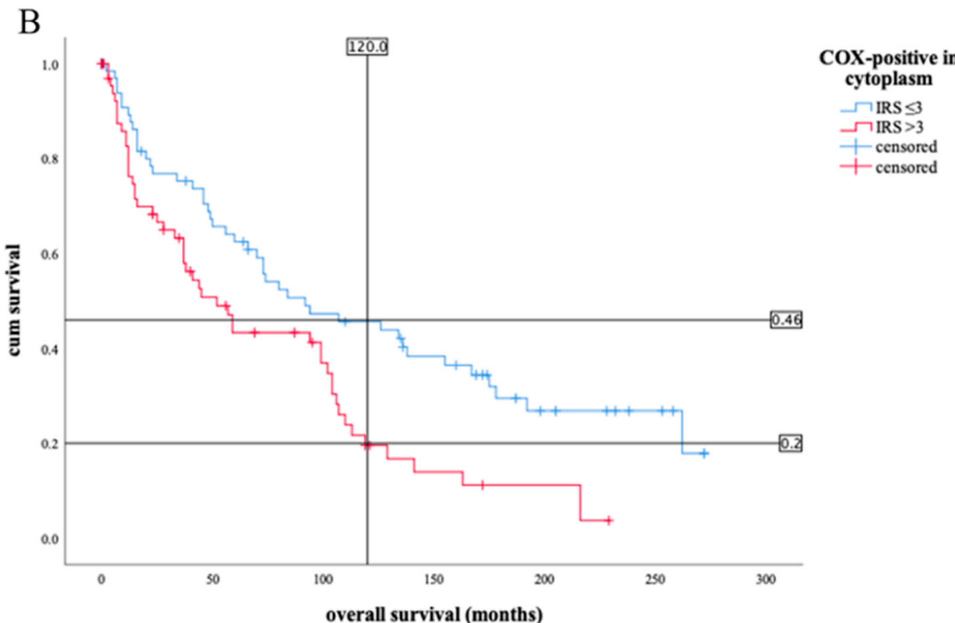


Figure 2. As the Kaplan-Meier curve shows, patients with cytoplasmatic COX-2 expression according to immunoreactive score (IRS) > 3 survive for a shorter time period than patients with a lower IRS ((**A**), * $p = 0.003$). The blue line shows the survival curve of patients with COX-2 expression of IRS level ≤ 3, the red line shows the survival curve of patients with COX-2 expression IRS level above. The 10 year survival ((**B**), 120 months signed with vertical line) of a patient with IRS > 3 is about half as high as that of a patient with lower IRS. The horizontal lines in (**B**) illustrate the points of intersection on the Kaplan-Meier curves: IRS values above 3 show that 20% of patients live after 10 years; IRS values below 3 demonstrate that 46% of patients survive after the same time.

A total of 49.3% of the tumor samples have an IRS > 3 for COX-2 in the cytoplasm, the remaining 50.7% were below this IRS value. As the Kaplan-Meier illustrates, the 10-year overall survival of patients with an IRS value >3 was 20%, but patients with a lower IRS value lived more than twice as long at 46% (Figure 2). This data shows a median survival advantage of patients with lower IRS values (≤3) compared to patients with higher IRS values (>3) at 40 months (Table 2).

Table 2. There is a clear difference in overall survival for patients with IRS values for COX-2 expression in the cytoplasm above 3. Patients with IRS values above 3 live with a median of 52 months, whereas patients with lower IRS values survive 92 months. As the table shows, the survival of patients of both groups differs by 40 months, i.e., more than three years. Even in the total group a survival difference is recorded: patients live a total of 73 months, but still lose 21 months of life with higher IRS values.

Median for Overall SuMedian for Overall Survival Time (Months)			
COX-2 IRS Value in Cytoplasm	Estimate	Lower 95% Confidence Interval	Upper 95% Confidence Interval
IRS ≤ 3	92.000	36.414	147.586
IRS > 3	52.000	31.292	72.708
Overall	73.000	44.681	101.319

The multivariate analysis revealed that grading (* p = 0.004), p16 status (* p = 0.001), and COX-2 (* p = 0.005) functioned as independent prognostic factors for overall survival. However, tumor stage, nodal status, and FIGO classification did not act as independent prognostic factors (Table 3).

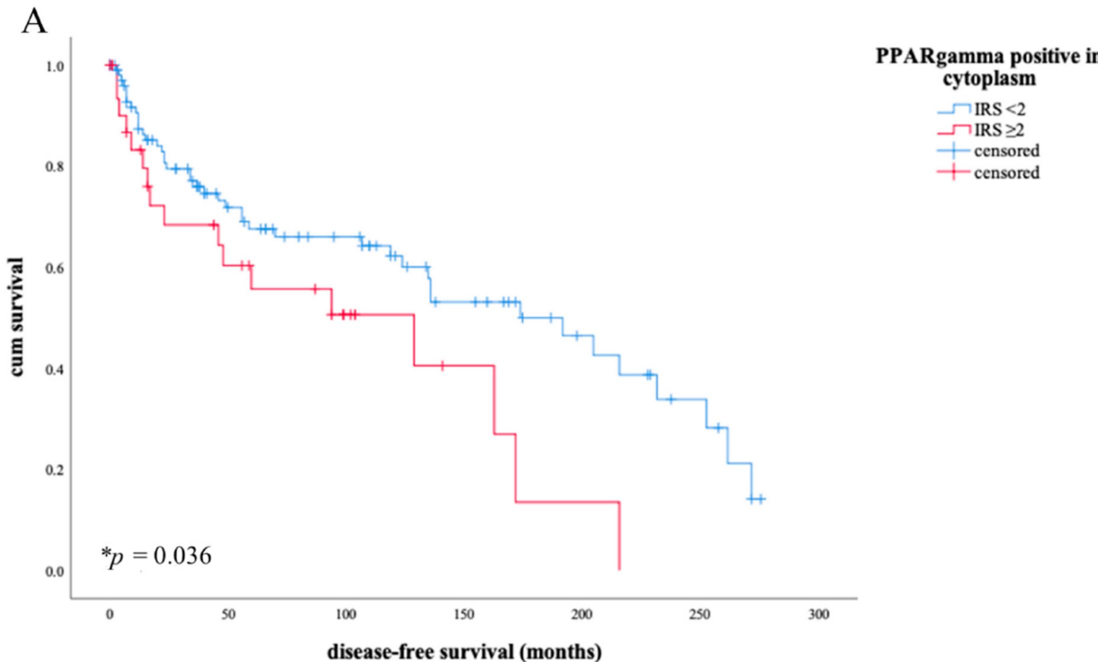
Table 3. Cox regression of clinical-pathological variables regarding overall survival in vulvar carcinoma patients.

Variable	**Significance**	**Hazard Ratio of Exp (B)**	**Lower 95% Confidence Interval of Exp (B)**	**Upper 95% Confidence Interval of Exp (B)**
COX-2 in cytoplasm	0.005	2.187	1.267	3.776
pT	0.488	1.275	0.642	2.535
pN	0.112	1.005	0.999	1.012
Grading	0.004	1.874	1.222	2.873
FIGO	0.199	1.336	0.858	2.081
p16 status	0.001	0.362	0.196	0.671

COX-2 cytoplasm = expression of COX-2 in cytoplasm with IRS > 3, pT = tumor stage, pN = nodal stage, FIGO = Classification of the International Federation of Gynecology and Obstetrics.

3.2. *PPARγ as a Negative Prognostic Factor for Disease-Free Survival in Cytoplasm*

A total of 78.4% of the tumor samples were positive for PPARγ in the cytoplasm, 21.6% showed no cytoplasmic staining. The intensity of PPARγ expression in the cytoplasm showed a positive correlation with the progression status (* p = 0.008) and the development of a local recurrence (* p = 0.016, all Spearman-Rho test). The Kaplan-Meier curve showed a significantly worse disease-free survival for patients with a PPARγ expression ≥ 2 in cytoplasm of the tumor tissue than for those whose IRS value is below 2 (* p = 0.036, Figure 3).



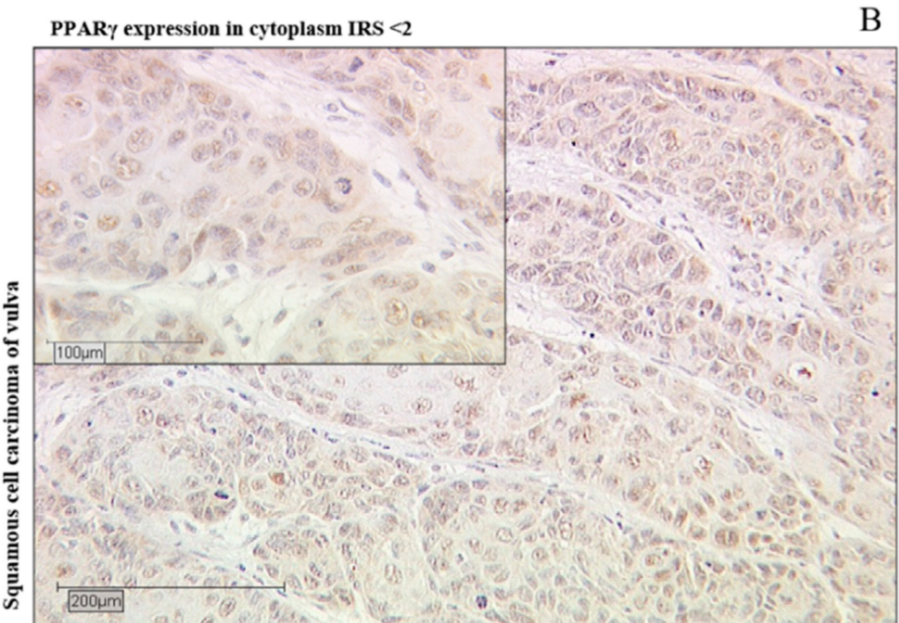


Figure 3. *Cont.*

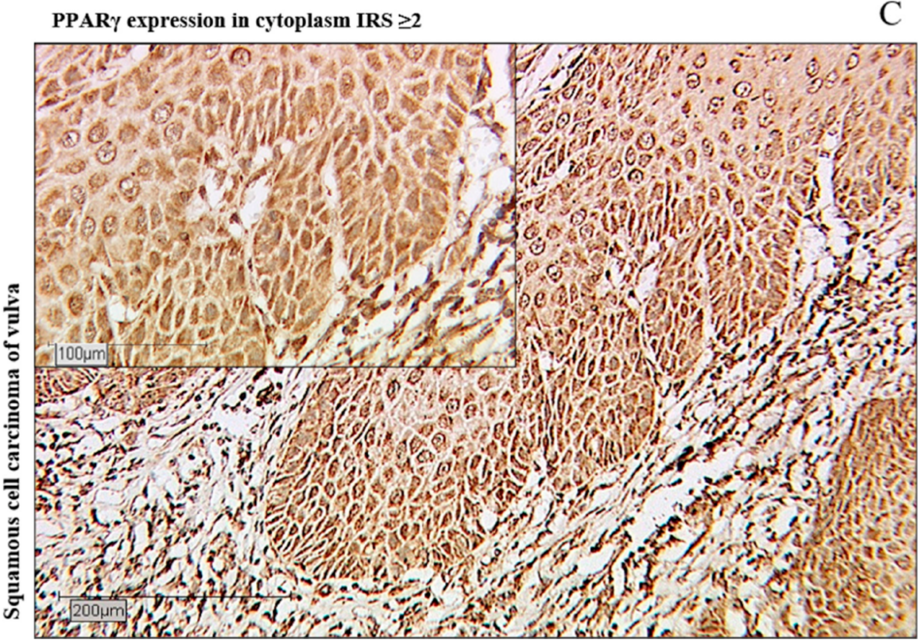


Figure 3. As the Kaplan-Meier curve (**A**) shows, patients with cytoplasmic PPARγ expression according to IRS ≥ 2 survive a shorter time than patients with a lower IRS (* $p = 0.036$). The blue line shows the survival curve of patients with PPARγ expression of IRS level under 2, the red line shows the survival curve of patients with PPARγ expression IRS level ≥ 2. (**B**) shows an example of low expression level of PPARγ in cytoplasm (IRS < 2), (**C**) represent a high expression level of PPARγ (IRS ≥ 2) in cytoplasm, in contrast.

After 10 years, 62% of tumor patients with an IRS value less than 2 lived disease-free. However, patients with a higher IRS value had a shorter disease-free survival (51% after 10 years). The median survival data showed an absolute survival advantage of 63 months for patients with IRS values below 2 (Table 4).

Table 4. The table shows that an expression of PPARγ in the cytoplasm from IRS values of 2 upwards is a survival disadvantage in disease-free survival. The tumor patients with IRS values of 2 and above live disease-free for a median of 129 months; Patients with lower IRS values, however, live 192 months and thus much longer. Overall the patients live 163 months. The difference between both IRS groups (from 2 or below) is a disease-free survival difference of 63 months, i.e., more than 5 years.

Medians for Disease-Free Survival Time (Months)			
PPARγ IRS Value in Cytoplasm	Estimate	Lower 95% Confidence Interval	Upper 95% Confidence Interval
IRS < 2	192.000	127.692	256.308
IRS ≥ 2	129.000	20.907	237.093
Overall	163.000	203.332	203.332

In 23.0% of the patients an IRS value ≥ 2 for PPARγ in the cytoplasm was found. Finally, the Cox regression analysis concluded that PPARγ was not an independent factor for disease-free survival in vulvar cancer (Table 5, $p = 0.626$). The independent prognostic factor in disease-free survival, though, was grading (Table 5, * $p = 0.009$).

Table 5. Cox regression of clinical-pathological variables regarding overall survival in vulvar carcinoma patients.

Variable	Significance	Hazard Ratio of Exp (B)	Lower 95% Confidence Interval of Exp (B)	Upper 95% Confidence Interval of Exp (B)
PPARγ in cytoplasm	0.626	1.193	0.588	2.418
pT	0.336	1.496	0.658	3.400
pN	0.403	0.996	0.985	1.006
Grading	0.009	2.016	1.190	3.413
FIGO	0.259	1.342	0.805	2.238
p16 status	0.061	0.481	0.224	1.034

PPARγ cytoplasm = expression of PPARγ in cytoplasm with IRS ≥ 2, pT = tumor stage, pN = nodal stage, FIGO = Classification of the International Federation of Gynecology and Obstetrics.

3.3. Nuclear PPARγ as a Positive Prognostic Factor for Overall Survival

Within our group of patients, a nuclear expression for PPARγ could be detected with the exception of only 2 cases. In total, 98.6% of the investigated patient group showed a positive expression pattern in the nucleus for PPARγ. The survival curve showed that the nuclear expression of PPARγ had a positive effect on overall survival at values ≥ 2 (Figure 4, image A, * $p = 0.019$). In comparison, the expression of PPARγ in the cytoplasm is associated with a negative trend in overall survival (Figure 4, image B, $p = 0.053$).

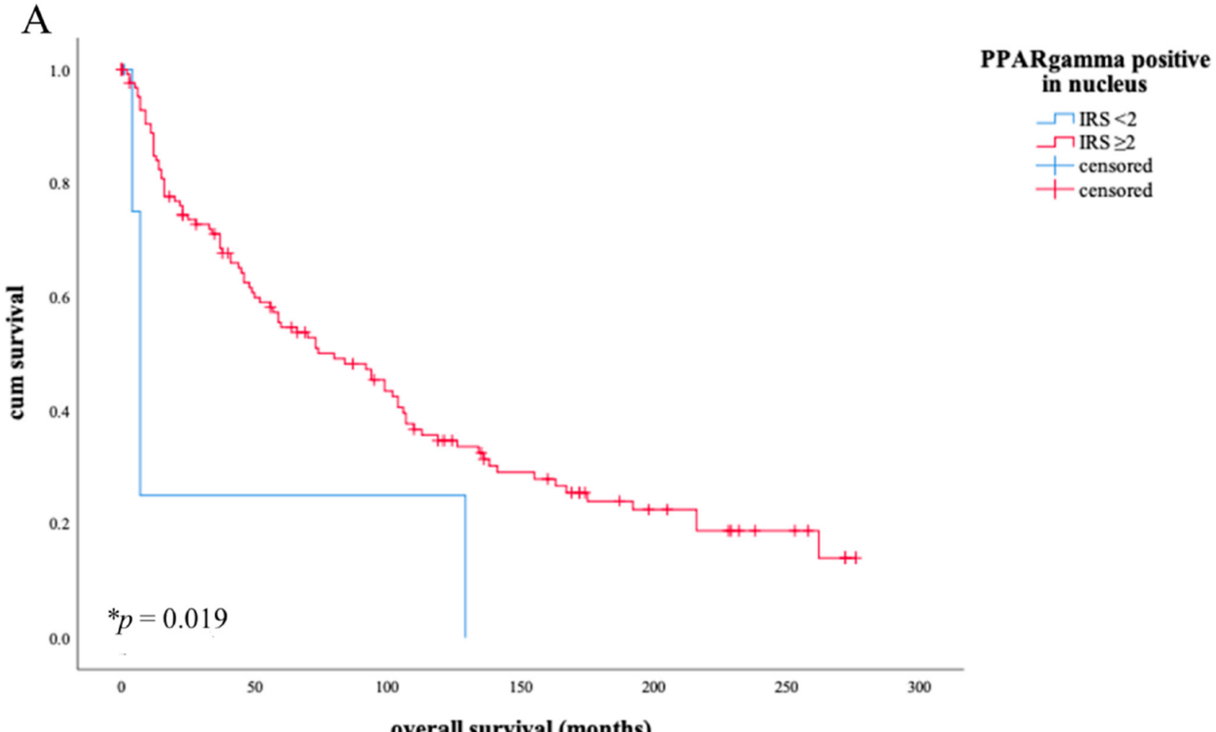


Figure 4. *Cont.*

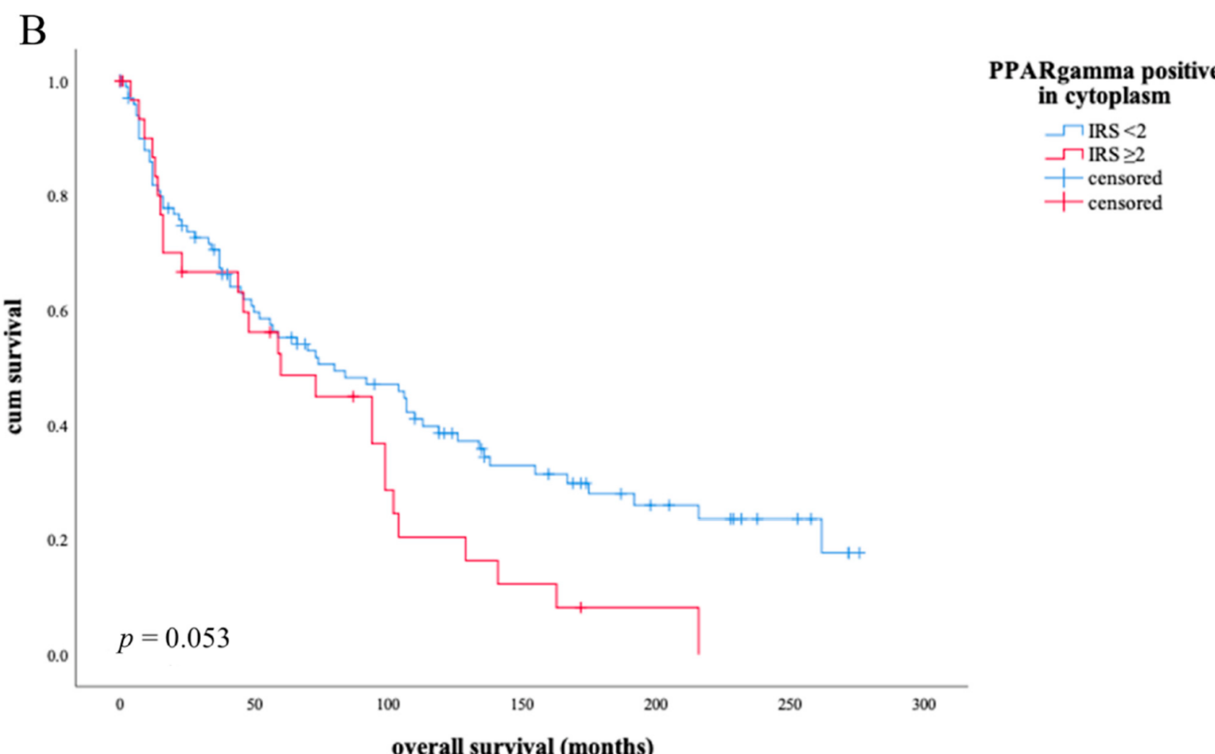


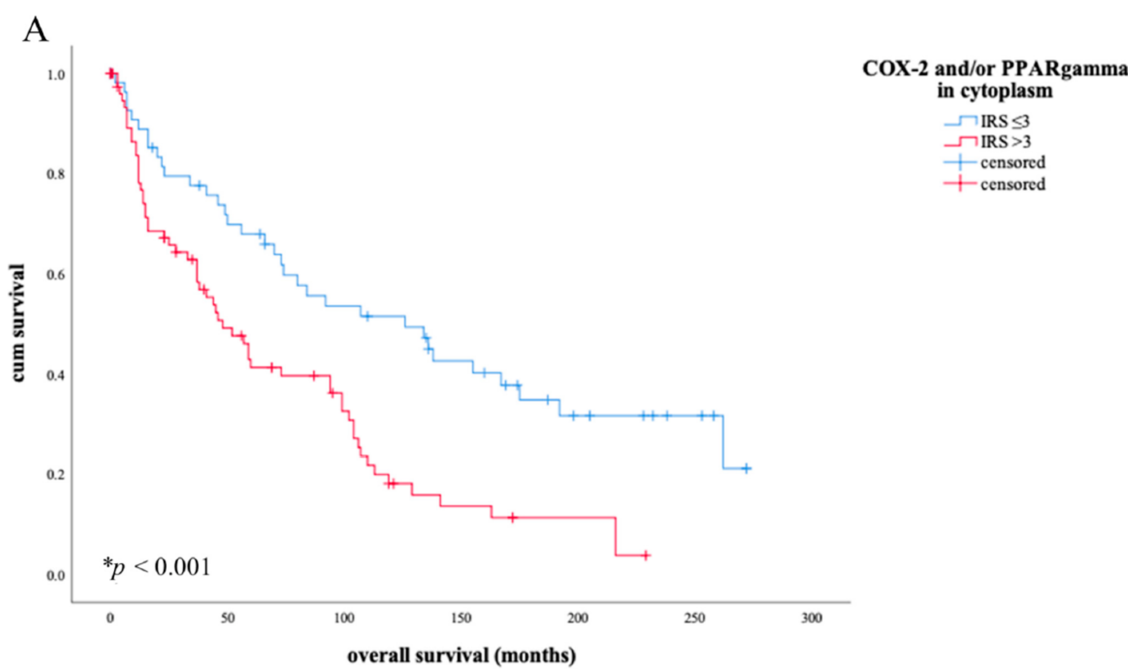
Figure 4. As the Kaplan-Meier curve illustrates, an IRS value ≥2 of the nuclear PPARγ expression is related to a longer overall survival than in patients with lower values ((**A**), * $p = 0.019$). Here, a prognostic survival advantage is shown in contrast to the contrary trend in the Kaplan-Meier curve in Figure 4B. The blue line in Figure 4A shows the survival curve of patients with nuclear PPARγ expression of IRS level under 2, the red line shows the survival curve of patients with nuclear PPARγ expression IRS level ≥2. (**B**) reveals an opposite trend in the overall survival curve as soon as PPARγ expression is detected in the cytoplasm rather than in the nucleus (**B**), $p = 0.053$). The blue line in (**B**) shows the survival curve of patients with PPARγ expression in cytoplasm of IRS level under 2, the red line shows the survival curve of patients with PPARγ expression in cytoplasm of IRS level ≥ 2.

3.4. Correlation between PPARγ and p16 Status

PPARγ (nuclear expression) shows a clear statistical negative correlation to the p16 status (* $p = 0.004$, Spearman-Rho test). Moreover, the Spearman-Rho test proved that the nodal status also has a negative correlation to the IRS value of PPARγ in the nucleus (* $p = 0.017$) which indeed underlines the survival advantage with higher nuclear expression of PPARγ in the nucleus.

3.5. Combined COX-2/PPARγ Expression as an Independent Prognostic Factor for Overall Survival

In addition to the individual studies of COX-2 and PPARγ regarding survival, a significantly stronger influence of both factors together was observed. This resulted in the association of a low to absent expression of one or both factors with the longest overall (** $p < 0.001$, Figure 5, image A) and disease-free survival (* $p = 0.006$, Figure 5, image B).



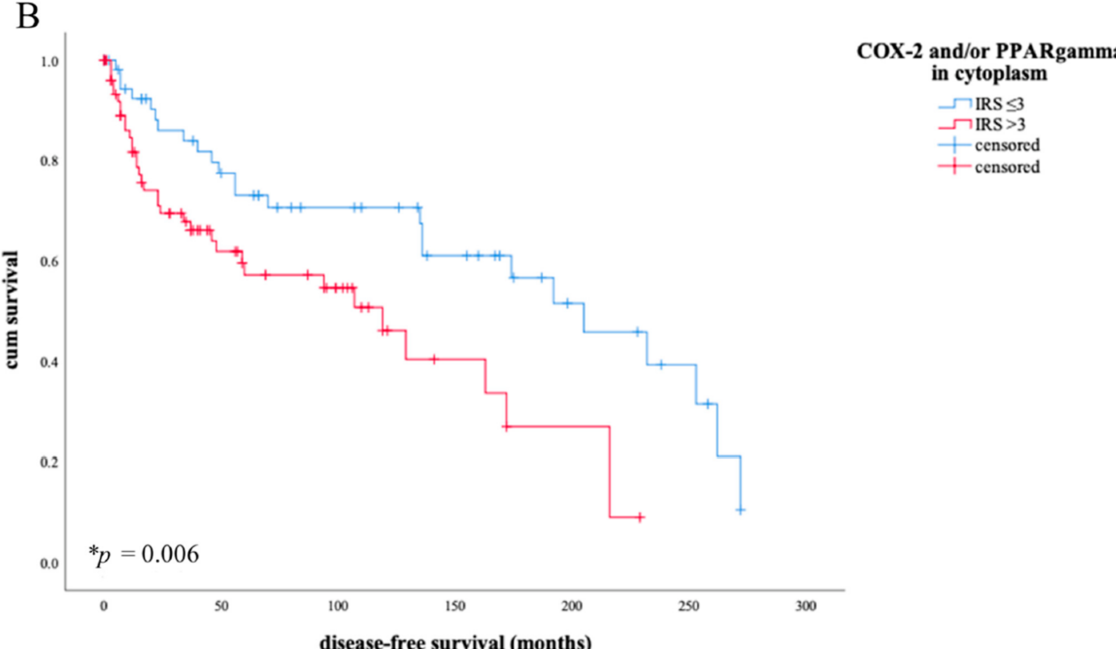


Figure 5. When COX-2/PPARγ is expressed with IRS in cytoplasm >3, a shorter overall survival ((**A**), * $p < 0.001$) but also with regard to disease-free survival (**B**), * $p = 0.006$) can be derived. The blue line in (**A**) shows the overall survival of patients with COX-2/PPARγ expression in cytoplasm of IRS level ≤ 3, the red line shows the survival curve of patients with COX-2/PPARγ expression in cytoplasm of IRS level above 3. The blue line in (**B**) shows disease-free survival of patients with COX-2/PPARγexpression in cytoplasm of IRS level ≤ 3, the red line shows the survival curve of patients with PPARγexpression in cytoplasm of IRS level above 3. After 10 years, more than twice as many patients (0.5) live with lower IRS values than patients with expression of one or both factors (0.21) after IRS > 3. The situation is similar in disease-free survival: lower expression of COX-2 and/or PPARγ shows longer disease-free survival (0.7) than with higher expression of one or both factors (0.49).

Low expression was determined below an IRS value of 3 in the cytoplasm. In comparison, 10-year survival is more than twice as long in patients with low IRS for COX-2 and/or PPARγ. Disease-free survival is also extended from 49% to 70% with values of IRS ≤ 3. Patients with an IRS value ≥ 3 live a median of 126 months, whereas patients with lower score values live only 48 months. The disease-free survival is similar: median disease-free survival is 205 months for patients with IRS values ≥ 3, while patients with lower IRS values spend 86 months less disease-free. A total of 57.7% of tumor patients were positive for COX-2 and/or PPARγ with a cytoplasmic IRS value >3; 42.3% did not have a value > 3 for either factor. The independence of COX-2 and PPARγ as a predictive factor for overall survival from other clinical pathological factors was tested by a cox regression analysis (* $p = 0.001$, Table 6) in comparison to tumor stage, nodal state, grading, FIGO-classification, and p16-state.

Table 6. Cox regression of clinical-pathological variables regarding overall survival in vulvar carcinoma patients.

Variable	Significance	Hazard Ratio of Exp (B)	Lower 95% Confidence Interval of Exp (B)	Upper 95% Confidence Interval of Exp (B)
PPARγ+/COX-2	0.001	2.615	1.460	4.683
pT	0.613	1.195	0.598	2.388
pN	0.85	1.006	0.999	1.013
Grading	0.012	1.738	1.129	2.676
FIGO	0.098	1.453	0.933	2.263
p16 status	0.002	0.380	0.208	0.694

PPARγ+/COX-2 = expression of PPARγ and/or COX-2 in cytoplasm (IRS), pT = tumor stage, pN = nodal stage, FIGO = Classification of the International Federation of Gynecology and Obstetrics.

4. Discussion

Our study proved that the expression of COX-2 and PPARγ and their combination in the tissue of the vulvar carcinoma has a strong impact on survival (Figures 2–5).

So far, there are only a few studies describing prognostic factors in vulvar carcinoma. The multicenter AGO-CaRE study [37] reported lymph node metastases as a decisive prognostic factor for patients with vulvar carcinoma [38]. Concerning potential prognostic markers showing significant relation to survival in vulvar cancer, only small studies addressed amongst others on p16 [39], p53 [40], ERβ [41], c-KIT [42], p14ARF [43].

COX-2 plays a decisive role in carcinogenesis. Acting COX-2 products, prostanoids, appear to be linked to the development and progression of a tumor disease. Processes including angiogenesis, invasion, apoptosis inhibition, growth, and aggressiveness of the tumor seem to depend strongly on COX-2 and its products [44–46]. Various cross-links to signaling pathways via NF-kB [47], Wnt/ß-catenin [48], PI3K/AKT [49], or activations of anti-apoptotic Bcl-2 [50] are established by COX-2. The NF-kB pathway regulates the expression of COX-2. As it has already been investigated in several studies, PPARγ in its activated form acts in the nucleus as an inhibitory factor on the transcription factor NF-kB. PPARγ thereby inhibits the expression of COX-2, which can be regarded as one important connection point between the two proteins [47,51,52].

Furthermore, blocking effects of prostaglandin E2 and prostaglandin F2α on the pro-apoptotic PPARγ have been reported [53,54]. However, there are some isolated studies to the contrary that pronounce COX-2 to have anti-tumor properties also [55,56].

Hence, our observation that COX-2 in the cytoplasm is a highly significant independent prognostic factor for the overall survival of our patient population is even more interesting.

Increased COX-2 expression was not only found in a variety of gynecological tumors such as endometrial carcinoma [57], breast carcinoma [58–60], ovarian carcinoma [61], or

cervical carcinoma [62], but also in a lot of other tumor entities [63–68]. Only a few studies exist regarding the expression of COX-2 in tumor tissue of the vulva [69,70].

In the study by Fons et al. [70], COX-2 has already been associated with poorer overall survival in vulvar cancer in a smaller number of cases (n = 50), but did not prove to be an independent factor. Comparing results with other tumor entities, a role as an independent prognostic factor was e.g., detected by Mrena et al. [71] in gastric carcinoma. Becker et al. [72] also reported a significant correlation between COX-2 levels in malignant melanoma and overall survival.

Apart from this, COX-2 has a direct relationship with tumor grading and tumor stage in our study. Lee et al. [73] observed an inverse relationship between grading and COX-2 levels in patients with vulvar cancer, where low grading stages had the highest COX-2 expression levels.

Our observation of a positive correlation between COX-2 and tumor stage and/or grading goes along with the results by Sheehan et al. [74] in colon cancer, Miyata et al. [75] in renal cell carcinoma, and Boland et al. [59] in DCIS of breast.

In terms of the prognostic significance of COX-2 in certain tumor entities, however, improved survival was also observed [76,77]. The prognostic significance of PPARγ in vulvar cancer is clearly different depending on its localization of expression. In the nucleus, PPARγ is actively involved in the regulation of gene expression in its role as a transcription factor [23,25]. Nevertheless, we also found that PPARγ can also be stained in the cytoplasm of vulvar carcinoma. Several other tumor tissues have been identified as having such a staining profile [78–80].

Due to the fact that PPARγ cannot act in the cytoplasm in its genomic function as a transcription factor, the expression of PPARγ in the cytoplasm is assumed to be associated with the lack of nuclear activity in gene regulation and a non-genomic functioning in cytoplasm. The translocation dynamics between cytoplasm and nucleus are getting in focus of scientific research to an increasing extent. Like other nuclear receptors such as progesterone receptor [81,82], glucocorticoid receptor [83,84], androgen receptor [85], or thyroid receptor [86], the nuclear-cytoplasmic shuttling is an important component of regulating the activity of these receptors. For now, there is no clearly identifiable shuttle protein involved in trafficking of PPARγ and the cytoplasmic function of PPARγ is widely unexplained, but often aim of scientific experiments [87–90]. Other factors influence the activity regulation of PPARγ like ubiquitination or the influence of natural ligands like PGJ2 or external ligands like thiazolidiones [91]. Additional influences by post-translational modification via phosphorylation by MAPK as well as activity modulation by traditional herbal medicine plants like V.trifolia demonstrate the diversity of possible modulatory pathways of the activation and expression pattern of PPARγ [92,93].

These findings would explain the opposite effect on survival when PPARγ is detected in the nucleus or in the cytoplasm: Nuclear expressed PPARγ is a prognostically favorable factor in overall survival, but detection in the cytoplasm is a prognostically unfavorable factor in disease-free survival and shows an unfavorable trend in overall survival. Shao et al. [94] described an unfavorable overall survival in breast cancer patients showing a high-expression level of PPARγ in cytoplasma. In some tumor entities, a positive prognostic influence of nuclear PPARγ expression on the survival of tumor patients has already been identified, but a difference between expression localization and relation to different patient outcome was not described [95,96]. In the present study, a predictive difference between nuclear expression and cytoplasmic expression of PPARγ in vulvar cancer patients is investigated and described for the first time.

Several in vitro and in vivo studies have suggested that PPARγ is effective as a tumor suppressor. Nicol et al. [97] demonstrated an increased rate of developed neoplasia in mammary, ovary and skin in PPARγ-deficient mice. Sarraf et al. [98] describes loss-of-function mutations of PPARγ in colon cancer tissue as a contributing factor to tumorigenesis. Furthermore, the pro-apoptotic and anti-proliferative effect in tumor cell lines with PPARγ agonists could be proven in some studies [34,99–102]. The tumor suppressing property of

PPARγ remains controversial. In contrast, Lefebvre [103] and Saez et al. [104] showed that ligand-induced activation of PPARγ in mouse experiments resulted in the occurrence of colonic polyps and an increased probability of degeneration.

Controversially discussed prognostic properties of PPARγ may be explained by tissue-specific effects and the concentration of the PPARγ ligands used. The detailed review of Clay et al. [105] showed that experiments with the PPARγ agonist PGJ2 induce carcinoma growth under low-dose conditions, but a decrease in proliferation behavior under high-dose conditions.

In studies of oropharyngeal squamous cell carcinoma, p16 is considered a surrogate marker for HPV positivity. Our research group has already investigated a proven positive effect of p16 on the prognostic outcome of patients with VSCC. p16 positive VSCC revealed a longer overall and progression free survival [106]. It also appears in some other studies that HPV-associated VSCC have a better clinical outcome as Sand et al. [107] reported in a recent review. However, our study revealed a negative correlation of nuclear PPARγ and p16. Therefore, p16 seems not to be involved in PPARγ translocation.

The combination of the cytoplasmic expression of COX-2 and PPARγ showed that both factors together have the strongest predictive power for a negative survival prognosis in overall and disease-free survival. COX-2 and PPARγ together function independent from other clinical pathological parameters as strongly significant prognostic factors for patients with vulvar cancer.

The enzyme COX-2 and the transcription factor PPARγ are interacting. PGE2 inhibits the activity of the pro-apoptotic active transcription factor and in turn underlines the carcinogenic effect of COX-2 [20,21,54]. Studies by Rothwell et al. using COX inhibitors showed impressive results in terms of improving the prognosis and reducing the incidence of colorectal cancer [108].

In addition, there is also an activating link between the two molecules via PGJ2, the natural ligand and agonist of PPARγ. Consequently, the anti-tumor effect of PPARγ is supported [30,32]. These two modes of activity illustrate clearly that the importance does not only lie in the anti-tumor or pro-tumor effects of molecules, but the respective predominance of a characteristic molecule in the individual and tissue-specific context. Factors that influence this balance, for example through shuttling with resulting activation or inactivation, require closer observation.

The limitations of our study are most likely the use of one method only to detect the expression level of COX-2 and PPARγ in the tissue sections. Immunohistochemistry was applied in our single-method approach due to the fact that this technique of advanced histopathological diagnostic is well established and renowned within the respective field of research. This limitation comes along with the retrospective design of the study. Only a highly limited number of patients included in this collective was alive during the examination of the tissue sections so that there would have been the possibility of applying a scan for cells within serum or primary cells.

However, this subjective form of evaluation was objectified by the use of two independent investigators who evaluated the expression pattern blinded.

There was no possibility to overview the process of the embedment of the tissue which leads to the fact that in the analyzed collective only tumorous tissue is accessible. Vulvar tissue patterns of patients with non-malignant vulvar diseases were tested by immunohistochemical expression level for COX-2 and PPARγ for clarifying differences between malignant and non-malignant tissue expression levels (Supplementary Figure S1 and S2).

In addition, only sections containing invasive vulvar carcinoma assessed by an experienced pathologist were evaluated. Furthermore, immunohistochemistry is an integral part of tumor diagnostics, which is still mainly used in gynecology and is highly appreciated as an economical and simple method in clinical routine.

The method of immunohistochemical staining used in this study reflected the expression level but is hardly meaningful regarding the activity of the stained proteins. Thus,

conclusions like the possible improvement of the outcome by using COX-2 inhibitors or PPARγ agonists cannot be drawn.

The data of the collective includes no information about the drug status of the patients at the time of tissue collection; therefore, no conclusion can be drawn about a possible intake of a COX inhibitor or PPARγ agonist. However, medication is unlikely to alter the expression pattern, as the drugs only affect the activity and not the expression.

In contrast to other immunohistochemical studies on vulvar cancer, we have a very large collective with real-time data from patients. In 2016, the Robert-Koch-Institute Germany reports regarding the epidemiology of vulvar carcinoma a medium age of 73 years, which is manifested in the highest burden of disease within the group of women over 70 [109]. In this case our collective represents an approximation of the frequency of this disease within the age groups. The aim of our study is to find a prognostic factor for all vulvar carcinoma patients. Due to the rarity of this type of carcinoma, especially in comparison to other types e.g., breast cancer, the sample size of our collective is unprecedented in common literature.

5. Conclusions

After demonstrating that COX-2 and PPARγ are prognostic factors for overall and disease-free survival in vulvar cancer, research should continue on further possible pathways linking COX-2 and PPARγ. Both molecules should be perceived as potential targets in the context of vulvar cancer therapy. Further in vitro experiments as well as a transfer into prospective clinical models must be reconsidered and their urgent necessity recognized. This would be a great opportunity for a patient collective that has so far received little attention, with the prospect of less invasive, more biomolecular, and individualized therapeutic approaches that could not only ensure survival but also improve the quality of life.

Supplementary Materials: The following are available online at https://www.mdpi.com/2075-4418/11/3/491/s1, Figure S1: COX-2 in non-malignant vulvar tissue, Figure S2: PPARγ in non-malignant vulvar tissue.

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

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

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Abbreviations

COX-2	Cyclooxygenase-2
DOAJ	Directory of open access journals
EGF	Epidermal growth factor
HPV	Human papilloma virus
IRS	Immunoreactive score
LD	linear dichroism
MDPI	Multidisciplinary Digital Publishing Institute
PPARγ	Peroxisome proliferator- activating receptor Gamma
TLA	Three letter acronym
TNF-α	Tumor necrosis factor alpha
VSCC	Vulvar squamous cell carcinoma

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3.2 Publikation 2

Titel:

Regulatory T cells with additional COX-2 expression are independent negative prognosticators for vulvar cancer patients

Autoren:

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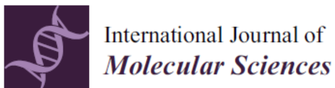
Abstract:

Vulvar cancer incidence numbers have been steadily rising over the past decades. In particular, the number of young patients with vulvar cancer has recently increased. Therefore, the need to identify new prognostic factors and, in addition, therapeutic options for vulvar carcinoma is more apparent. The aim of this study was to analyze the influx of COX-2 positive tumor-infiltrating lymphocytes and monocytes and their influence on prognosis. Using subtyping by immunofluorescence, the majority of COX-2 expressing immune cells were identified as FOXP3-positive regulatory T cells. In addition, peri- and intra-tumoral macrophages in the same tumor tissue were detected simultaneously as M2-polarized macrophages. COX-2 positive immune cells were independent negative prognostic markers in long-term overall survival of patients with vulvar cancer. These results show an influence of immune cell infiltration for vulvar carcinoma patients. Immune cell infiltration and immune checkpoint expression may, therefore, become interesting targets for further research on new vulvar cancer treatment strategies.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Article

Regulatory T Cells with Additional COX-2 Expression Are Independent Negative Prognosticators for Vulvar Cancer Patients

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Abstract: Vulvar cancer incidence numbers have been steadily rising over the past decades. In particular, the number of young patients with vulvar cancer has recently increased. Therefore, the need to identify new prognostic factors and, in addition, therapeutic options for vulvar carcinoma is more apparent. The aim of this study was to analyze the influx of COX-2 positive tumor-infiltrating lymphocytes and monocytes and their influence on prognosis. Using subtyping by immunofluorescence, the majority of COX-2 expressing immune cells were identified as FOXP3-positive regulatory T cells. In addition, peri- and intra-tumoral macrophages in the same tumor tissue were detected simultaneously as M2-polarized macrophages. COX-2 positive immune cells were independent negative prognostic markers in long-term overall survival of patients with vulvar cancer. These results show an influence of immune cell infiltration for vulvar carcinoma patients. Immune cell infiltration and immune checkpoint expression may, therefore, become interesting targets for further research on new vulvar cancer treatment strategies.

Keywords: vulvar cancer; regulatory T cells; COX-2; tumor-infiltrating lymphocytes; M2-polarized macrophages

1. Introduction

In 2020, more than 17,000 women worldwide died from vulvar cancer. The number of new cases has been steadily increasing over recent decades, with a worldwide incidence of 45,240 new cases [1]. An additional threat is the fact that a continuous increase in new cases in young women has been observed [2,3]. A total of 90% of vulvar carcinomas are squamous cell carcinomas (VSCC). Non-keratinized squamous cell carcinomas are often human papilloma virus (HPV)-associated and mainly affect younger women [4,5]. Essential for malignant transformation in HPV-dependent carcinogenesis is the inactivation of p53 and the retinoblastoma tumor-suppressor gene product by the viral gene products E6 and E7 [6]. In contrast, keratinized squamous cell carcinomas are usually HPV-independent and, due to chronic genital inflammatory disease, such as lichen sclerosus, affect older women [7,8]. Beside HPV, the development of vulvar carcinomas is associated with other risk factors: immunosuppression, smoking [9], and sexually transmitted diseases, such as herpes simplex virus 2 infections [5], are associated with an increased risk of this disease. Radical surgical

interventions are predominantly used for therapy, often ending in vulvectomy in the case of extensive involvement. To date, the mental consequences of such a serious and extensive intervention have not been studied extensively. Sexual behavior restrictions, micturition problems, or even mental effects that impair quality of life are long-term consequences of this radical form of therapy [10,11]. As for prevention, HPV vaccination, for example, has been seen as a new hope in fighting against HPV-related tumors such as cervical, anal, and vulvar cancers [12,13]. In 2016, the EURO Vaccination Meeting listed Belgium as the top country with a vaccination rate of 84%, while in Germany the vaccination rate reached a critical 31% in 2015 [14]. Based on the low vaccination rate and an aging population in Germany it can be assumed that the need for newly found prognostic factors for vulvar carcinoma is even more apparent. Our group showed negative prognosticators in vulvar cancer patients, such as LDOC1 [15] or combined expression of COX-2 and PPARγ in cytoplasm of vulvar cancer tissue [16]. An increasing number of new cases, younger patients, radical therapy, and a lack of comprehensive prevention due to the low vaccination rates, at least in this country, are concerning observations. Cyclooxygenase-2 (COX-2) features as a long-standing object of scientific interest in the context of carcinogenesis in several tumor entities [17–21]. COX-2, in contrast to the constitutive housekeeping enzyme COX-1, is inductively expressed as a known inflammatory enzyme [22,23]. Tissues of the brain, kidney, testis, and tracheal epithelium are exceptions with respect to constitutive COX-2 expression [24,25]. The inducing of the COX-2 enzyme is triggered by cell damage or inflammation by the release of various factors, such as growth factors like epidermal growth factor (EGF) [26], prostaglandins, or chemokines like TNF-γ [27]. Affecting COX-2 products, the prostanoids appear to be associated with the development and progression of tumor disease. Factors such as angiogenesis, invasion, apoptosis inhibition, growth, and aggressiveness of the tumor seem to be highly dependent on COX-2 and its products [28,29]. It is thought that products of COX-2, such as prostaglandin E2 (PGE2), critically influence the development of tumors e.g., in angiogenesis [30,31]. COX-2 is also known to be active in cancer-associated immune cells [32,33]. We used the abbreviation sTILs (stromal tumor-infiltrating lymphocytes) in our study for these cells. They have become important players in immuno-oncology with regard to predicting prognosis in cancer patients [34–37]. Already in the treatment of breast carcinomas, the presence of sTILs is taken into account in the interpretation of tumor biology and ultimately in the decision-making process of the optimal therapy according to guidelines [38]. Due to the diverse cell populations within sTILs, the subgroups are not fully understood in their prognostic role and are potentially conceivable as biomarkers in the future [39].

Types of sTILs and iTILs form a specific group of immune cells called tumor-infiltrating lymphocytes (TILs) and were assessed based on the recommendations of the International TIL Working Group (ITILWG). According to this working group, lymphocytes organized in tumor nests are defined as intratumoral TILs (iTILs). They making cell-to-cell contact without intervening stroma or interacting directly with carcinoma cells. Hence, stromal TILs (sTILs) are located in the stroma among carcinoma cells and have no direct contact with carcinoma cells [40].

In this study, we specifically investigated sTILs and no iTILs. Our study analyzed and characterized sTILs expressing COX-2 as mainly Treg cells in tissues of VSCC, their relevance as a prognostic factor, and in addition, the subtyping and polarization of infiltrating macrophages in the tumor microenvironment.

2. Results

2.1. High COX-2 Intensity of sTILs as an Independent Negative Prognostic Factor in Long-Term Overall Survival

Figure 1A,B show the sTILs with low and high intensity of COX-2. The Kaplan–Meier curve shows a significant survival disadvantage for patients whose sTILs have a COX-2 intensity > 2 in overall survival, especially in long-term survival from 60 months (Figure 1C). A total of 47% of the tumor samples have a COX-2 intensity > 2 for COX-2 in sTILs; the

remaining 41% were below this intensity value. As the Kaplan–Meier test illustrates, the 10-year overall survival rate of patients with an IRS value > 2 was 52%, but patients with a lower COX-2 intensity lived longer at 79% (Figure 1C). These data show a median survival advantage for patients with lower intensity values (≤2) compared with patients with higher intensity values (>2) at 87 months (Table 1). Multivariate analysis revealed that COX-2 expression in the sTILs of vulvar cancer patients who were alive at 60 months acted as an independent prognostic factor for overall survival (* $p = 0.007$, Table 2). However, tumor stage, nodal status, grading, p16 status and FIGO classification did not act as independent prognostic factors (Table 2).

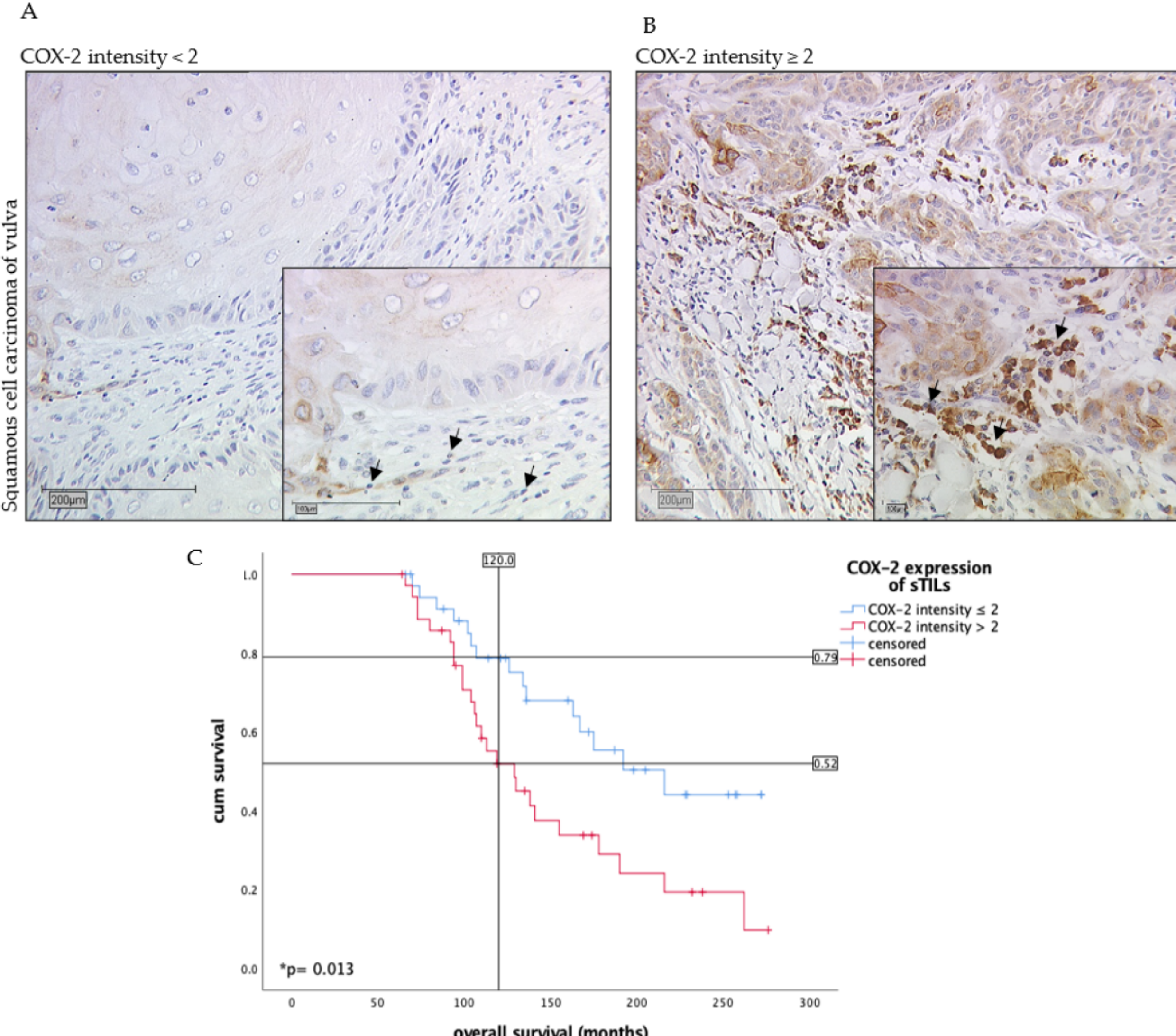

Figure 1. Immunohistochemistry staining of COX-2 (10× and 25× magnification) in vulvar cancer tissue showing expression of stromal sTILs with intensity of COX-2 expression < 2 (**A**) and ≥2 (**B**). The arrows in both figures mark exemplarily sTILs (**A**,**B**). The Kaplan–Meier curve shows a significantly worse overall survival rate for the patients with a strong COX-2 intensity of sTILs > 2 in long- term survival of 10 years (* $p = 0.013$, (**C**)). (**C**) is labeled with the percentage of patients with vulvar carcinoma still alive after 10 years: In the group of patients with COX-2 expression of sTILs ≤ 2 of vulvar carcinoma, 79% are still alive, whereas among the diseased women with higher COX-2 expression of sTILs, only 52% are still alive in comparison.

Table 1. There is a clear difference in long-term overall survival after 10 years for patients with intensity values for COX-2 expression in sTILs > 2. Patients with intensity values > 2 live with a median of 129 months, whereas patients with lower IRS values survive 216 months. As the table shows, the survival of patients of both groups differs by 87 months, i.e., more than 7 years. Even in the total group, a survival difference is recorded: patients live a total of 163 months, but still lose 34 months of life with higher intensity values.

Median for Long-Term Overall Survival Time (Months) after 10 Years			
COX-2 Intensity in sTILs	**Estimate**	**Lower 95% CI**	**Upper 95% CI**
IRS ≤ 2	216.000	37.953	290.388
IRS > 2	129.000	14.736	157.882
Overall	163.000	21.286	204.720

Table 2. Cox regression of clinical–pathological variables regarding long-term overall survival after 10 years in VSCC patients.

Variable	Significance	Hazard Ratio of Exp (B)	Lower 95% CI of Exp (B)	Upper 95% CI of Exp (B)
COX-2 intensity in sTILs	0.007	4.731	1.525	14.676
pT	0.063	6.576	0.192	2.463
pN	0.633	0.996	0.980	1.012
Grading	0.078	2.204	0.914	5.312
FIGO	0.565	0.687	0.192	2.463
p16 status	0.024	0.243	0.071	0.831

In addition, the calculations demonstrated for this patient collective that the intensity of COX-2 expression of sTILs has positive correlations with the general percentage of COX-2 expression in tumor tissue (* $p = 0.001$), the IRS score of COX-2 expression in tumor tissue (* $p = 0.014$), and the combined cytoplasmic expression of COX-2 and PPARγ (* $p = 0.011$). An analysis of total COX-2 high stroma cell expression was also performed, although without significant differences. The results are presented as Supplementary Figure S1. In addition, we also analyzed the influence of tumor COX-2 and sTILs infiltration and found that COX-2 is a negative prognosticator in cases with high stromal COX-2 intensity (Supplementary Figure S2).

2.2. Significant Majority of COX-2 Positive sTILs Are FOXP3 Positive Treg Cells

The immune cell subpopulations were quantified by counting CD56-, CD68-, and FOXP3-positive cells per field of view (20× magnification). Subtyping COX-2 expressing sTILs by immunofluorescence staining revealed parallel expression of FOXP3 in a clear majority (Figures 2 and 3).

Because the transcription factor FOXP3 is considered a specific marker of natural CD4 + CD25 + Treg cells, the FOXP3 + COX-2 + sTILs are scored as stromal regulatory T- cells. 70.2% of all counted COX-2 positive sTILs showed concomitant expression of FOXP3 and thus could be detected as Treg cells. There were also in 20.3% CD56 positive sTILs, specific marker for NK cells, and in 9.4% CD68 positive sTILs, macrophages, detected in the subtyping. However, the proportion of these two subtypes was shown to be much lower compared with the proportion of FOXP3 positive sTILs. (** $p < 0.001$, Figure 3).

2.3. M2-Polarized Macrophages Are Mainly Located on and in Tumor Tissue

In a further step, the CD68-positive macrophages were differentiated into M2-polarized and non-M2-polarized macrophages using PPARγ as specific marker. In fact, it was already reported that PPARγ plays an essential role as a nuclear receptor for the maturation of alternatively activated M2 macrophages [41] and also primes monocytes into M2 macrophages [42].

M2-polarized macrophages were found to be located peri- to intratumorally and those without M2-polarization remained in the stroma. (Figure 4A,C).

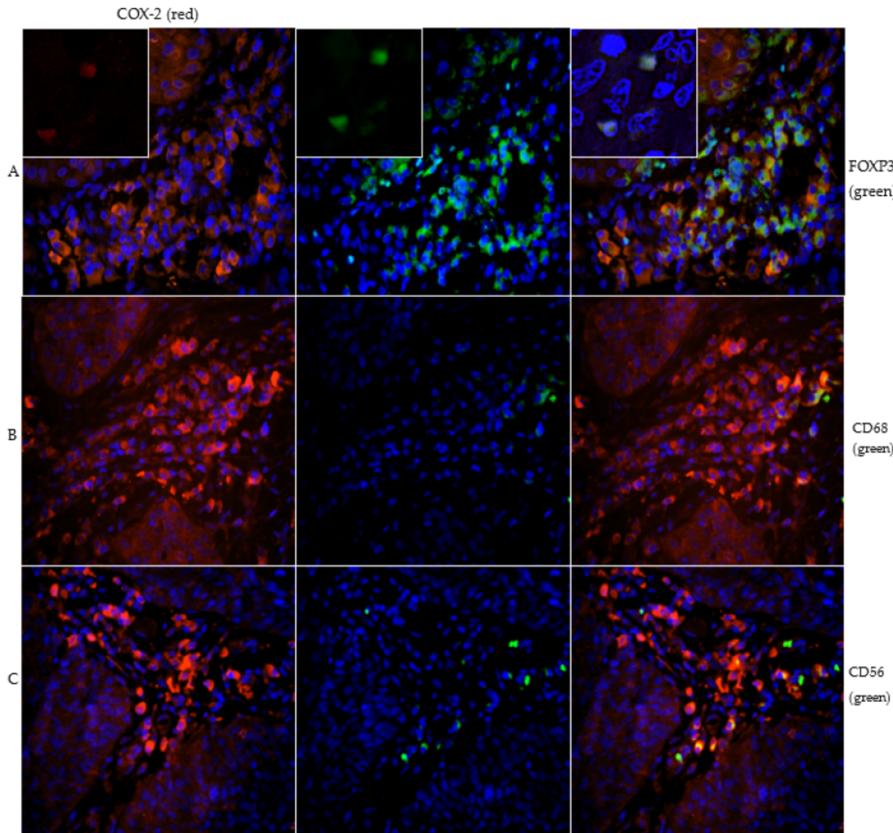

Figure 2. Image series (**A**) demonstrates the double staining of COX-2 (red) and FOXP3 (green) with inserts of magnification 40× to show nuclear staining of FOXP3. The majority of COX-2 positive immune cells peritumorally are FOXP3 positive and thus detected as Treg cells. Image series (**B**) presents the double staining of COX-2 (red) and CD68 (green). The subtyping shows that some macrophages are COX-2 positive. Figure series (**C**) shows the double staining of COX-2 (red) and CD56 (green). The CD56 positive NK cells are weakly pronounced and only singly distributed in the stroma.

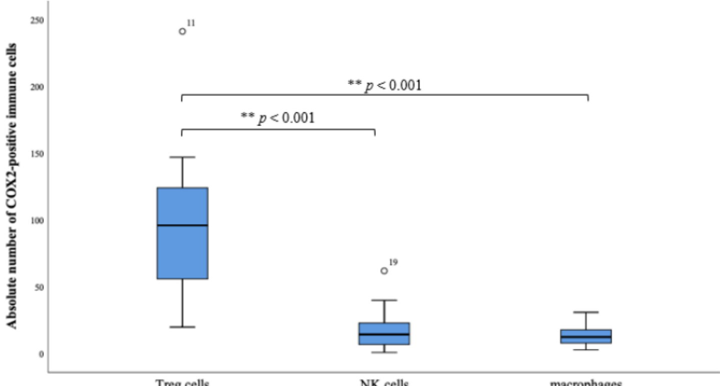

Figure 3. The boxplots reveal the absolute number of COX-2 positive Treg cells (FOXP3+), NK cells (CD56+) and macrophages (CD68+). There is a clear distribution in the direction of the Treg cells. The box plots indicate mild outliers, which are marked with circles. These outliers (case numbers 11 and 19) show an interquartile distance to the third quartile of values that is less than three times higher than the third quartile of values. The difference of the occurrence of the subtypes Treg cells to NK cells, as well as Treg cells to macrophages, is highly significant (** $p < 0.001$).

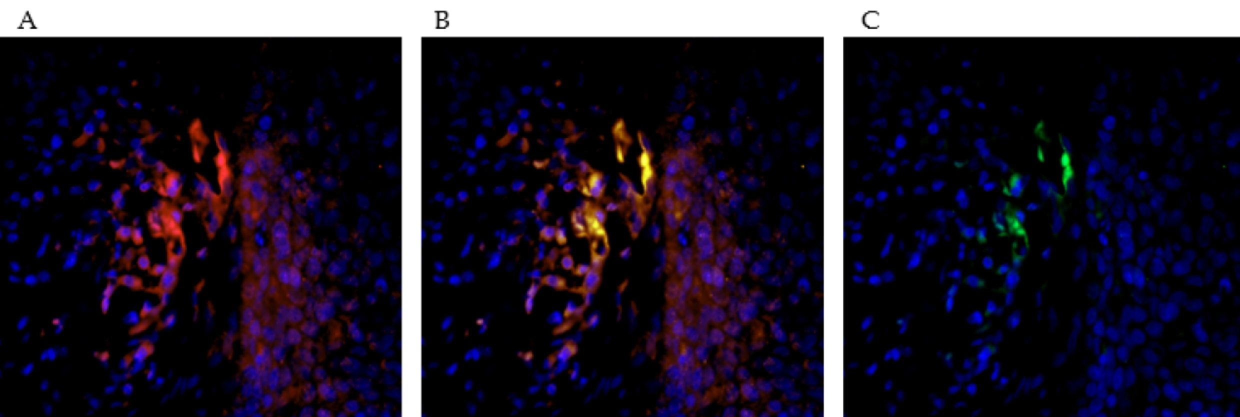

Figure 4. The picture series (**A**–**C**) demonstrate the double staining with PPARγ (red) and CD68 (green). This allows a subtyping of CD68-positive macrophages into M2-polarized and M2-unpolarized macrophages. PPARγ acts as a marker for the M2-polarization of the macrophages. In the selected tissue sections, it was found that more doubly stained, M2-polarized macrophages are resident in the immediate environment and within the tumor association than in the extratumoral stroma.

3. Discussion

Our study observed COX-2 positive Treg cells to be an independent negative prognosticator in long-term overall survival for vulvar cancer patients (Table 2); also detected in this context are M2-polarized macrophages, which have been previously described as negative influencing factors in several tumor disease [43–45].

Regulatory T cells, also known as Treg cells, are also increasingly becoming the focus of research in the field of tumor immunology. As a subset of immunosuppressive T cells, they account for approximately 4–8% of CD4+ T cells in peripheral blood and are characterized by the transcription factor FOXP3 as a specific intracellular marker. Sakaguchi et al. discovered the function of Treg cells as a key role in human immune self-tolerance: a depletion of this cell population in mice showed an induction of autoimmune disease due to their inhibitory effect on CD8+ cytotoxic T cells. [46,47] As already demonstrated in several tumor entities, the expression of Treg cells is found to be a determinant of survival and prognosis of affected patients—but still without definitive knowledge of the pathways in which Treg cells are involved.

Immunological processes in the context of tumorgenesis and maintenance of tumor growth rely on enhancing effect as tumor promotion and on inhibitory effects as tumor suppression. For M2-polarized macrophages and Treg cells in the tumor environment, a tumor-promoting effect was reported [48].

Other studies observed that population of Treg cells in sTILs is significantly higher in carcinoma tissue than in normal healthy tissue [49,50]. Our study shows a clear majority of FOXP3-positive Treg cells with 70.2% (Figures 2 and 3) within the sTILs, so a negative impact on long-term survival in patients with VSCC is assumed. A significant impact of immunologic processes on long-term survival is already known and has been described before [51], but for ovarian cancer Yuan et al. [52] showed that an increased number of Treg cells occur in the tumor microenvironment in patients with gastric carcinoma. There is also an elevated expression of FOXP3 in tumor-infiltrating Treg cells which correlates with up-regulation of COX-2 and its product PGE2 [52]. The COX-2 positivity of the Treg cells can be explained in terms of the COX-2/PGE2 pathway (Figure 5). As Baratelli et al. have established, there is an upregulation of Treg cells by PGE2, an important product of COX-2 [53]. COX-2 as a significant negative prognostic factor on overall survival of the studied patient population has already been demonstrated in our previous study [16].

Studies by Rothwell et al. using COX-inhibitors showed impressive results in terms of improving the prognosis and reducing the incidence of colorectal cancer [54].

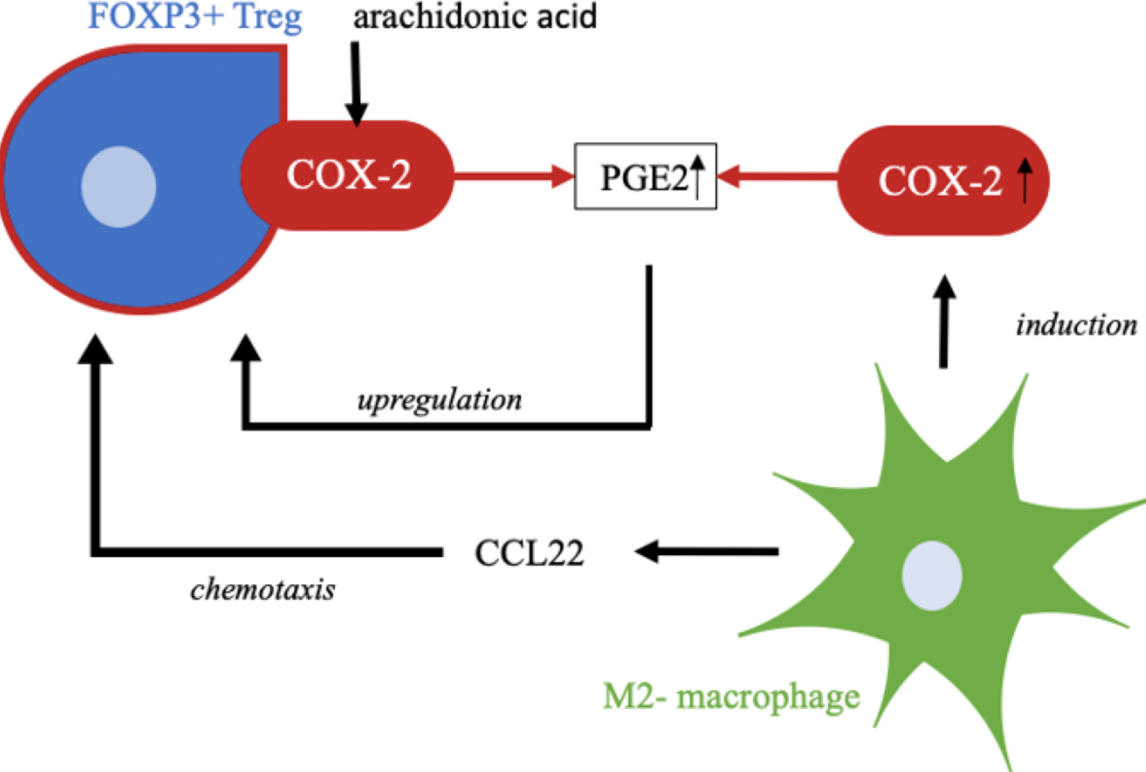

Figure 5. COX-2 produces PGE2, a prostaglandin, from the substrate arachidonic acid as part of lipid metabolism. Upregulation of Treg cells is affected by this prostaglandin. M2-polarized macrophages attract regulatory T cells by chemotaxis, e.g., by CCL22. In addition, M2-polarzied macrophages induce expression of COX-2, which in turn leads to higher enzyme activity and thus higher accumulation of products, such as PGE2 [27,28,48,54,55].

Besides Treg cells, we also identified tumor infiltrating macrophages with COX-2 expression. Macrophages are an important component of the innate immune system and are involved in many immunological processes [55]. In cancer research, these immune cells are gaining increasing attention. Tumor-associated macrophages, so-called TAMs, resemble macrophages in regenerating and growing tissue [56]. The different polarization of macrophages, and thereby functional distinction into M1-polarized, tumor-inhibiting macrophages, and M2-polarized, tumor-promoting macrophages, shows the controversial role of this cell group in immunology. Subtyping is indicated by different stimuli. M2-polarization is indicated by Th2 cytokines such as IL-4 and IL-13 and leads to macrophage functionalization in the context of anti-inflammatory processes, tissue repair, and immunoregulatory and tumor-promoting processes [57]. Martinez et al. showed that M2-polarized macrophages exert a major influence on lipid metabolism; this includes induction of COX-2 activity and thus increased production of its enzyme products, such as prostaglandins [58]. For cervical cancer, our group could identify TAMs as a major source of CCL22, a chemokine responsible for Treg cell infiltration [59] (Figure 5).

One of the limitations of our study is the retrospective design. Only a highly limited number of patients included in this collective was alive during the examination of the tissue sections so that there would have been the possibility of applying a scan for cells within serum or primary cells. The evaluation of the tissue sections was not performed by an objectifiable measurement tool; but tissue sections were objectified by two independent

investigators who evaluated the expression pattern blinded. There was no possibility to overview the process of the embedment of patient tissue, which leads to the fact that in the analyzed collective only tumorous tissue is accessible.

Double staining of FOXP3 and COX-2 in the immunofluorescence revealed evidence of nuclear expression of FOXP3, but also evidence of partially cytoplasmic expression of the transcription factor FOXP3. In the Human Protein Atlas, the reference images also show partial cytoplasmic expression, which is described as expression in the nucleoplasm [60].

The data of the collective do not contain information about the medication status of the patients at the time of tissue sampling; therefore, no conclusion can be drawn about a possible intake of a COX- inhibitor. However, it is unlikely that medication alters the expression pattern because drugs only affect activity and not expression. In addition, the serological test results of the patients are not available and subsequent blood diagnostics are not possible.

Comparable studies with such a high number of examined tissue sections from vulvar cancer patients do not currently exist. Only Sznurkowski et al. reported the results of their study of the influence of Treg cells in vulvar carcinoma; using a patient collective of 110 patients, they observed that Treg cells had no effect on overall survival [61].

In addition, a strong prognostic factor for survival of vulvar carcinoma patients is the nodal status [62]. In our study, Cox regression did not identify the nodal status as an independent prognostic factor. This can be explained by a high number of nodal-negative patients (42.6%), as well as an equally high number of unknown lymph node status (29.8%). The missing data mainly concerned patients whose date of diagnosis was decades ago. Because of this time gap between diagnosis and the analysis for this study, there were missing values in some cases.

Due to the rarity of this cancer, especially compared with other cancers such as breast cancer, the sample size of our collective in the current literature is a very large one. The increasing rate of new cases again highlights the greater relevance of the need for new knowledge regarding prognostic factors and linkage to tumor immunology in a world where the role of immunotherapy is rapidly gaining importance.

4. Materials and Methods

A total of 177 patients with VSCC primarily diagnosed in the period from 1990 to 2008 were included in this study. The entire patient group was treated at the department of Obstetrics and Gynecology of the Ludwig-Maximilians-University in Munich, Germany. Surgically obtained tissue samples were histopathologically processed and specified. All follow-up and survival data were provided by the Munich Cancer Registry (MCR) from the Munich Tumour Centre (TZM—Munich Tumour Centre, Munich, Germany).

For immunohistochemical staining, 157 of the 177 samples were available. During the evaluation, a further 16 tissue samples were excluded, as the incisions did not contain a tumor, but only precancerous stages of the carcinoma. Therefore, in the end a collective of 141 slides of VSCC was assessed, one slide per staining and case.

The median age of the investigated collective was 70 years, ranging from 20 to 96 years, with 72 of the 141 patients younger than 70 years (=51.8% of the collective). All relevant clinic–pathologic parameters are listed in Table 3 below. The collective is the same as described by Ansorge et al. in previous studies by our research group [16].

4.1. Ethical Approval

All patients data were completely anonymized and the study was performed according to the standards set in the Declaration of Helsinki 1975. The examined tissues were residual material that had been collected in the first instance for histopathological diagnostic procedures. The actual study was approved in writing by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 367-16). The authors were blinded for clinical information during the experimental analysis.

Table 3. Clinicopathological parameters of VSCC patients' collective.

Clinicopathologic Parameters	*n*	Percentage (%)
Histology		
keratinizing	134	95
warty/basaloid	7	5
Tumor size		
T1	51	36.2
T2	74	52.5
T3	9	6.4
unknown	7	5
Nodal status		
N0	60	42.6
N1	31	22
N2	8	5.7
unknown	42	29.8
FIGO		
I	45	31.9
II	45	31.9
III	36	25.5
IV	9	6.4
unknown	6	4.3
Grading		
G1	24	17
G2	87	61.7
G3	29	20.6
NOS/unknown	1	0.7
p16 status		
positive	34	24.1
negative	57	40.4
unknown	50	35.5
COX-2 expression of sTILs		
Positive	136	96.5
negative	4	2.8
unknown	1	0.7
Progression status		
positive	61	43.3
negative	79	56
unknown	1	0.7
Local recurrence status		
positive	35	24.8
negative	105	74.5
unknown	1	0.7

4.2. Immunohistochemistry

The already formalin-fixed and paraffin-embedded samples were then cut by microtome to 4µm from the paraffin block and mounted on SuperFrost Plus microscope slides (Menzel Glaeser, Braunschweig, Germany). To deparaffinize the tissue, samples were processed with xylol for 20 min and washed by 100% ethanol. All slides were prepared with 3% hydrogen peroxide diluted in methanol for 20 min to stop activity of endogenous peroxidase. Afterwards, rehydration took place in a descending alcohol series (100%, 70%, 50%) and the samples were washed with distilled water. The samples were then heated with citric acid buffer in a pressure cooker to uncover antigen epitopes. Furthermore, slides were washed two times with phosphate buffered saline (PBS). A Zytochem-Plus HRP Polymer-kit (Zytomed, Berlin, Germany) was utilized for blocking and antibody staining. After saturating the electrostatic charges in the tissue with blocking solution for 5 min, either

the polyclonal rabbit IgG anti- COX-2 antibody (Sigma, St. Louis, MI, USA, SAB4502491) or the polyclonal rabbit IgG anti-PPARγ antibody (abcam, Cambridge, United Kingdom, ab59256) was applied on tissue specimens. Anti-COX-2- antibody was diluted at a ratio of 1:400 and anti-PPARγ antibody at a ratio of 1:100. The incubation time of both antibodies amounted to 16 h at 4 °C in a humidity chamber. Slides were incubated by post-block reagent for 20 min and thereafter by HRP-Polymer for 30 min at room temperature in the humidity chamber. After each application with the antibody, post-block, and HRP-Polymer, the samples were washed two times with PBS. 3,3′-Diaminobenzidine (Dako, Hamburg, Germany) catalyzed the peroxidase substrate staining so that the color precipitation was detectable with a light microscope. Finally, the slides were counterstained with hemalum, washed again using 100% ethanol, and covered with glass. Both antibodies were stained in placenta tissue as a positive control to validate the staining method. The staining was considered positive in the case of cytoplasmic positivity of COX-2, and in the case of cytoplasmic and nuclear positivity of PPARγ. The semi quantitative immunoreactive score (IRS) by Remmele and Stegner [63] was used to evaluate immunostaining, together with a light microscope (Leitz, Wetzlar, Germany). For this purpose, a product of two factors, the intensity and the proportion of staining in the tumor tissue, was formed. The intensity was classified into 0 = no, 1 = weak, 2 = moderate, 3 = strong; the proportion of tumor tissue was also categorized: 0 = no staining, 1 $\leq$ 10%, 2 = 11% to 50%, 3 = 51% to 80%, 4 $\geq$ 81%. The antibodies showed expressions in cytoplasm and in nucleus, so both expression templates were examined independently by IRS. Patient data were correlated by IRS and by its two IRS-forming factors of staining intensity and percentage of positively stained cells. Neither HPV testing nor an immunohistochemical survey of p16 status was performed as part of this study; information on this was obtained from archives.

4.3. Immunofluorescence

The same formalin-fixied and paraffin-embedded samples were placed in xylol for 20 min for deparaffinization. Subsequently, the sections were panned in ethanol in order of descending concentrations (100%, 70%, 50%) and washed in distilled aqua. Unmasking of antigens was performed simultaneously with the immunohistochemistry protocol by a 5 min heat pretreatment in a pressure cooker in the citrate buffer-use solution described previously. After washing in distilled water and PBS for 4 min, incubation with UltraV block solution (Labvision, Fremont, CA, USA) was performed. The solution was tipped off after 15 min and the primary antibodies were applied. COX-2 was stained at a ratio of 1:400 together with CD56 (Bio-Rad, Oxford, United Kingdom, MCA591) at a ratio of 1:100, CD68 (Sigma, St. Louis, MO, USA, AMAb90874) at a ratio of 1:8000, or FOXP3 (Abcam, Cambridge, United Kingdom, ab20034) at a ratio of 1:300 in dilution medium (Dako, Glostrup, Denmark, S3022) to differentiate cells in a double staining procedure. CD56 is a known structural protein of natural killer (NK) cells [64], CD68 is known as a structural protein of macrophages, and FOXP3 is expressed specifically by regulatory T (Treg) cells [65–67]. Double staining with PPARγ and CD68 was performed to subtype macrophages [68]. Here, a concentration of 1:100 was chosen for the PPARγ antibody (Abcam, Cambridge, United Kingdom, ab27649) and CD68 was added, as in the double staining with COX-2.

Incubation was performed for 16 h at 4 °C. After washing in PBS, the experimental room was darkened and the mixed secondary antibodies were applied: Cy-2- conjugated antibody at a ratio of 1:100, which later fluoresced green (Dianova, Hamburg, Germany, 115-546-062) or Cy-3- conjugated antibody at a ratio of 1:500, which fluoresced red (Dianova, Hamburg, Germany, 111-225-144). After 30 min of incubation, the excess secondary antibodies were washed off in PBS. In the dark, the preparations dried at room temperature and were cover slipped with Mounting medium for fluorescence with DAPI, which stains the cell nuclei as a blue light impression.

During the performance of immunofluorescence double staining, the primary antibodies CD56, CD68 and FOXP3 appear green in the fluorescence microscope and COX-2

is perceived as red fluorescence. The double staining was evaluated and assessed using a fluorescence microscope (Zeiss, Oberkochen, Germany). A total of 18 tumor microenvironments were examined in a sampling from the previously described patient population using this described method, and based on this, subtyping of stromal tumor-infiltrating lymphocytes was performed.

4.4. Statistical Analysis

For statistical analysis, SPSS Statistic version 25 (IBM Corp., Armonk, NY, USA) was used. The non-parametric Kruskal–Wallis test was used to compare between and among groups. Correlation analyses were performed using the Spearman rank correlation coefficient. Kaplan–Meier curves were generated from the collected survival data of patients with vulvar carcinoma. The differences between these curves of sTILs with high and with low COX-2 expression were tested with the log-rank test. The identification of these sTILs was analyzed by specific markers of Treg cells, macrophages and NK cells. Cox regression models were applied for multivariate analysis. Patient-specific pairwise analysis of significance differences in immune cell subtypes of Treg cells, NK cells, and macrophages was performed using the Wilcoxon test. The level of statistical significance was accepted at $p \leq 0.05$ and all tests were two-sided.

5. Conclusions

In this study, COX-2 positive sTILs were observed to be an independent prognostic factor in long-term overall survival in the patient population studied. Furthermore, subtyping by immunofluorescence revealed that most sTILs are Treg cells. This is the first time that COX-2 positive Treg cells have been observed to have a significant impact in the context of long-term survival in patients with vulvar cancer. This finding might help on the way towards individualized immunotherapy and could eventually lead to further investigations of new immune checkpoints. Further research goals are in vitro experiments to reliably demonstrate a direct causal relationship between COX-2 and Treg cells and the planning of a prospective study model using COX-2 inhibition to add an important further approach to the limited therapy options and prognosis of vulvar cancer.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23094662/s1.

Author Contributions: Conceptualization, N.A., U.J. and S.F.; methodology and experiments, H.H.H. and A.V.; software and visualization, C.D. and U.J.; data analyses, S.F., U.J. and N.A.; writing—original draft preparation, N.A. and S.F.; data interpretation, H.H.H., A.V., E.S., M.B., B.C., S.M. and U.J.; writing—Review and Editing, H.H.H., A.V., M.B., B.C., S.M. and U.J.; supervision, S.M., U.J. and S.F.; project administration, U.J.; funding acquisition, S.M., U.J. and S.F. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Ludwig-Maximilians-Universität, Munich, Germany (Approval number 367-16, 29 December 2016).

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

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

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Abbreviations

COX-2	Cyclooxygenase-2
EGF	Epidermal growth factor
FOXP3	forkhead-box-protein P3
HPV	Human papilloma virus
IRS	Immunoreactive score
ITILWG	International TIL Working Group
MDPI	Multidisciplinary Digital Publishing Institute
NK cells	natural killer cells
PPARγ	Peroxisome proliferator-activating receptor Gamma
iTILs	intratumoral tumor infiltrating lymphocytes
sTILs	stromal tumor infiltrating lymphocytes
TAMs	Tumor-associated macrophages
TLA	Three letter acronyme
Treg cells	Regulatory T cells
TNF-γ	Tumor necrosis factor Gamma
VSCC	Vulvar squamous cell carcinoma

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4. Zusammenfassung

Immer mehr und zunehmend jüngere Frauen leiden an einem Vulvakarzinom. Die Inzidenzen steigen weiter an. Prognostische Faktoren für diese Tumorerkrankung existieren bislang nicht.

In unserer Studie wurde untersucht, welche Bedeutung das Enzym COX-2 und der Transkriptionsfaktor PPARγ hinsichtlich der Prognose der Patientinnen mit Vulvakarzinom haben und welche prognostische Rolle den regulatorischen T-Zellen, die COX-2 exprimieren, zuteil wird.

Ein Patienteninnenkollektiv von 157 Frauen, die zwischen 1990 und 2008 an einem Vulvakarzinom erkrankten und an der Frauenklinik der LMU München behandelt wurden, wurde mittels immunhistochemischer Färbungen und anhand Subtypisierung der TILs analysiert und vorwiegend auf die Einflussnahme auf das Überleben der Betroffenen untersucht.

Wir konnten zeigen, dass sowohl die Expression von COX-2 als auch von PPARγ einen signifikanten Effekt auf das Gesamt- bzw. krankheitsfreie Überleben hat, die Kombination beider Faktoren kann als unabhängiger Prognosefaktor für das Gesamtüberleben (*$p<0.001$, Publikation 1) und das krankheitsfreie Überleben (*$p=0.006$, Publikation 1) herangezogen werden.

Im Rahmen dieser immunhistochemischen Untersuchungen zeigte sich außerdem eine Expression der COX-2 in den umliegenden sTILs, die ein weiterer unabhängiger Prognosefaktor im Langzeitüberleben der Patientinnen darstellt (*$p=0.007$, Publikation 2). Zur spezifischen Detektion jener Immunzellen erfolgte die Subtypisierung anhand Immunfluoreszenz-Färbungen. Diese ergab den überwiegenden Nachweis von Tregs, aber auch NK-Zellen und Monozyten. Hierunter fanden sich ebenfalls Makrophagen mit COX-2-Expression. Anhand weiterer Spezifizierung konnten diese den M2- polarisierten Makrophagen zugeordnet werden.

Wie stehen diese Erkenntnisse zusammen?

Mögliche Verknüpfungspunkte sind die Produkte der COX-2, die sog. Prostaglandine, z.B. PGE2. Diese beeinflussen nicht nur die Prozesse der Inflammation, sondern auch entscheidende Prozesse der Angiogenese und Immunantwort im Rahmen der Karzinogenese. Hier entsteht die Verbindung zu Immunzellen wie den Tregs oder den Makrophagen. Das Prostaglandin PGJ2, ein Produkt der COX-2, ist als natürlicher Agonist für PPARγ bekannt und reiht sich in die Zahl vieler bekannter, aber auch unbekannter Zusammenhänge ein.

Das Feld der Tumorimmunologie und das Verständnis der Verknüpfungen einzelner Reaktionswege ist mittlerweile in der spezifischen Immuntherapie nicht wegzudenken. Patientinnen mit Karzinomen wie dem malignen Melanom, aber auch dem Mammakarzinom, profitieren bereits durch dein therapeutischen Einsatz jener Erkenntnisse an Lebensqualität und an Lebenszeit.

Daher ist die Notwendigkeit einer Studie, die prognostische Einflussfaktoren einer Erkrankung untersucht, deren bisherige Therapien teils zu massiven Einschränkungen der Lebensqualität führt, ein großer Zugewinn zum aktuellen Forschungsstand und kann so der Impulsgeber werden; weg von der radikal chirurgischen Therapie hin zur individuellen, zielgerichteten Therapielösung.

5. Summary

The incidence of vulvar cancer is increasing and the number of women, especially of younger women, suffering from this disease is constantly rising. Prognostic factors for this tumor disease do not exist yet.

In our study, we investigated the influence of the enzyme COX-2 and the transcription factor PPARγ on the prognosis of patients with vulvar carcinoma as well as the role of COX-2 expressing regulatory T cells in this context.

A patient collective of 157 women who developed vulvar carcinoma between 1990 and 2008 and were treated at the Women's Hospital of the LMU Munich was investigated by immunohistochemical staining and by subtyping of TILs and predominantly examined for their impact on survival.

We demonstrated that both COX-2 and PPARγ have a significant effect on overall and disease-free survival, whereas the combination of both factors may be considered an independent prognostic factor for overall survival (*p<0.001, publication 1) and disease-free survival (*p=0.006, publication 1).

These immunohistochemical studies also identified the expression of COX-2 in surrounding sTILs, which is another independent prognostic factor in long-term patient survival (*p=0.007, publication 2). For specific detection of those immune cells, subtyping was performed using immunofluorescence staining. This resulted in the predominant detection of Tregs, but also NK cells and monocytes. Here, macrophages with COX-2 expression were also found. Based on further specification, these could be classified as M2-polarized macrophages.

But how do these findings interact?

Possible points of connection are the products of COX-2, the prostaglandins, e.g., PGE2. These influence not only the processes of inflammation, but also crucial processes of angiogenesis and immune response in the context of carcinogenesis. This is where the link to immune cells such as Tregs or macrophages arises. The prostaglandin PGJ2, a product of COX-2, is known as a natural agonist for PPARγ and joins the number of many known but also unknown connections.

The field of tumor immunology and understanding the linkages of individual pathways has become indispensable in specific immunotherapy. Patients with carcinomas such as malignant melanoma and breast cancer are already benefiting from the therapeutic application of these findings in terms of quality of life and longevity.

Therefore, the need for a study that investigates prognostic factors of a disease, whose previous therapies sometimes lead to massive restrictions in the quality of life, is a great gain to the current state of research and can thus become a major impulse; away from radical surgical therapy towards an individual immunotherapeutic solution.

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