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Laboratory markers associated with endothelial injury in prediction of severity of acute pancreatitis in the early phase of the disease

Wskaźniki laboratoryjne związane z uszkodzeniem śródbłonka w przewidywaniu ciężkości przebiegu ostrego zapalenia trzustki we wczesnej fazie choroby

Praca doktorska

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powstała dzięki zaangażowaniu całego zespołu badawczego.

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Spis treści

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Wstęp

Ostre zapalenie trzustki jest najczęstszą przyczyną hospitalizacji z powodu ostrych schorzeń przewodu pokarmowego: zachorowalność wynosi 10-100 przypadków / 100 000 / rok i w ostatnich dekadach odnotowano jej wzrost [1,2]. Jest to choroba zapalna, charakteryzująca się zróżnicowanym przebiegiem klinicznym, od postaci łagodnej, ustępującej samoistnie, do ciężkiej, obarczonej znaczną śmiertelnością.

Obecnie obowiązująca zmodyfikowana klasyfikacja Atlanta z 2012 roku definiuje łagodne, średnio-ciężkie (umiarkowane) oraz ciężkie ostre zapalenie trzustki [3]. Łagodne ostre zapalenie trzustki (*mild acute pancreatitis,* MAP) rozpoznaje się u pacjentów bez niewydolności narządowej, powikłań ogólnoustrojowych i miejscowych. Wystąpienie przemijającej niewydolności narządowej (ustępującej przed upływem 48 godzin), powikłań miejscowych (ostry okołotrzustkowy zbiornik płynowy, torbiel rzekoma, ostry zbiornik martwiczy, odizolowana martwica) lub ogólnoustrojowych (zaostrzenie współwystępujących chorób przewlekłych) jest podstawą rozpoznania średnio-ciężkiego ostrego zapalenia trzustki (*moderately severe acute pancreatitis,* MSAP). Przetrwała niewydolność narządowa (utrzymująca się ponad 48 godzin) jest kryterium rozpoznania ciężkiego ostrego zapalenia trzustki (*severe acute pancreatitis,* SAP) [3]. W tej postaci choroby śmiertelność może sięgać 50% [3]. Rozwój niewydolności narządowej i wielonarządowej uznano za główny wyznacznik ciężkości choroby. Niewydolność narządowa rozwijająca się w wyniku uogólnionego stanu zapalnego jest też główną przyczyną zgonów we wczesnej fazie ostrego zapalenia trzustki [3]. Wśród chorych zmarłych z powodu ostrego zapalenia trzustki najczęściej diagnozowano ostrą niewydolność oddechową, niewydolność sercowo-naczyniową (wstrząs), ostrą niewydolność nerek, niewydolność wątroby, jelit oraz zaburzenia układu hemostazy (klinicznie manifestujące się zarówno krwawieniami jak i zakrzepicą) [4].

Definicje MAP, MSAP i SAP przedstawione w klasyfikacji Atlanta z 2012 roku można w pełni zastosować *post hoc*, biorąc pod uwagę cały przebieg ostrego zapalenia trzustki. Jednak w opiece nad pacjentami kluczowe jest jak najwcześniejsze rozpoznanie ciężkiej postaci choroby. Pacjenci ze spodziewanym ciężkim przebiegiem ostrego zapalenia trzustki powinni być możliwie szybko objęci intensywnym nadzorem medycznym [3,5]. Obecnie w praktyce klinicznej kluczową rolę pełni częsta, regularna kontrola stanu pacjenta, zwłaszcza pod kątem rozwijającej się niewydolności narządowej [3,5], jednak badania wskazują, że sama ocena kliniczna nie zapewnia zadowalającej czułości diagnostycznej we wczesnym rozpoznaniu SAP [6]. Ocenę tę mogą wspomóc badania laboratoryjne: najszerzej wykorzystuje się oznaczenia stężenia białka C-reaktywnego (CRP) w surowicy, jednak największą dokładność diagnostyczną test ten osiąga dopiero po 48 godzinach od wystąpienia objawów choroby (lub nawet od przyjęcia pacjenta do szpitala) [6]. W ocenie pacjentów stosuje się także skale prognostyczne, uwzględniające zarówno parametry kliniczne, jak i laboratoryjne, m.in. skalę APACHE II (*Acute Physiology and Chronic Health Evaluation II*), skalę Glasgow oraz nowszą skalę BISAP (*Bedside Index of Severity in Acute Pancreatitis*).

Poszukiwanie wczesnych wskaźników prognostycznych ciężkiej postaci ostrego zapalenia trzustki jest od lat przedmiotem badań naukowych. Obiecujące wyniki uzyskiwano dla innych niż CRP białek ostrej fazy (surowiczy amyloid A, pentraksyna 3), prokalcytoniny oraz cytokin zapalnych (zwłaszcza

interleukiny-6) [6]. Obserwowano również związki między ciężkością ostrego zapalenia trzustki a stężeniami markerów aktywacji i dysfunkcji śródbłonka, m.in. E-selektyny i innych cząstek adhezyjnych, trombomoduliny, a nawet cząsteczek mikro RNA pochodzenia śródbłonkowego [7–9]. Ponadto wśród wczesnych wskaźników prognostycznych SAP należy wymienić laboratoryjne wskaźniki aktywacji układu krzepnięcia, w tym wysokie stężenia D-dimerów, kompleksów trombina-antytrombina i niską aktywność antytrombiny [10]. Wspólnym mianownikiem dla wymienionych markerów prognostycznych SAP wydaje się uogólniony proces zapalny prowadzący do aktywacji i uszkodzenia komórek śródbłonka, zwiększonej przepuszczalności naczyń i utraty antykoagulacyjnych właściwości śródbłonka. W ostatnich latach w kontekście zespołu zwiększonej przepuszczalności naczyń w ostrych stanach badano również białka związane z angiogenezą, w tym angiopoetynę-2 oraz fms-podobną kinazę tyrozynową-1 (*fms-like tyrosine kinase 1*, Flt-1).

Angiopoetyna-2 należy do angiogennych czynników wzrostu. Białko to jest wytwarzane przez komórki śródbłonka i przechowywane w ciałkach Weibel-Palade'a, skąd może być uwalniane w wyniku aktywacji lub uszkodzenia śródbłonka, m.in. pod wpływem mediatorów zapalnych [11]. Według większości autorów angiopoetyna-2 hamuje wiązanie angiopoetyny-1 z receptorem Tie-2 na komórkach śródbłonka, co prowadzi do wzrostu przepuszczalności naczyń i nasilenia stanu zapalnego [11,12]. Wysokie stężenia angiopoetyny-2 w osoczu i surowicy korelowały z wystąpieniem niewydolności wielonarządowej i śmiertelnością u pacjentów z sepsą [12], a także z wystąpieniem ostrego uszkodzenia nerek u pacjentów wymagających intensywnej terapii z różnych przyczyn [13]. We wczesnej fazie ostrego zapalenia trzustki obserwowano dodatnie korelacje między stężeniem angiopoetyny-2 w surowicy i osoczu pacjentów a ciężkością choroby i rozwojem niewydolności wielonarządowej [14,15].

Flt-1 jest receptorem dla czynnika wzrostu śródbłonka naczyniowego (*vascular endothelial growth factor,* VEGF) oraz dla łożyskowego czynnika wzrostu (*placental growth factor*, PlGF). Rozpuszczalna forma receptora, sFlt-1, powstająca w wyniku alternatywnego składania mRNA dla Flt-1, wiąże VEGF i PlGF we krwi [16]. U pacjentów z sepsą obserwowano wysokie stężenia zarówno VEGF, jak i sFlt-1. Stężenia sFlt-1 w osoczu dodatnio korelowały z ciężkością sepsy [17–19]. U pacjentów z ostrym zapaleniem trzustki również obserwowano podwyższone stężenia VEGF w osoczu [20,21]. Wykazano, że VEGF zwiększa przepuszczalność śródbłonka [22], jednak w ostrym zapaleniu trzustki postulowano ochronną rolę VEGF w odniesieniu do niewydolności narządowej [20,23]. Stężenia sFlt-1 nie były intensywnie badane w ostrym zapaleniu trzustki. Espinosa i wsp. [24] w 2013 roku oznaczali sFlt-1 w niewielkiej grupie chorych z ostrym zapaleniem trzustki, jednak nie wykazali związku osoczowych stężeń sFlt-1 z ciężkością choroby. W cytowanym badaniu wykorzystano test immunoenzymatyczny wykonywany na płytce 96-dołkowej, obecnie jednak dysponujemy zautomatyzowaną metodą, wystandaryzowaną na potrzeby diagnostyki preeklampsji [25], umożliwiającą szybkie wykonanie oznaczeń w rutynowych laboratoriach medycznych.

Cele pracy

Przeprowadzone badania miały na celu ocenę:

- stężeń nowych markerów dysfunkcji śródbłonka: angiopoetyny-2 i rozpuszczalnej formy fms-podobnej kinazy tyrozynowej-1 (sFlt-1) u chorych we wczesnej fazie ostrego zapalenia trzustki (w ciągu pierwszych 72 godzin od wystąpienia objawów choroby);
- związku pomiędzy stężeniami angiopoetyny-2 i sFlt-1 a rozwojem powikłań narządowych we wczesnej fazie ostrego zapalenia trzustki, w szczególności rozwojem ostrego uszkodzenia nerek;
- związku między stężeniami angiopoetyny-2 i sFlt-1 a rozwojem zaburzeń krzepnięcia we wczesnej fazie ostrego zapalenia trzustki;
- związku pomiędzy stężeniami angiopoetyny-2 i sFlt-1 a dotychczas stosowanymi laboratoryjnymi markerami ciężkości ostrego zapalenia trzustki (stężenie białka C-reaktywnego, stężenie D-dimerów), skalą BISAP oraz klinicznymi wykładnikami ciężkiego przebiegu ostrego zapalenia trzustki;
- użyteczności diagnostycznej stężeń angiopoetyny-2 i sFlt-1 w prognozowaniu ciężkości ostrego zapalenia trzustki ocenianej zgodnie ze zmodyfikowaną klasyfikacją Atlanta z 2012 roku.

Metody

Pacjenci

Do badania włączono pacjentów z rozpoznaniem ostrego zapalenia trzustki, przyjętych w ciągu 24 godzin od wystąpienia objawów choroby, leczonych w Oddziale Chirurgicznym Szpitala Powiatowego w Suchej Beskidzkiej.

Ostre zapalenie trzustki diagnozowano w oparciu o zmodyfikowane kryteria Atlanta z 2012 roku [3], w przypadku spełnienia dwóch z trzech poniższych kryteriów:

- wystąpienia ostrego, utrzymującego się bólu w nadbrzuszu;
- podwyższonej aktywności lipazy lub amylazy w surowicy przekraczającej trzykrotność górnej granicy przedziału referencyjnego;
- charakterystycznych dla ostrego zapalenia trzustki zmian obserwowanych w badaniach obrazowych (tomografii komputerowej z kontrastem, tomografii rezonansu magnetycznego lub badaniu ultrasonograficznym jamy brzusznej).

Kryteria wyłączenia z badania obejmowały:

- brak świadomej zgody na udział w badaniu, potwierdzonej podpisem pacjenta,
- rozpoznanie następujących chorób: przewlekłe zapalenie trzustki, aktywny nowotwór złośliwy, przewlekłe choroby wątroby (marskość, wirusowe zapalenie wątroby).

Dodatkowo w przypadku prac, w których oceniano funkcję nerek oraz oznaczano stężenie lipokaliny związanej z żelatynazą neutrofili (*neutrophil gelatinase-associated lipokalin,* NGAL) w moczu (Artykuły nr 2 i 3) z grupy badanej wyłączono pacjentów, u których w badaniu ogólnym moczu stwierdzono leukocyturię, ze względu na możliwe zawyżenie stężeń NGAL w moczu. W przypadku pracy obejmującej badania układu krzepnięcia (Artykuł nr 4) dodatkowe kryterium wyłączenia stanowiły epizody zakrzepowe przebyte w ciągu 3 miesięcy poprzedzających badanie.

W sumie do grupy badanej włączono 70 pacjentów, jednak ze względu na wymienione wyżej kryteria wyłączenia, w poszczególnych artykułach opisano grupy liczące od 65 do 69 pacjentów.

Protokół badania

Badanie prowadzono zgodnie z protokołem, który został pozytywnie zaopiniowany przez Komisję Bioetyczną Uniwersytetu Jagiellońskiego (nr 122.6120.242.2015). Rekrutacja pacjentów odbyła się na podstawie wcześniejszej zgody Komisji Bioetycznej Uniwersytetu Jagiellońskiego (nr KBET/247/B/2013).

W ramach badania od pacjentów pobierano próbki krwi i moczu trzykrotnie: po 24, 48 i 72 godzinach od wystąpienia objawów ostrego zapalenia trzustki, przede wszystkim bólu brzucha. Pobrane próbki krwi posłużyły do wykonania rutynowych badań oraz zabezpieczenia porcji surowicy w celu wykonania oznaczeń angiopoetyny-2 i sFlt-1. W próbkach moczu wykonywano ogólne badanie moczu oraz oznaczenia stężeń NGAL, albuminy i kreatyniny.

W ciągu całej hospitalizacji gromadzono dane kliniczne pozwalające na ocenę ciężkości ostrego zapalenia trzustki. Zebrane dane kliniczne obejmowały wiek i płeć pacjentów, dane o chorobach towarzyszących, ocenę funkcji narządów (nerek, płuc, układu sercowo-naczyniowego, wątroby, układu nerwowego), dane na temat prowadzonego leczenia (antybiotykoterapia, leczenie chirurgiczne) i długości hospitalizacji, dane dotyczące wyników badań obrazowych, a także ocenę stanu pacjentów w skalach prognostycznych (BISAP, Glasgow).

Ciężkość ostrego zapalenia trzustki oceniono według klasyfikacji Atlanta z 2012 roku [3], biorąc pod uwagę przebieg choroby w ciągu całej hospitalizacji. Na tej podstawie wyróżniono trzy podgrupy pacjentów: z łagodnym, średnio-ciężkim i ciężkim ostrym zapaleniem trzustki. Ostre uszkodzenie nerek diagnozowano na podstawie kryteriów *Kidney Disease: Imroving Global Outcomes* (KDIGO) [26], zaś niewydolność nerek w oparciu o zmodyfikowaną skalę Marshalla [3]. Zespół rozsianego wykrzepiania wewnątrznaczyniowego rozpoznawano w oparciu o wytyczne Międzynarodowego Stowarzyszenia Zakrzepicy i Hemostazy (*International Society on Thrombosis and Haemostasis,* ISTH) [27].

Wykorzystane w pracy kryteria diagnostyczne oraz skale kliniczne przedstawiono szczegółowo w Załączniku nr 1.

Oznaczenia laboratoryjne

Rutynowe badania laboratoryjne wykonywano w moczu (badanie ogólne moczu), krwi pełnej pobranej na K_2EDTA (morfologia krwi obwodowej), surowicy (aktywność amylazy, stężenia albuminy, wapnia, glukozy, kreatyniny, mocznika, CRP) oraz osoczu cytrynianowym (czas protrombinowy, czas aktywowanej częściowej tromboplastyny, stężenia fibrynogenu i D-dimerów) w dniu pobrania materiału od pacjentów. Oznaczenia stężenia NGAL w moczu wykonano w dniu pobrania próbek z użyciem metody chemiluminescencyjnej z mikrocząsteczkami na platformie analitycznej Architect (Abbott Diagnostics, Lake Forrest, IL, USA). Stężenia albuminy i kreatyniny oznaczone w moczu posłużyły do wyliczenia wskaźnika albumina/kreatynina.

Stężenia angiopoetyny-2, sFlt-1, cystatyny C i NGAL oznaczono w surowicy uzyskanej w wyniku odwirowania krwi pobranej na skrzep w ciągu 1 godziny od pobrania. Surowicę porcjowano i przechowywano w temperaturze -70 °C do czasu wykonania oznaczeń (nie dłużej niż 3 miesiące). Stężenia angiopoetyny-2 i NGAL w surowicy oznaczano metodami immunoenzymatycznymi

z użyciem zestawów Quantikine ELISA Human Angiopoietin-2 (R&D Systems, McKinley Place, MN, USA) i Human Lipocalin-2 / NGAL ELISA (BioVendor, Brno, Czechy). Stężenia sFlt-1 oznaczono metodą elektrochemiluminescencyjną na analizatorze Cobas 8000 (Roche Diagnostics, Mannheim, Niemcy); sFlt-1 oznaczono jedynie w próbkach pobranych po 24 i 48 godzinach od wystąpienia objawów ostrego zapalenia trzustki. Stężenia cystatyny C oznaczono immunonefelometrycznie na analizatorze Nephelometer BN II (Siemens Healthcare, Erlangen, Niemcy).

Przedziały referencyjne dla wykorzystanych w pracy badań laboratoryjnych podano w Załączniku nr 2.

Analiza statystyczna

Dane jakościowe przedstawiono jako liczbę pacjentów (odsetek odpowiedniej grupy). Rozkład zmiennych ilościowych oceniono testem Shapiro-Wilka. Dane ilościowe przedstawiono jako średnią ± odchylenie standardowe lub medianę (dolny-górny kwartyl), odpowiednio dla zmiennych o rozkładzie normalnym i różnym od normalnego. W analizie tabel liczności wykorzystano test chi-kwadrat. Różnice między grupami oceniano testem t-Studenta, Manna-Whitney'a lub z użyciem jednoczynnikowej analizy wariancji i testu Kruskala-Wallisa (zależnie od liczby grup i rozkładu zmiennych). W badaniu korelacji wykorzystano współczynnik korelacji Persona lub Spermana. Analizę regresji wielokrotnej wykorzystano do oceny, czy badane związki są niezależne od zmiennych towarzyszących. Regresję liniową stosowano po wstępnym zlogarytmowaniu zmiennych prawoskośnych w celu normalizacji rozkładów tych zmiennych. W regresji logistycznej wykorzystano zmienne oryginalne. W ocenie użyteczności diagnostycznej ocenianych badań laboratoryjnych wykorzystano analizę krzywych ROC (*receiver operating characteristic*). Wyniki uznawano za istotne statystycznie przy $p \leq 0{,}05$. W analizie statystycznej wykorzystano oprogramowanie Statistica 12.0 (StatSoft, Tulsa, USA) wraz z Zestawem Medycznym (StatSoft Polska, Kraków, Polska).

Zakres tematyczny artykułów wchodzących w skład cyklu i główne wyniki

Artykuł nr 1

Dumnicka P., Maduzia D., Ceranowicz P., Olszanecki R., Drożdż R., Kuśnierz-Cabala B. The interplay between inflammation, coagulation and endothelial injury in the early phase of acute pancreatitis: clinical implications. *Int. J. Mol. Sci.* 2017; 18: 354. doi: 10.3390/ijms18020354

Artykuł jest pracą przeglądową i stanowi obszerny wstęp do niniejszej pracy doktorskiej. Przedstawia szczegółowo zagadnienia związane z lokalną i uogólnioną aktywacją i dysfunkcją śródbłonka we wczesnej fazie ostrego zapalenia trzustki. W pracy podkreślono związki pomiędzy ostrym stanem zapalnym, dysfunkcją śródbłonka i aktywacją układu hemostazy. Dokonano przeglądu doniesień dotyczących użyteczności diagnostycznej laboratoryjnych markerów aktywacji i uszkodzenia śródbłonka oraz badań laboratoryjnych układu hemostazy jako wskaźników prognostycznych ciężkiego przebiegu ostrego zapalenia trzustki. Wśród badań laboratoryjnych charakteryzujących się dobrą użytecznością diagnostyczną we wczesnym prognozowaniu ciężkiego przebiegu ostrego zapalenia trzustki należy wymienić stężenia angiopoetyny-2 w surowicy, D-dimerów w osoczu i aktywność antytrombiny w osoczu. W pracy dokonano także przeglądu badań eksperymentalnych i klinicznych, w których podjęto próby zapobiegania i leczenia ostrego zapalenia trzustki za pomocą leków przeciwzakrzepowych. Mimo obiecujących wyników

eksperymentów z udziałem zwierząt laboratoryjnych, w większości badań klinicznych nie uzyskano zadowalających wyników takiego leczenia. Wyjątkiem są dwa badania kliniczne z zastosowaniem heparyn drobnocząsteczkowych, ich wyniki wymagają jednak potwierdzenia w innych ośrodkach.

Artykuł nr 2

Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembiński A., Stępień E., Walocha J., Drożdż R., Kuźniewski M., Kuśnierz-Cabala B. Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. *Mediators Inflamm.* 2016; 5780903: 1–7. doi: 10.1155/2016/5780903

Praca jest pierwszym polskim doniesieniem dotyczącym związku między stężeniem angiopoetyny-2 w surowicy pacjentów we wczesnej fazie ostrego zapalenia trzustki a ciężkością choroby. Szczególną uwagę poświęcono ostremu uszkodzeniu nerek i niewydolności nerek w przebiegu ostrego zapalenia trzustki. W grupie 65 pacjentów z ostrym zapaleniem trzustki (5 z SAP, 14 z MSAP i 46 z MAP) stężenie angiopoetyny-2 oznaczone po 24, 48 i 72 godzinach od wystąpienia objawów ostrego zapalenia trzustki było istotnym czynnikiem prognostycznym cięższego przebiegu choroby: wystąpienia SAP, MSAP i SAP, zespołu uogólnionej odpowiedzi zapalnej oraz wyników ≥ 3 punktów w skali BISAP. Ponadto stężenia angiopoetyny-2 korelowały dodatnio z laboratoryjnymi wskaźnikami funkcji nerek: stężeniem kreatyniny, mocznika, NGAL w surowicy, NGAL w moczu i wskaźnikiem albumina/kreatynina w moczu. Ostre uszkodzenie nerek zdiagnozowano u 11 chorych, zaś niewydolność nerek (według zmodyfikowanej skali Marshalla) u 6 chorych. Wysokie stężenia angiopoetyny-2 były istotnie skorelowane z wystąpieniem powikłań nerkowych, niezależnie od wieku, płci i chorób współistniejących.

Artykuł nr 3

Dumnicka P., Sporek M., Mazur-Laskowska M., Ceranowicz P., Kuźniewski M., Drożdż R., Ambroży T., Olszanecki R., Kuśnierz-Cabala B. Serum soluble fms-like tyrosine kinase 1 (sFlt-1) predicts the severity of acute pancreatitis. *Int. J. Mol. Sci.* 2016; 17: 2038. doi: 10.3390/ ijms17122038

Zgodnie z wiedzą autorki, artykuł jest pierwszym doniesieniem, w którym wykazano związek między stężeniem sFlt-1 w surowicy pacjentów we wczesnej fazie ostrego zapalenia trzustki a ciężkością choroby. Stężenia sFlt-1 oznaczono przy użyciu zautomatyzowanej metody laboratoryjnej w próbkach pobranych w pierwszych 24 godzinach i po 48 godzinach od wystąpienia objawów ostrego zapalenia trzustki w grupie 66 chorych (5 z SAP, 15 z MSAP, 46 z MAP). Najwyższe stężenia sFlt-1 odnotowano u pacjentów z SAP w pierwszych 24 godzinach ostrego zapalenia trzustki. Stężenia sFlt-1 korelowały dodatnio z nasileniem stanu zapalnego (CRP, liczba leukocytów) oraz wskaźnikami uszkodzenia nerek (kreatynina, mocznik, cystatyna C, NGAL w surowicy, wskaźnik albumina/kreatynina i NGAL w moczu). Wyższe stężenia sFlt-1 oznaczone w pierwszych 24 godzinach ostrego zapalenia trzustki pozwalały przewidzieć cięższy przebieg choroby (rozpoznanie MSAP i SAP, wyniki ≥ 3 punktów w skali BISAP, zespół uogólnionej odpowiedzi zapalnej, niewydolność narządową przemijającą lub przetrwałą oraz niewydolność nerek), niezależnie od wieku i chorób towarzyszących. Stężenia sFlt > 139 pg/ml w pierwszych 24 godzinach od wystąpienia objawów ostrego zapalenia trzustki umożliwiały rozpoznanie MSAP i SAP z czułością 94% i swoistością 63%, a wielkość pola pod krzywą ROC dla sFlt-1 była nieco wyższa niż dla CRP, D-dimerów i angiopoetyny-2.

Artykuł nr 4

Dumnicka P., Kuśnierz-Cabala B., Sporek M., Mazur-Laskowska M., Gil K., Kuźniewski M., Ceranowicz P., Warzecha Z., Dembiński A., Bonior J., Drożdż R. Serum concentrations of angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) are associated with coagulopathy among patients with acute pancreatitis. *Int. J. Mol. Sci.* 2017; 18: 735. doi: 10.3390/ijms18040753

Artykuł uzupełnia obserwacje przedstawione w Artykułach nr 2 i 3 na temat korelacji surowiczych stężeń angiopoetyny-2 i sFlt-1 z ciężkością ostrego zapalenia trzustki. W publikacji opisano związki między stężeniami angiopoetyny-2 i sFlt-1 a aktywacją układu hemostazy obserwowaną w ciągu pierwszych 48 godzin ostrego zapalenia trzustki. W grupie 69 pacjentów (5 z SAP, 15 z MSAP i 49 z MAP) wykonano rutynowe badania układu hemostazy (liczba płytek we krwi obwodowej, czas protrombinowy, czas aktywowanej częściowej tromboplastyny, stężenie fibrynogenu i D-dimerów w osoczu) w pierwszych 24 godzinach i po 48 godzinach od wystąpienia objawów ostrego zapalenia trzustki. Nieprawidłowe wyniki badań wskazujące na aktywację krzepnięcia ze zużyciem składników układu hemostazy (w tym niską liczbę płytek, przedłużone czasy krzepnięcia, niskie stężenia fibrynogenu i wysokie stężenia D-dimerów) obserwowano u wszystkich badanych chorych. U 6 chorych nasilenie zaburzeń układu hemostazy uprawniało do rozpoznania zespołu rozsianego wykrzepiania wewnątrznaczyniowego według kryteriów ISTH. Stężenia angiopoetyny-2 i sFlt-1 korelowały z wynikami badań układu hemostazy, w szczególności obserwowano dodatnie korelacje z czasem protrombinowym, stężeniem D-dimerów i punktacją w skali ISTH. Korelacje badanych markerów uszkodzenia śródbłonka ze stężeniem D-dimerów były niezależne od stężenia CRP. W pierwszych 24 godzinach ostrego zapalenia trzustki stężenia obu markerów śródbłonkowych oraz wyniki wszystkich ocenianych badań układu hemostazy wykazywały użyteczność diagnostyczną w rozpoznaniu SAP. Najwyższą wartość pola pod krzywą ROC uzyskano dla angiopoetyny-2, punktacji w skali ISTH oraz stężenia D-dimerów. Natomiast stężenie sFlt-1 było najlepszym wskaźnikiem prognostycznym dla rozpoznania MSAP i SAP.

Ograniczenia badania

Ograniczona liczba pacjentów biorących udział w badaniu, a szczególnie niewielka liczba chorych z ciężką postacią ostrego zapalenia trzustki (pięć osób z SAP) powoduje, że uzyskane wyniki, zwłaszcza dotyczące użyteczności diagnostycznej badanych wskaźników laboratoryjnych w prognozowaniu SAP należy traktować ostrożnie. Jednocześnie należy zaznaczyć, że odsetek pacjentów z SAP w badanej grupie (ok. 7% wszystkich chorych) nie odbiega od wartości podawanych w aktualnych badaniach epidemiologicznych [3,28]. Potwierdzenie wyników niniejszej pracy wymaga oceny większej grupy chorych; planowane jest przeprowadzenie takich badań.

W pracy nie badano innych niż angiopoetyna-2 i sFlt-1 wskaźników związanych z aktywacją i uszkodzeniem śródbłonka, a zastosowany protokół badania obserwacyjnego nie pozwala na prześledzenie związków patofizjologicznych między czynnikami stymulującymi stan zapalny, aktywacją i uszkodzeniem śródbłonków, wzrostem przepuszczalności naczyń i aktywacją układu hemostazy. Wyniki prac eksperymentalnych omówionych w Artykule nr 1 potwierdzają istnienie takich zależności. Celem niniejszej pracy była przede wszystkim ocena użyteczności diagnostycznej badanych markerów w prognozowaniu ciężkości przebiegu ostrego zapalenia trzustki.

Wnioski oraz implikacje praktyczne pracy

Wyniki przeprowadzonych badań potwierdzają wysoką użyteczność diagnostyczną oznaczeń angiopoetyny-2 w surowicy w prognozowaniu rozwoju ciężkiego ostrego zapalenia trzustki we wczesnej fazie choroby. Wyniki te są zgodne z wcześniejszymi doniesieniami. Zastosowanie oznaczeń angiopoetyny-2 w praktyce klinicznej wymagałoby jednak opracowania zautomatyzowanych i wystandaryzowanych metod laboratoryjnych, umożliwiających szybkie wykonanie badania w warunkach rutynowego laboratorium medycznego.

Obiecujące wyniki uzyskano w przypadku oznaczeń rozpuszczalnej fms-podobnej kinazy tyrozynowej-1 (sFlt-1) z użyciem metody automatycznej, stosowanej dotąd w diagnostyce preeklampsji u kobiet ciężarnych. Stężenia sFlt-1 w surowicy wykazują dobrą użyteczność diagnostyczną w prognozowaniu wystąpienia niewydolności narządowej przemijającej i przetrwałej w przebiegu ostrego zapalenia trzustki, zwłaszcza jeśli oznaczenia wykonywane są wcześnie, w ciągu pierwszych 24 godzin od wystąpienia objawów choroby. Wyniki te wymagają jednak potwierdzenia w badaniach z udziałem większej grupy pacjentów, najlepiej w badaniach wieloośrodkowych. Oznaczenie sFlt-1 mogłoby stać się dodatkowym testem wykonywanym przy przyjęciu pacjenta z ostrym zapaleniem trzustki; wysokie stężenia sFlt-1 przemawiałyby za objęciem pacjenta intensywnym nadzorem medycznym.

Wyniki rutynowych badań układu hemostazy, przede wszystkim stężenie D-dimerów w osoczu, powinny być brane pod uwagę we wczesnej ocenie ciężkości przebiegu ostrego zapalenia trzustki. Obserwowana w niniejszej pracy wysoka użyteczność diagnostyczna D-dimerów w prognozowaniu SAP jest zgodna z danymi z piśmiennictwa. W świetle prezentowanych badań wysokie stężenie D-dimerów można traktować jako pośredni dowód uszkodzenia śródbłonka we wczesnej fazie ciężkiego ostrego zapalenia trzustki.

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Artykuł nr 1

Paulina Dumnicka, Dawid Maduzia, Piotr Ceranowicz, Rafał Olszanecki, Ryszard Drożdż, Beata Kuśnierz-Cabala

The interplay between inflammation, coagulation and endothelial injury in the early phase of acute pancreatitis: clinical implications.

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Review

The Interplay between Inflammation, Coagulation and Endothelial Injury in the Early Phase of Acute Pancreatitis: Clinical Implications

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Abstract: Acute pancreatitis (AP) is an inflammatory disease with varied severity, ranging from mild local inflammation to severe systemic involvement resulting in substantial mortality. Early pathologic events in AP, both local and systemic, are associated with vascular derangements, including endothelial activation and injury, dysregulation of vasomotor tone, increased vascular permeability, increased leukocyte migration to tissues, and activation of coagulation. The purpose of the review was to summarize current evidence regarding the interplay between inflammation, coagulation and endothelial dysfunction in the early phase of AP. Practical aspects were emphasized: (1) we summarized available data on diagnostic usefulness of the markers of endothelial dysfunction and activated coagulation in early prediction of severe AP; (2) we reviewed in detail the results of experimental studies and clinical trials targeting coagulation-inflammation interactions in severe AP. Among laboratory tests, D-dimer and angiopoietin-2 measurements seem the most useful in early prediction of severe AP. Although most clinical trials evaluating anticoagulants in treatment of severe AP did not show benefits, they also did not show significantly increased bleeding risk. Promising results of human trials were published for low molecular weight heparin treatment. Several anticoagulants that proved beneficial in animal experiments are thus worth testing in patients.

Keywords: acute pancreatitis; coagulation; endothelial injury; inflammation; laboratory markers

1. Introduction

Acute pancreatitis (AP) is the most common cause of acute hospital admissions among gastrointestinal diseases, with the incidence of about 10–100 per 100,000 population [1–3]. Increasing incidence has been recently reported in the USA and many European countries [1,4]. The disease is characterized by the spectrum of severity: most cases are mild and self-limiting; however, about 30% of cases are classified as moderately severe, and about 10% as severe according to the 2012 revision of the Atlanta classification [5,6]. Organ failure is the main determinant of severity and the main cause of early mortality, while secondary infections, including infected pancreatic necrosis and sepsis,

are responsible for the late deaths [5]. Overall mortality in AP is about 3%–6%, whereas in severe AP (SAP), it reaches 30% [1,6]. The high mortality is associated with the lack of specific treatment; however, a decrease in mortality has been achieved thanks to improved intensive care and less invasive surgical management in severe cases [1,6,7]. As indicated in current clinical guidelines [7], early (within first 24 h from admission) and adequate fluid resuscitation decreases the rates of persistent systemic inflammatory response syndrome (SIRS), organ failure and mortality.

Although the etiology of AP is complex [8], the two most common causes are biliary tract diseases and excessive alcohol consumption [6]. Premature activation of digestive enzymes (most importantly, trypsinogen into trypsin) within acinar cells is the key event in early pathogenesis of AP, leading to destruction (autodigestion) of the pancreas [9,10]. Unconjugated bile acids and fatty acid ethyl esters (the products of non-oxidative alcohol metabolism) cause mitochondrial injury and sustained increase in intracellular Ca^{2+} concentrations in acinar cells, leading to inhibition of zymogen secretion and premature activation of digestive enzymes [11,12]. Recent studies have shown that acinar cells form a functional unit with ductal cells. Low doses of bile acids or alcohol cause increased secretion of bicarbonate-rich fluid by pancreatic ductal cells that may protect acinar cells from the contact with toxic substances. To the contrary, high concentrations of unconjugated bile acids and alcohol inhibit secretion of bicarbonate-rich fluid by pancreatic ducts. Thus, the initial events in AP involve both ductal and acinar cells [13,14].

Irrespective of the causative factor, acinar injury is associated with early inflammatory reaction within the pancreas, characterized by nuclear factor κB (NFκB) activation and cytokine production in acinar cells, at least partially independent of trypsinogen activation [15–17]. As a consequence, inflammatory cells, including neutrophils and monocytes, are activated and recruited to the pancreas, exaggerating the damage of the gland as well as the inflammation [18]. In particular, neutrophils are the source of tissue-degrading enzymes, reactive oxygen species, and further inflammatory cytokines [19]. Most recently, the formation of neutrophil extracellular traps has been documented within pancreatic ducts, which enhances premature activation of trypsinogen [20,21]. Another consequence of local inflammation is vascular injury within the pancreas, manifesting as endothelial activation and endothelial injury, increased vascular permeability, activation of coagulation, and increased leukocyte rolling, sticking and transmigration to pancreatic tissue [22,23]. In mild AP, the inflammatory response is local and self-limiting, whereas in SAP, excessive systemic inflammation develops. The levels of proinflammatory cytokines and acute phase proteins in systemic circulation correlate positively with the severity of AP [17,18,24]. In SAP, trypsin, damage-associated molecular patterns, and proinflammatory cytokines released from the inflamed pancreas lead to systemic vascular injury with vascular leak syndrome and cardiovascular, kidney and lung failure [22,25]. Systemic endothelial dysfunction may also manifest itself as diffuse activation of coagulation, with clinically significant thrombotic complications observed in a part of patients with SAP [26,27].

Despite recent progress in understanding the early events in AP, more research is needed to enable faster and more accurate prediction of a severe course of the disease as well as more specific and better targeted treatment [3]. At present, prediction of SAP is based on clinical assessment at admission and during the treatment [7]. The laboratory markers of trypsinogen activation or inflammation [24], the severity scores based on computer tomography imaging, and the multi-parameter severity scores such as bedside index of severity in AP (BISAP) or acute physiology and chronic health evaluation (APACHE) have been proposed for prediction of SAP [28,29]. While they are helpful, they are far from perfect. The biomarkers associated with systemic vascular injury may prove a useful alternative or supplementation.

The purpose of the review is to summarize current evidence regarding the interplay between inflammation, coagulation and endothelial dysfunction in the early phase of AP. Practical aspects are underscored, such as the possibilities to use the markers of endothelial dysfunction and activated coagulation in early prediction of SAP, as well as the possibilities of targeting coagulation-inflammation interactions in the treatment of SAP.

2. Interrelations between Coagulation and Inflammation

Coagulation and inflammation clearly show reciprocal connections. Activation of coagulation leads to stimulation of inflammatory mechanisms [30,31]. The contact of factor VII with tissue factor (TF) is the main trigger for activation of coagulation. The complex formed by TF and activated factor VII (VIIa) in the presence of factor X stimulates protease-activated receptors (PARs) [32,33]. Also thrombin as a serine protease activates PARs [34]. PARs are expressed by platelets and by numerous immune cells such as monocytes, lymphocytes, macrophages, dendritic cells and mast cells, as well as by endothelial cells [35]. Once thrombin affects PARs present on the membranes of platelets, platelet activation occurs with shape change and the release of granules' content, including adenosine diphosphate, serotonin, thromboxane and chemokines [30]. Activation of platelets occurs as well in the platelet phase of local hemostasis. Platelet stimulation leads to the release of soluble ligand for CD40 receptor (sCD40L) [36,37]. CD40 molecule belongs to the tumor necrosis factor (TNF) receptor family. Soluble CD40L causes stimulation of TF production and the release of proinflammatory cytokines [38]. Acting on vascular endothelial cells, sCD40L, in addition to chemokine release, causes expression of adhesion proteins, leading to rolling and sticking of leukocytes to the vascular endothelium, their subsequent migration through the vascular wall and inflammatory infiltration of tissues [39]. Moreover, thrombin may directly stimulate vascular endothelial cells, leading to increased vascular permeability, expression of the adhesion proteins such as P-selectin, release of von Willebrand factor (vWF), as well as increased production and release of cytokines [30].

The role of thrombin in the activation of inflammatory process is also related to its chemotactic activity on monocytes, mitogenic activity on lymphocytes and stimulating effect on the production and release of proinflammatory cytokines, particularly TNF-α, interleukin (IL)-1β and IL-6 [34]. In addition, thrombin is capable of activating the complement system that plays an important role in humoral innate immune response and modifies the specific immune response [40]. Thrombin can cause formation of complement fragment C5a, an anaphylatoxin not related to the classic, alternative or lectin pathway of complement activation [41].

Other serine proteases of the coagulation system, including a complex formed by TF and activated factor VII, act directly on endothelial cells, macrophages and monocytes stimulating their proinflammatory mechanisms, such as production of free radicals and expression of adhesion proteins [30,42]. Also, fibrin formed as a result of activated coagulation as well as fibrin/fibrinogen-degradation products act in a proinflammatory manner via activation of leukocytes [43,44] and influence the vascular endothelial cells, which are stimulated to produce proinflammatory cytokines [45].

The relationships between coagulation and inflammation are two-way and are based on positive feedbacks. Therefore, development of inflammation leads to activation of coagulation [30,31]. Trauma, tissue injury, hypoperfusion, hemodilution, hypothermia and acidosis induce acute posttraumatic coagulopathy. Inflammation is an important causative factor [46]. The inflammatory process activates the coagulation system, reduces the activity of natural anticoagulants and disturbs functioning of the fibrinolysis system, leading to (microvascular) thrombosis [31]. Cytokines play an important role in activation of the coagulation system and formation of fibrin, through their action in the extrinsic coagulation pathway, i.e., induction of TF expression on endothelial cells and monocytes [47,48].

Recently, the contact system (or intrinsic pathway) has garnered increasing interest. Although the deficiencies of factor XII (Hageman's anomaly), plasma prekallikrein or high-molecular weight kininogen do not lead to bleeding disorders, activation of the contact system has been implicated in thrombosis [49]. Animal studies indicate that the inhibition of factor XII prevents thrombosis without exerting increased bleeding risk [50]. Activation of the contact system also leads to the production of inflammatory mediators, in particular bradykinin, a vasoactive substance with the potential to increase vascular leakage. The contact system may be activated in vivo by inorganic polyphosphates released from dense granules of activated platelets, or by neutrophil extracellular traps [51]. Interestingly, recent studies suggest that activated endothelial cells may also form a surface that is capable of activating the contact system [52].

Increased activity of the coagulation system in the course of inflammation results not only from direct activation of coagulation, but is also a consequence of the increase in plasma fibrinogen concentrations and increased expression of endothelial TF and P-selectin [31]. Moreover, inflammation leads to increased levels of C-reactive protein (CRP) and platelet activation with the exposure of procoagulant phospholipids [31,53]. Influence of CRP on coagulation is associated with facilitating interaction between monocytes and vascular endothelial cells [54], as well as with increased production of TF by monocytes [55] and thus activation of the extrinsic pathway [56]. Hypercoagulability observed in inflammation is also a result of inhibition of natural anticoagulants and decreased fibrinolytic activity. CRP increases the expression of plasminogen activator inhibitor-1 (PAI-1) by endothelial cells [57], while the production of prostacyclin is reduced [58]. This results in a reduction of the plasminogen activation and increased platelet aggregation.

Antithrombin inactivates thrombin as well as factors Xa, IXa, and VIIa linked with TF [31], resulting in the inhibition of fibrin formation. In the case of free antithrombin, this effect is weak; however, the thousand-fold increase of the inhibitory effect of antithrombin on thrombin and Xa occurs after complexing with heparin or glycosaminoglycans present on the surface of endothelial cells [47]. In the inflammatory state, accelerated degradation and inhibition of antithrombin activity occurs [31,59] as well as reduced expression and accelerated degradation of endothelial cells' glycosaminoglycans [60].

Moreover, the anticoagulant pathway of protein C is inhibited in inflammation [31]. Endotoxin, IL-1β and TNF-α inhibit the expression of thrombomodulin and the endothelial cell protein C receptor, thus reducing the formation of activated protein C and inhibiting its anticoagulant activity [61,62].

3. Endothelial Cells at the Interface of Coagulation and Inflammation

Endothelial cells have several important functions above being a barrier between blood and tissues. They control vascular pressure and permeability, activation and adhesion of platelets and leukocytes, coagulation and fibrinolysis. Their precise characteristics differ between organs (with special characteristics of brain, lung and kidney vessels), and between venous and arterial vascular beds. Endothelium is a dynamic tissue, quickly responding to various stimuli [63].

Endothelial cells are important for both hemostasis and inflammation. Healthy endothelium under resting conditions exerts both anti-inflammatory and antithrombotic functions. Upon activation by inflammatory factors, such as IL-1β and TNF-α, endothelial cells increase expression of adhesion molecules, i.e., intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E- and P-selectins, leading to increased adhesion of leukocytes and platelets and transmigration of leukocytes through vascular wall. Inflammatory signals result in more procoagulant phenotype of endothelial cells, with TF and factor V expression, production of PAI-1, downregulation of thrombomodulin, and decrease of protein C synthesis [64]. Also, multiple stimuli, including thrombin, histamine, leukotrienes, superoxides, complement components (C5a, C5b-9), vascular endothelial growth factor (VEGF), vasopressin or epinephrine lead to degranulation of Weibel-Palade bodies and the release of large multimers of vWF as well as other proteins (including P-selectin, IL-8, eotaxin-3, angiopoietin-2, endothelin-1 and osteoprotegerin) [65].

Recently, the importance of angiogenic signaling pathways for vascular permeability became evident. Two main signaling systems are known, namely VEGFs/VEGF receptors and angiopoietins/Tie receptors. VEGF, also known as vascular permeability factor, is capable of strongly increasing vascular permeability [66]. In systemic inflammation, high concentrations of VEGF are observed in blood [67]. Angiopoietin-2 is stored in Weibel-Palade bodies and rapidly released upon stimulation; its binding to Tie-2 receptor leads to destabilization of the endothelium and increased vascular permeability [68].

4. Vascular Involvement in Acute Pancreatitis

In early pathogenesis of AP, microvascular abnormalities play an important role; in particular, SAP is associated with early impairment of pancreatic blood flow (reviewed in [23]). In mild AP, pancreatic capillary blood flow increases; however, SAP is associated with substantial early decrease in capillary blood flow, with complete capillary stasis observed in almost 40% of pancreatic capillaries [69].

Numerous clinical and experimental studies have shown that pancreatic ischemia plays an important role in the development of AP and in the progression of the disease to severe necrotizing pancreatitis [70–72]. As shown in a porcine model of severe AP, microcirculatory derangements are responsible for the decreased pancreatic tissue oxygenation and tissue damage, and the severity of microvascular disturbance is positively associated with mortality [73]. In AP evoked by pancreatic ischemia followed by reperfusion, disturbance of pancreatic blood flow is a primary cause of this disease. Numerous animal studies have indicated that pancreatic ischemia may be a causal factor in the pathogenesis of AP [71,72,74,75]. A vascular mechanism plays an essential role in the development of AP in some experimental settings [76]. Also, AP develops in clinical situations associated with ischemia of the pancreas, such as shock, cardiac surgery, or pancreatic transplantation [23,70]. In human necrotizing AP of various etiologies, microcirculatory derangements were confirmed by histopathological studies revealing microcirculatory intravascular thrombosis, intravascular stasis and endothelial desquamation, as well as parenchymal swelling of the pancreas thus reflecting the increased microvascular permeability preceding the development of pancreatic necrosis [77].

Moreover, improvement of pancreatic blood flow inhibits the development of AP and accelerates the recovery [78–80]. In recent experimental animal studies, where AP was induced by cerulein and ischemia/reperfusion, the course of acute pancreatitis was significantly milder after administration of digestive tract hormones such as obestatin and ghrelin [81–83]. Administration of these hormones improved pancreatic blood flow and, by their anti-inflammatory effect, led to acceleration of recovery from AP.

Microcirculatory changes in early SAP are not confined to the pancreas, but are also well documented in other organs, i.e., colon and ileum, liver, lungs, kidneys, heart and brain [84–87]. In the early phase of organ failure due to SAP (only 3 h post induction of AP by taurocholate in rats), edema, leukocyte adhesion to capillary walls and infiltration in tissues were observed in histopathological examination of liver, kidney, lung, intestine, and spleen. Areas of necrosis were detected in kidneys, intestine, spleen and lymph nodes. In pancreas, liver and kidneys, these changes were also accompanied by microvascular thrombosis [87]. Experiments using intravital microscopy confirmed increased capillary permeability and leukocyte rolling and sticking in distant organs in the early phase of SAP, leading to decreased blood flow velocity [84,86]. Recently, the disturbances and heterogeneity of capillary blood flow have been underscored as a cause of diminished blood oxygenation in lungs and oxygen supply to tissues, despite preserved total organ perfusion [88].

Several vasoactive substances have been associated with microcirculatory impairment in AP, including nitric oxide, bradykinin, endothelins, and platelet-activating factor (PAF) [89]. The role of nitric oxide in AP is still controversial and has been extensively reviewed elsewhere [90]. Other mediators were with some success targeted in experimental AP. In severe porcine AP and in various rat models of AP, pretreatment with the inhibitor of bradykinin B2 receptor (icatibant) showed beneficial effects [91–94]. Endothelin-1, a potent vasoconstrictor, was shown to be up-regulated in pancreatic endothelial cells by inflammatory stimuli, including cytokines, thrombin, and trypsin, which was associated with impaired splanchnic microcirculation in SAP [95]. Inhibition of endothelin receptor A attenuated reduced functional capillary density associated with experimental SAP in rats, and ameliorated platelet-endothelial and leukocyte-endothelial cell interactions, reducing numbers of stagnant platelets and leukocytes in pancreatic postcapillary venules [96]. PAF is a pleiotropic phospholipid mediator, with roles in hemostasis (platelet activation), endothelial cell activation (increase of capillary permeability), and inflammation (induction of cytokines, including IL-1β, TNF-α and IL-6) [97]. Several PAF receptor antagonists were beneficial in experimental AP as reviewed by Chen et al. [98]. Unfortunately, despite encouraging results of phase II human trials [99,100], in randomized phase III trial, PAF antagonist lexipafant did not prevent new organ failure or ameliorate systemic inflammatory response syndrome (SIRS) in patients with predicted SAP [101]. More recently, pretreatment with recombinant PAF acetylhydrolase was evaluated for the prevention of post-endoscopic retrograde cholangiopancreatography (post-ERCP) pancreatitis in randomized

multicenter trial. No beneficial effects on the incidence or severity of AP was shown, despite high number of patients enrolled (600 patients) [102].

5. Laboratory Markers of Endothelial Activation and Injury in Acute Pancreatitis

Destabilization of the vascular endothelium, increased vascular permeability, disrupted vasomotor regulation, and activated coagulation lead to early complications of acute pancreatitis [103,104]. Fluid sequestration in patients with AP within the first 48 h from admission is significantly associated with SIRS criteria and the subsequent development of multiorgan failure [105].

VEGF is one of the most potent mediators capable of increasing vascular permeability. Several groups have studied the involvement of VEGF in AP. The results of both animal and human studies are, however, discrepant to some extent. Increased expression of VEGF was detected in the inflamed pancreas and associated with the increased vascular permeability observed in the early phase of AP [106,107]. The tyrosine kinase inhibitor of VEGF signaling attenuated almost completely the increased vascular permeability in the pancreas during experimental AP [107]. In rats with mild AP and severe necrotizing AP, serum VEGF concentrations were higher than in control animals [108]. However, infusion of VEGF in rats with SAP partially inhibited apoptosis in small intestine, kidney and liver, without affecting water content of the lung, the volume of ascitic fluid or hematocrit, suggesting a protective role of VEGF against endothelial injury in distant organs [108,109]. In the study of Ueda et al. [109], serum VEGF in the early phase of AP was higher among patients with moderately severe and severe AP, and was positively associated with kidney and liver failure, although not with mortality. Conversely, Mentula et al. [110] did not observe differences in VEGF concentrations between patients with AP who developed organ failure and those who did not.

A decoy VEGF receptor, soluble fms-like tyrosine kinase 1 (sFlt-1), has been strongly associated with the severity of sepsis [111]. We observed positive correlations between soluble sFlt-1 and the severity as well as complications of AP, such as acute kidney injury and activated coagulation [112].

Angiopoietin-2 has been proposed as a causative factor and a laboratory marker of endothelial cells' destabilization and increased vascular permeability. In patients with AP, higher angiopoietin-2 predicted SAP, multiorgan failure, infectious complications and bowel ischemia as well as mortality [113,114]. Increased angiopoietin-2 was positively associated with the severity of AP, particularly kidney injury in the early phase of AP [104]. In recent years, angiopoietin-2 emerged as one of the most promising biomarkers for the early prediction of AP severity (Table 1).

Angiopoietin-2 is known to be stored in Weibel-Palade bodies of endothelial cells. The main protein of Weibel-Palade bodies, namely vWF, is also increased in plasma during SAP. In rats, SAP was associated with increased plasma vWF and soluble endothelial protein C receptor, as well as with increased endothelial cell apoptosis in the aorta as compared to the mild AP [115]. Increased plasma vWF was also reported in humans with SAP: its concentrations correlated positively with the severity of organ failure, APACHE III and sequential organ failure assessment (SOFA) scores, and significantly predicted acute lung injury [116] (Table 1). Several reports were published showing coincidence between AP and thrombotic thrombocytopenic purpura [117–119]. Morioka et al. [117] found highly increased concentrations of vWF (mean on admission 402%) coinciding with low activities of a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS-13) among 13 SAP patients without disseminated intravascular coagulation (DIC). Mean ADAMTS-13 activity on admission was 37%, decreased to 32% on day 2 and subsequently increased. ADAMTS-13 activity negatively correlated with the APACHE II score as well as with markers of inflammation (IL-6, IL-8, fibrinogen, CRP, leukocytes) and pancreatic enzymes (serum amylase, elastase 1, trypsin and lipase). No ADAMTS-13 inhibitor was detected. Consequently, ultra large vWF multimers were detected in patients with the highest vWF/ADAMTS-13 ratios.

Also, serum concentrations of osteoprotegerin, another protein stored and released from Weibel-Palade bodies upon endothelial activation, are significantly higher in patients with SAP as compared to mild AP [120].

Table 1. Diagnostic accuracy of laboratory markers of endothelial activation or injury measured in serum or plasma for the prediction of severity, complications and mortality of acute pancreatitis (AP).

Laboratory Test	Studied Group	Time of Blood Collection	Outcome Variable (Number of Cases)	Values Associated with Outcome Variable	Se., % [1]	Sp., % [2]	AUC [3]	Ref. [4]
Angiopoietin-2	28 patients with AP from University of Pittsburgh Medical Center	Within 3 days from the onset of pain [5]	Severe AP (persistent organ failure >48 h or death) (6 patients form Pittsburg and 14 from Greifswald)	>1.91 ng/mL	83	91	0.940	[113]
	123 patients with AP from Greifswald University			>2.94 ng/mL	93	63	0.790	
	25 patients with AP	At 12 h from admission (admission within 72 h from the onset of pain)	Severe AP according to 1992 Atlanta classification (7 patients)	>10 ng/mL	100	88	0.970	[121]
	115 patients with AP (subsample from PROPATRIA trial cohort)	Within 5 days from admission (median 3 days from the onset of pain)	Severe AP (organ failure or pancreatic necrosis) (37 patients)	>4.56 ng/mL	81.1	73.2	0.851	[114]
			Multiorgan failure (18 patients)	>5.01 ng/mL	72.2	73.2	0.784	
			Infectious complications of AP (39 patients)	>4.51 ng/mL	79.5	76.3	0.816	
Soluble fms-like tyrosine kinase 1	66 consecutive adult patients with AP	At 24 h from the onset of pain	Severe and moderately severe AP according to 2012 Atlanta classification (20 patients)	>139 pg/mL	94%	63%	0.808	[112]
		At 48 h from the onset of pain		>120 pg/mL	78	77	0.791	
Soluble E-selectin	56 patients with AP	At admission (≤48 h from the onset of pain)	Severe AP according to Ranson's score and Balthazar CT grading (28 patients)	increased	NR [6]	NR [6]	0.802	[122]
	15 consecutive patients with AP	At admission (≤6 h from the onset of pain) and on two subsequent days (pooled results)	Severe AP according to 1992 Atlanta classification (5 patients)	>3.92 ng/mL	60	90	0.780	[123]
	69 adult patients with severe AP	At admission (≤48 h from the onset of pain)	Acute respiratory distress syndrome in the course of severe AP (39 patients)	>165.6 ng/mL	48.3	86.7	0.704	[116]
Soluble ICAM-1	15 consecutive patients with AP	At admission (≤6 h from the onset of pain) and on two subsequent days (pooled results)	Severe AP according to 1992 Atlanta classification (5 patients)	>80.4 ng/mL	73.3	70	0.684	[123]
	69 adult patients with severe AP	At admission (≤48 h from the onset of pain)	Acute respiratory distress syndrome in the course of severe AP (39 patients)	>711.2 ng/mL	61.5	93.3	0.787	[116]
Soluble thrombomodulin	73 patients with AP	On day 3 from the onset of pain	Death (12 patients)	<75 ng/mL	100	77	NR [6]	[124]
	104 patients with AP	At 48 h from the onset of pain	Pancreatic necrosis (32 patients)	>71.5 µg/L	75	99	0.949	[125]
	27 patients with AP	At admission	Death (5 patients)	>32 TU/mL	80	91	0.876	[126]
von Willebrand factor (antigen)	69 adult patients with severe AP	At admission (≤48 h from the onset of pain)	Acute respiratory distress syndrome in the course of severe AP (39 patients)	>169.2%	43.2	93.3	0.686	[116]

[1] diagnostic sensitivity; [2] diagnostic specificity; [3] area under receiver operating characteristic curve; [4] reference number; [5] the onset of pain due to AP is considered the onset of the disease; [6] not reported.

Adhesion proteins, including E- and P-selectins, ICAM-1 and VCAM-1 have been studied as markers of endothelial activation or injury in AP. E-selectin is synthesized de novo by endothelial cells stimulated by IL-1, TNF-α, endotoxin and oxidative stress. Its soluble form occurs due to shedding from the surface of activated endothelial cells. P-selectin is stored in Weibel-Palade bodies, and thus may be rapidly released upon stimulation of endothelial cells, e.g., by thrombin or histamine. Additionally, upon stimulation by TNF-α and IL-1β, endothelial cells synthesize P-selectin. In experimental AP, prophylactic inhibition of P-selectin resulted in reduced platelet activation, platelet-endothelium and leukocyte-endothelium interactions and reduced pancreatic tissue inflammation and necrosis [127]. Both P- and E-selectins are overexpressed in lung tissue during experimental AP. Their up-regulation was associated with increased sequestration of neutrophils and pulmonary injury observed in histological examination [128]. ICAM-1 is constitutively expressed by endothelial cells, but the expression highly increases upon inflammatory stimulation. VCAM-1 expression is specific to endothelium; together with ICAM-1, it is involved in leukocyte adhesion and rolling. Increased ICAM-1 expression was reported in lungs of rats with SAP [129]. Blocking ICAM-1 with an antibody resulted in reduced neutrophil sequestration, decreased microvascular permeability and improved lung histology [129]. Frossard et al. [130] also found increased ICAM-1 in serum, pancreas and lungs of mice with AP induced with cerulein or with choline-deficient, ethionine-rich diet. Both pancreatitis and lung injury were diminished but not completely prevented in mice with ICAM-1 deficiency. In porcine model of SAP, increased expression of adhesion proteins was shown: platelet-endothelial cell adhesion molecule-1 in liver, kidney and pancreas, VCAM-1 in kidney, and P-selectin in liver [131].

Increased soluble E-selectin has been proposed as a marker of severe AP in several studies (Table 1). Wereszczynska-Siemiatkowska et al. [122] reported increased soluble E-selectin during first 10 days from admission among patients with SAP, as compared to those with mild AP and to patients with non-AP acute abdominal pain (mainly acute biliary tract diseases). At admission of AP patients, strong correlations were observed between soluble E-selectin and IL-6 concentrations, polymorphonuclear elastase activity, as well as oxidative stress markers (serum malondialdehyde and 4-hydroxyalkenals) [122]. Of note, patients with severe AP had also increased IL-10 serum concentrations, especially during the first two days from admission, and positive correlation was found between E-selectin and IL-10 [122]. In another study, soluble E-selectin, ICAM-1, TF and vWF were shown to be significantly higher in SAP associated with acute respiratory distress syndrome; all studied endothelial markers correlated positively with APACHE III and SOFA scores, and negatively with oxygenation index (PaO_2/FiO_2) during the first 10 days of hospital stay due to AP [116]. Also, Powell et al. [132] and Ida et al. [126] reported higher soluble E-selectin in patients with SAP, especially in those who subsequently died, as compared to mild AP. Moreover, during 3 days from admission, E-selectin concentrations were increasing in severe disease in contrast to mild AP [132]. Nakae et al. [133] observed positive correlation between soluble E-selectin and TNF-α in the early phase of human AP; both mediators were positively associated with AP severity. Hynninen et al. [134] reported similarly increased soluble E-selectin in patients with SAP and with severe sepsis, positively correlated with SOFA scores. However, there are also contradictory reports. It was suggested that the peak soluble E-selectin concentrations are observed late (after 72 h from the onset of AP symptoms) and can therefore not be used as an early marker of AP severity [135,136]. During first 6 h from the onset of pain, Pezzilli et al. [123] did not observe higher soluble E-selectin in AP patients compared to healthy controls; however, it was higher in SAP than in mild AP. Ida et al. [126] did not show significant difference in soluble E-selectin on admission and on subsequent days in those who died from AP compared to survivors. Nonetheless, both mild and severe AP were associated with concentrations above the reference limit.

Reports regarding soluble P-selectin in patients with AP are scarce. In a small study of Powell et al. [132], serum soluble P-selectin concentrations during 3 days from admission did not differ between mild and severe AP, but were significantly higher in non-survivors than survivors.

To the contrary, Pezzilli et al. [123] reported lower soluble P-selectin in SAP patients' sera as compared to those with mild AP and healthy controls.

Soluble ICAM-1 has been associated with the severity of human AP (Table 1). Pezzilli et al. [123] observed higher concentrations of soluble ICAM-1 in patients with SAP versus mild AP. This result is, however, depreciated by the fact that the levels in patients with AP were not significantly different compared to healthy volunteers. Siemiatkowski et al. [116] have shown that soluble ICAM-1 may serve as a marker of AP-associated lung injury. In this study, strong positive correlations were observed between plasma-soluble ICAM-1 and severity of organ dysfunction (APACHE III and SOFA scores) in SAP patients. Nakae et al. [133] observed higher soluble ICAM-1 in patients who died from AP than in survivors.

Studies of soluble VCAM-1 in patients with AP are inconclusive. Serum VCAM-1 in AP patients on admission was lower than in controls and did not correlate with AP severity in one study [123], while it was higher among non-survivors of SAP and positively correlated with ICAM-1 and TNF-α in another study [133].

Elevated plasma concentrations of thrombomodulin in inflammation are caused by shedding of membrane-bound thrombomodulin from endothelial cells by neutrophil elastase. In a small study, Ida et al. [126] have shown that increased plasma concentrations of soluble thrombomodulin was positively associated with the severity of AP and was higher in patients who died. Mantke et al. [124] studied soluble thrombomodulin during the first 28 days after the onset of symptoms of AP: starting from day 3, non-survivors had significantly higher concentrations than survivors. The clinical studies consistently reported positive association between plasma thrombomodulin in the early phase of AP and more severe disease [124–126] (Table 1). Plasma tissue factor pathway inhibitor (TFPI) in human AP was shown to be higher in patients with SAP as compared to mild AP, and was positively correlated with inflammatory mediators, thrombomodulin and PAI-1, consistent with the assumption that plasma TFPI levels reflect endothelial injury rather than anticoagulation [137].

Recently, levels of endothelial-specific microRNAs (miR-551-5p and miR-126a-5p) were associated with the severity of human AP [138].

6. Disturbances of Hemostasis in Relation to Inflammation in Acute Pancreatitis

In experimental and human AP, abnormalities were reported regarding all aspects of hemostasis [26]. Decreased numbers of platelets and increased platelet activation were observed in the early phase of AP [139–143]. Plasma TF concentrations were increased [116,144,145]. The levels of prothrombin, fibrinogen and factor X gradually decreased [146], and prolonged clotting times (prothrombin time, activated partial thromboplastin time and thrombin time) were observed [139,146,147]. Decreased concentrations of natural anticoagulants, especially protein C and antithrombin, were consistently reported [139,148–150]. Activity of tissue plasminogen activator (tPA) and PAI-1 was increased [151,152]. A complex of α2-plasmin inhibitor with plasmin was increased in patients with the most severe AP [142]. These changes are consistent with the activation of the coagulation system, following local and systemic inflammation, leading to consumptive coagulopathy. The degree of coagulation abnormalities in AP depends on the severity of inflammation [146,147]. In mild pancreatitis, thrombosis may be limited to pancreatic microcirculation. In severe systemic inflammation, DIC may occur [139,153]. Activation of fibrinolysis secondary to activated coagulation results in increased concentrations of fibrin/fibrinogen degradation products, including D-dimer, that are significantly correlated with inflammatory markers and AP severity [142,143,147,150,154].

The classic Virchow's triad of factors predisposing to thrombosis, i.e., procoagulant changes in the blood components, procoagulant properties of the vessel wall and decreased blood flow velocity, can be observed in SAP. Consequently, various clinically relevant thrombotic complications are observed in human AP, ranging from localized thrombosis [155–158] and pulmonary thromboembolism [159] to DIC [153,160]. Both thrombotic and hemorrhagic complications were associated with deaths due to AP [27].

Even in the absence of clinically significant thrombotic complications, laboratory tests reveal the activation of coagulation and fibrinolysis, related to the severity of AP. Maeda et al. [142] reported significant correlations between laboratory parameters of DIC and the severity of AP measured in the five-stage Japanese scoring system: more severe AP was associated with lower platelet counts and antithrombin concentrations as well as higher levels of D-dimer, fibrin/fibrinogen degradation product E, and thrombin-antithrombin complexes. All the parameters were also significantly associated with mortality (Table 2); and the diagnosis of DIC was much more prevalent among patients who died (79% versus 10% of patients) [142]. Also, longer PT, APTT, higher fibrinogen and D-dimer, lower protein C and antithrombin, lower plasminogen, and higher PAI-1 on admission and 24 h thereafter were associated with organ failure (pulmonary, kidney or cardiovascular) in the course of AP in humans [161] (Table 2). Of note, high D-dimer concentrations in SAP were observed already at admission (1–2 days from the onset of symptoms), but persisted during the subsequent week and even during remission of AP [143,147,150]. Also, patients with moderately severe AP were characterized by higher D-dimer and lower protein C levels as compared with mild AP [162]. Plasma TF concentrations were significantly increased in SAP during the first 10 days from admission, and significantly correlated with APACHE III and SOFA scores as well as with PaO_2/FiO_2 [116]. The admission TF concentrations significantly predicted AP-associated lung injury [116] (Table 2). In patients who eventually died from AP as compared to patients who survived, natural anticoagulants (antithrombin and protein C, but not protein S) were lower, and increased levels of PAI-1 and D-dimer were observed during the preceding period [150,161,163]. In patients with SAP as defined by the original 1992 Atlanta classification (organ failure and/or local complications), the development of organ failure was associated with decreased protein C concentrations as well as decreased activated protein C [148]. Protein C and activated protein C levels correlated with the numbers of activated monocytes [148].

The results of the studies that reported diagnostic accuracy of laboratory markers of hemostasis are summarized in Table 2. Most reliable diagnostic accuracy was consistently reported for D-dimer measured at admission for the prediction of (multi)organ failure [161,164] and antithrombin for the prediction of death [142] (Table 2).

Table 2. Diagnostic accuracy of laboratory markers of hemostasis measured in whole blood (platelet count) or plasma (other markers) for the prediction of severity, complications and mortality of acute pancreatitis (AP).

Laboratory Test	Studied Group	Time of Blood Collection	Outcome Variable (Number of Cases)	Values Associated with Outcome Variable	Se., % [1]	Sp., % [2]	AUC [3]	Ref. [4]
Platelet count	139 consecutive patients with AP	At admission	Death (14 patients)	$<92 \times 10^3/\mu L$	75	71	0.850	[142]
D-dimer	139 consecutive patients with AP	At admission	Death (14 patients)	>6.1 μg/mL	85	67	0.783	[142]
	91 consecutive patients with AP	At admission	Organ failure: pulmonary or kidney failure, or shock (24 patients)	>0.414 μg/mL	90	89	0.908	[161]
		24 h from admission		>0.551 μg/mL	90	81	0.916	
	38 consecutive patients with AP	At admission	Organ failure (23 patients)	>0.4 μg/mL	81.7	54.2	0.683	[150]
			Death (14 patients)	>0.4 μg/mL	90.9	58.3	0.708	
	45 consecutive adult patients with severe AP	Day 0–2 from admission (mean value)	Multiorgan dysfunction syndrome (16 patients)	>0.812 μg/mL	81	90	0.899	[154]
			Pancreatic infection (14 patients)	>0.762 μg/mL	100	87	0.968	
		Day 0–2 from admission (maximum value)	Multiorgan dysfunction syndrome (16 patients)	>0.975 μg/mL	81	79	0.885	
			Pancreatic infection (14 patients)	>0.975 μg/mL	93	81	0.935	
	36 pediatric patients with AP (aged 1–17 years)	At admission	Multiorgan failure (4 patients)	>1.189 μg/mL	100	87.5	0.914	[164]
	173 adult patients with AP	At admission (≤96 h from the onset of pain [5])	Critical AP (persistent organ failure plus infected necrosis) (47 patients)	>0.67 μg/mL	83	68	0.810	[165]
	106 patients with mild to moderately severe AP	Within 24 h from admission (≤48 h from the onset of pain)	Moderately severe AP according to 2012 Atlanta classification	>0.91 μg/mL	62.2	84.1	0.747	[162]
Fibrin/fibrinogen degradation product-E	139 consecutive patients with AP	At admission	Death (14 patients)	>894 ng/mL	93	73	0.873	[142]
Antithrombin	139 consecutive patients with AP	At admission	Death (14 patients)	<69%	81	86	0.926	[142]
	91 consecutive patients with AP	24 h from admission	Organ failure: pulmonary or kidney failure, or shock (24 patients)	<75.5%	62	89	0.770	[161]
	38 consecutive patients with AP	At admission	Organ failure (23 patients)	≤71%	66.7	78.6	0.748	[150]
			Death (14 patients)	≤71%	70.8	81.8	0.830	
Protein C	38 consecutive patients with AP	At admission	Organ failure (23 patients)	≤60%	62.5	64.3	0.683	[150]
			Death (14 patients)	≤60%	70.8	63.6	0.691	
Thrombin-antithrombin complex	139 consecutive patients with AP	At admission	Death (14 patients)	>11 ng/mL	79	72	0.768	[142]
Tissue factor	19 patients with alcoholic SAP	At admission (≤48 h from the onset of pain)	Pancreatic necrosis (11 patients)	>350 pg/mL	60	100	0.773	[144]
	48 consecutive patients with AP	At inclusion into study (median duration of pain 34 and 25 h in mild and severe AP)	Severe AP according to 1992 Atlanta classification (21 patients)	>32 pg/mL	86	48	0.679	[145]
				>46 pg/mL	62	74		
	69 adult patients with severe AP	At admission (≤48 h from the onset of pain)	Acute respiratory distress syndrome in the course of severe AP (39 patients)	>168.4 pg/mL	61.1	90.0	0.757	[116]

[1] diagnostic sensitivity; [2] diagnostic specificity; [3] area under receiver operating characteristic curve; [4] reference number; [5] the onset of pain due to AP is considered the onset of the disease.

On the other hand, activated platelets and coagulation have been shown to drive inflammation in AP. Platelets enhance leukocyte rolling, sticking and transmigration in pancreatic venules in the early phase of AP [96,166]. Platelet P-selectin appears crucial for leukocyte recruitment and rolling in inflamed venules of the pancreas [167,168]. PAR-2 signaling was implicated in AP pathogenesis; its inhibition protected mice against experimental biliary pancreatitis and AP-associated lung injury [169,170].

These observations have led to the assumption that anticoagulant or antithrombotic treatment in AP may result not only in reduction of (micro) thrombosis and improved microcirculation but also in reduced local and systemic inflammation.

7. Therapeutic Effects of Anticoagulants in Acute Pancreatitis

7.1. Heparin

Heparin as a cofactor of antithrombin inhibits thrombin activity as well as factor Xa activity and thus thrombin formation. Heparin attenuates not only procoagulant but also proinflammatory effects of thrombin. Moreover, heparin in a complex with antithrombin or heparin cofactor II is able to reduce activity of trypsin and chymotrypsin as well as the conversion of trypsinogen into trypsin [171,172]. Various experimental studies have shown the anti-inflammatory effect of heparin, administered both in a protective and therapeutic manner in AP [171,173–178]. Earlier studies have been summarized by Hackert et al. in 2004 [179].

In humans, several trials explored the utility of unfractioned or low molecular weight heparin (LMWH) to prevent post-ERCP pancreatitis in high-risk patients [180–183]. Neither of the studies demonstrated reduced AP or SAP ratios in the treatment groups. A meta-analysis [177] performed in 2011 that included the four trials cited above (1438 patients in total) did not show significant benefit of prophylactic heparin in prevention of post-ERCP pancreatitis (relative risk 0.67, 95% confidence interval 0.44–1.03, $p = 0.07$) or post-ERCP SAP (relative risk 0.62, 95% confidence interval 0.15–2.60, $p = 0.51$). There were no differences between unfractioned and low molecular weight heparin. Of note, no increased bleeding risk was shown as well (relative risk for ERCP-related hemorrhage 0.84, 95% confidence interval 0.34–2.03, $p = 0.69$).

Heparin has been used in the treatment of SAP caused by severe hypertriglyceridemia. Such treatment is justified by the ability of heparin to stimulate lipoprotein lipase activity [184]. Several case reports or case series suggested effectiveness of heparin, usually administered in conjunction with insulin, in lowering triglyceride concentrations in such patients [184–187]. The results of a clinical trial evaluating the effects of LMWH and intensive insulin therapy in SAP were published in 2014 [188]. The trial included 134 adult patients with SAP treated in single-center (General Hospital of PLA, Beijing, China), randomly assigned to four groups: control group treated conventionally, intensive insulin therapy group, LMWH group (5000 U every 12 h) and combined treatment group (insulin plus LMWH), in addition to conventional therapy. Authors reported reduced lengths of stay, incidence of multiorgan failure, need for surgery and mortality in treatment groups, with best results of combined treatment. Four patients (12%) died in the control group (conventional treatment), as compared to one death (3%) in the intensive insulin therapy group, one death (3%) in the LMWH group and no deaths in the group administered combined therapy.

The use of LMWH for treatment of SAP was also evaluated in a multicenter randomized trial that recruited 265 patients from four hospitals from China [189,190]. LMWH was administered in dose 100 μg/kg/day starting at admission, until day 7 of the hospital stay. Balthazar computed that the tomography scores at the end of the first and second week of the hospital stay were better in the treatment group than in the control group (conservative treatment), as well as APACHE II score for week 2. The incidence of acute respiratory distress syndrome, pancreatic encephalopathy, multiorgan failure, and mortality (10.4% versus 30.6%) was lower in the treatment group.

7.2. Activated Protein C

In experimental SAP, treatment with activated protein C (APC) resulted in decreased inflammation (decreased expression of pancreatic TNF-α and IL-1β proteins, decreased serum TNF-α, IL-8 and IL-6), increase in pancreatic expression of endothelial protein C receptor and thrombomodulin, and reduced severity of pancreatic morphological changes, including necrosis [149,191,192]. Bacterial translocation to mesenteric lymph nodes and to pancreas was reduced in APC-treated rats with SAP [192]. However, a contradictory report was also published, illustrating that administration of APC did not result in improved histopathologic scores of the pancreas and, in fact, was associated with significantly higher serum IL-6 [193]. In the study of Alsfasser et al. [194], despite no difference in the histopathologic scores of the pancreas, rats with SAP that were treated with APC presented reduced pancreatic and pulmonary inflammation (reduced myeloperoxidase activity) and improved survival.

A small clinical trial was undertaken to evaluate safety and efficacy of the treatment with APC in AP (APCAP study) [195]. A prospective double-blind randomized study included 32 patients with SAP and no sepsis from a single center (Helsinki University Central Hospital, Helsinki, Finland). Patients were admitted within 96 h from the onset of pain. APC was administered intravenously for 96 h in a fixed dose of 24 μg/kg/h; physiologic saline was used as placebo. All patients received the treatment according to the initial randomization. No significant difference was observed between treatment and placebo groups regarding the primary efficacy endpoint, i.e., the change in SOFA score between the start of the study drug (day 0) and day 5. In fact, a small non-significant difference in advantage of placebo was found. Three deaths due to multiple organ failure occurred in the treatment group and none in the placebo group (autopsy excluded bleeding as a cause of the deaths). The only significant difference was the increase in total and conjugated bilirubin in the treatment group. No serious bleeding occurred in treated patients; four patients had minor bleeding (from mouth, nose, or urinary tract) versus two patients in the placebo group. Recent analysis revealed that APC did not alleviate coagulative disorders in patients included in the APCAP study; rather, the treatment with APC was associated with retarded recovery from coagulopathy [196].

7.3. Soluble Thrombomodulin

Eguchi et al. [197] performed a retrospective analysis of 54 adult patients with SAP diagnosed according to Japanese severity scoring system, treated in a single center (Osaka Saiseikai Nakatsu Hospital, Osaka, Japan), of whom 24 developed DIC and were therefore treated with recombinant human soluble thrombomodulin (rTM). The study included patients in whom treatment started within the first 48 h from the onset of pain. Patients who were subsequently treated with rTM had on average more severe disease, with higher APACHE II and SOFA scores on admission, i.e., before treatment. Acute necrotizing collections within the pancreas were equally prevalent in both treated and untreated groups at admission. rTM was administered in dose 380 or 130 U/ kg/day for patients on hemodialysis. The treatment was introduced in those who were diagnosed with DIC and continued until remission of DIC. Other aspects of AP treatment did not differ between the groups. The length of hospital stay, need for intensive care, length of intensive care unit stay, incidence of persistent organ failure, or mortality did not differ between the groups. Walled-off necrosis at 4 weeks from admission or later was significantly less prevalent in the treated group (29% versus 57% of patients). No serious adverse events (e.g., bleeding) were recorded in the treatment group.

7.4. Other Anticoagulants

In a recent experimental study, pretreatment with low doses of acenocoumarol, a vitamin K antagonist, attenuated ischemia/reperfusion-induced AP and was associated with reduced leukocyte inflammatory infiltration of the pancreas as well as diminished pancreatitis-induced increase in serum IL-1β concentrations [198]. The results were confirmed in another model of experimental AP, i.e., cerulein-induced AP in rats [199]. Since acenocoumarol is a commonly used antithrombotic drug

with well-known safety profile, its usefulness in prevention of AP among patients with pancreatic circulation disorders would be worth studying.

Pretreatment with antithrombin was shown to ameliorate cerulein-induced AP in rats [200]. Edema, inflammation and necrosis of the pancreas were partially reduced and serum concentrations of IL-6, TNF-α, and high-mobility box group 1 protein were diminished in mice pretreated with antithrombin. Similar results were obtained with pretreatment with low molecular weight heparinoid, danaparoid sodium, that was also shown to inhibit cerulein-induced NFκB activation [201].

Andersson et al. [202] evaluated the effects of pretreatment with active side-inactivated factor VIIa in rats. AP was then induced by infusion of sodium taurodeoxycholate into common bile duct. Myeloperoxidase activity was significantly reduced in ileum and lungs of pretreated animals, and serum concentrations of inflammatory markers were lowered.

8. Conclusions

The interplay between inflammation, coagulation and endothelial activation is involved in the earliest local events in acute pancreatitis (AP), and is associated with the early phase of systemic disease in severe AP (SAP), although many aspects remain unknown. From the current evidence on this subject, there are several practical conclusions: (1) Systemic inflammation as seen in SAP is not rarely associated with thrombotic disorders, and the activation of coagulation may further aggravate inflammation. Laboratory tests in SAP often reveal abnormalities of coagulation, while clinically relevant disorders of coagulation in AP are associated with significantly worse prognosis; (2) Markers of coagulation/fibrinolysis measured early in the course of AP, in particular D-dimer, are significantly associated with AP severity, and may therefore be used to assist treatment decisions and prognosis; (3) Markers of endothelial dysfunction, in particular angiopoietin-2, may prove even more useful; however, we need robust, routine and standardized laboratory tests, currently available only for sFlt-1; (4) There are severe difficulties in translation between animal experiments using anticoagulant or antithrombotic medications and their use in humans. There may be several reasons. Patients with AP form a much more heterogeneous group than the experimental animals, both regarding the causes of AP and the time from the disease onset to the start of treatment. In substantial proportion of patients, systemic inflammation and organ failure are present already at admission. Also, there are difficulties in recruiting enough patients for clinical trials; (5) Nevertheless, some reports from clinical trials with low molecular weight heparin treatment are promising. However, the results have to be corroborated by other groups before this treatment can be recommended; (6) Although the use of anticoagulants was rarely associated with significant benefits in clinical trials, there were also no significant bleeding complications. This fact encourages the use of such drugs in the treatment of thrombotic complications of AP.

Further clarification of the relationships between inflammation, pancreatic blood flow, coagulation system, endothelial involvement and the development and course of AP is needed. Several anticoagulant or antithrombotic drugs have ameliorated AP severity in experimental designs, and their therapeutic potential is still worth being tested in patients.

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Abbreviations

AP	acute pancreatitis
NFκB	nuclear factor κB
SAP	severe acute pancreatitis
TF	tissue factor
PAR	protease-activated receptor
sCD40L	soluble CD40 ligand
TNF	tumor necrosis factor
vWF	von Willebrand factor
IL	interleukin
CRP	C-reactive protein
PAI-1	plasminogen activator inhibitor-1
ICAM-1	intercellular adhesion molecule-1
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
PAF	platelet activating factor
ERCP	endoscopic retrograde cholangiopancreatography
SIRS	systemic inflammatory response syndrome
sFlt-1	soluble fms-like tyrosine kinase 1
APACHE	acute physiology and chronic health evaluation
SOFA	sequential organ failure assessment
ADAMTS-13	disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13
DIC	disseminated intravascular coagulation
CT	computed tomography
TFPI	tissue factor pathway inhibitor
tPA	tissue plasminogen activator
LMWH	low molecular weight heparin
APC	activated protein C
rTM	recombinant human soluble thrombomodulin

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Artykuł nr 2

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Research Article

Angiopoietin-2 Is an Early Indicator of Acute Pancreatic-Renal Syndrome in Patients with Acute Pancreatitis

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Within the first week of the disease, acute kidney injury (AKI) is among the most common causes of mortality in acute pancreatitis (AP). Recently, serum angiopoietin-2 (Ang-2) has been associated with hyperdynamic state of the systemic circulation. The aim of this study was to examine the associations between Ang-2 and the clinical AP severity during the first 72 hours of the disease, and organ disfunction, including AKI. *Methods*. Study included patients admitted to the surgery ward, diagnosed with AP. AKI was diagnosed according to KDIGO guidelines and renal failure according to modified Marshall scoring system. Ang-2 was determined in serum with ELISA. *Results*. AP was classified as mild (MAP) in 71% of patients, moderately severe (MSAP) in 22%, and severe (SAP) in 8%. During the first 72 hours of AP, 11 patients developed AKI and 6 developed renal failure. Ang-2 at 24, 48, and 72 hours following the onset of AP symptoms significantly predicted SAP and MSAP, as well as AKI and renal failure. Also, Ang-2 significantly correlated with acute phase proteins as well as with the indicators of renal disfunction. *Conclusions*. Serum Ang-2 may be a relevant predictor of AP severity, in particular of the development of AP-renal syndrome.

1. Introduction

Destabilization of the vascular endothelium as well as escape of fluid from the vascular bed is one of the main factors leading to vasodilatation, decrease in blood pressure, and the development of early complications of acute pancreatitis (AP), which clinically manifest themselves as shock and developing organ failure [1, 2]. The patient's transfer to the intensive care unit, initiation of intensive fluid resuscitation within the first 12–24 hours of the onset of severe acute pancreatitis (SAP), close monitoring of organ function, and, in case of acute kidney injury (AKI) in circulatory unstable patients, implementation of continuous blood purification techniques (continuous venovenous hemofiltration or continuous venovenous hemodiafiltration) may have a decisive impact on the survival in this group of patients [1, 3, 4].

Such procedures are particularly justified because, in nearly half of patients classified as SAP, organ failure is diagnosed on admission, whereas the other 50% of patients develop symptoms of organ failure in the first four days of

hospitalization [5, 6]. Moreover, more than a half of deaths in SAP patients occur within the first week of the disease [7]. AKI and acute respiratory distress syndrome are the most common causes of mortality (70–80%) in this group of patients [4, 8].

Currently, for the diagnostics of AKI, the risk, injury, failure, loss, and end stage (RIFLE) and the Kidney Disease Improving Global Outcomes (KDIGO) criteria are used. Both criteria are based on the increase in serum creatinine and the decrease in diuresis [4, 9]. In the treatment of AKI, it is critical to quickly determine the cause of kidney injury (prerenal, intrinsic, or postrenal). Unfortunately, an increase in serum creatinine concentration usually occurs about 1-2 days after kidney injury, which often delays the possibility of taking action in the reversible phase of the disease. The limited value of serum creatinine in the early phase of AKI as well as a burdensome necessity to monitor diuresis requiring (invasive) urinary catheterization urges us to search for new biomarkers of kidney injury. Early indicators include urine concentrations of neutrophil gelatinase-associated lipocalin (uNGAL), kidney injury molecule-1 (KIM-1), and interleukin-18 (IL-18) [9].

One of the promising prognostic biomarkers of acute pancreatitis is angiopoietin-2 (Ang-2) [10]. Studies by Whitcomb et al. [11] showed that Ang-2 concentration is considerably increased already at the time of the diagnosis of AP and is significantly higher in patients with developing persistent organ failure [11]. Watanabe et al. [12] demonstrated a correlation between Ang-2 concentrations and perfusion parameters using computed tomography [12]. Increased Ang-2 concentrations in blood are associated with hyperdynamic state of the systemic circulation, and Ang-2 monitoring may be helpful in the evaluation of the response to systemic treatment, that is, fluid resuscitation, or antithrombotic therapy [12].

Ang-2 belongs to a new class of angiogenic growth factors that exerts a selective effect on the endothelium by binding with Tie-2 receptor. It was demonstrated that Ang-2 has an inhibitory effect on angiopoietin-1 binding to Tie-2, which leads to destabilization of the vascular endothelium, increased fluid leakage, and leukocyte adhesion [2, 12, 13]. Under physiologic conditions, Ang-2 is stored within Weibel-Palade bodies, along with P-selectin, von Willebrand factor, CD63, interleukin-8, and endothelin-1 [14]. Factors such as hypoxia, inflammation, or mechanical injury may cause the rapid release of Ang-2 into the systemic circulation [13, 14]. Ang-2 was proposed to be a link between angiogenic and inflammatory pathway [14]. Serum concentrations of Ang-2 seem to reflect the extent of endothelial activation [2] and may be useful in early clinical assessment of patients with acute conditions, including AP.

The aim of this preliminary study was to assess Ang-2 serum concentrations in patients with AP of various severity in the early phase of the disease, that is, during the first 72 hours of the disease, as well as to examine the associations between Ang-2 and the clinical measures of the severity of AP, in particular organ dysfunction, including AKI.

2. Materials and Methods

The prospective observational study included 65 consecutive adult patients (men and women) admitted with AP, hospitalized, and treated in the Department of Surgery, District Hospital in Sucha Beskidzka, Poland, between January and December 2014. AP was diagnosed according to the 2012 revision of the Atlanta classification, that is, when at least two of the three following features were present: abdominal pain consistent with AP (acute onset and persistent and severe and epigastric pain); serum lipase or amylase activity at least three times greater than the upper reference limit; and the characteristic findings of AP on contrast-enhanced computer tomography or magnetic resonance imaging or transabdominal ultrasonography [15]. Only adult patients who signed the informed consent for the study were included. Patients with symptoms of AP lasting longer than 24 hours were excluded. Also, the exclusion criteria were chronic pancreatitis, neoplasms, and chronic liver diseases such as cirrhosis or viral hepatitis.

On the basis of the clinical evaluation of the severity of AP, the patients were assigned to one of the 3 groups: with mild acute pancreatitis (MAP), moderately severe acute pancreatitis (MSAP), or severe acute pancreatitis (SAP). The MAP group included patients who did not show any organ failure or local complications. Patients with organ failure lasting less than 48 hours (transient organ failure), local complications (necrosis, acute necrotic collection, and walled-off pancreatic necrosis), and/or exacerbation of comorbidity were assigned to MSAP. Patients with persistent organ failure (lasting more than 48 hours) and ≥1 local complications were assigned to SAP [15].

AKI was diagnosed according to KDIGO guideline [16], that is, when serum creatinine increased ≥26.5 μmol/L within 48 hours or ≥1.5 times which is known or presumed to occur within 7 days, or urine volume <0.5 mL/kg/h for 6 hours. Organ failure was diagnosed according to the modified Marshall scoring system (MMSS), as cited in 2012 revision of the Atlanta classification [15]; in particular, renal failure was diagnosed in patients with serum creatinine ≥170 μmol/L.

In order to determine serum Ang-2 concentrations in healthy persons, a control group was recruited which included 21 healthy volunteers (men and women) at the age similar to that of the study group, without any pancreatic, liver, or renal diseases.

The study protocol was approved by the Bioethics Committee of the Jagiellonian University (Approval number KBET/247/B/2013).

2.1. Laboratory Tests. Blood and urine for laboratory tests were collected at 24, 48, and 72 hours from the onset of symptoms of AP. The routine laboratory tests and the measurements of uNGAL, urine albumin, and urine creatinine were performed at the day of collection, while serum samples for serum NGAL (sNGAL) and Ang-2 measurements were frozen in aliquots and stored in −70°C. The routine tests included complete blood count, urinalysis, serum amylase, total calcium, albumin, urea, creatinine, glucose, C-reactive protein, plasma fibrinogen, and D-dimer. All the routine

Table 1: Clinical characteristics of patients included in the study.

Variable	Patients with acute pancreatitis, $n = 65$
Male sex, *n* (%)	34 (52)
Age, years	62 ± 16
Etiology:	
Gallstones, *n* (%)	33 (51)
Alcohol, *n* (%)	18 (28)
Hypertriglyceridemia, *n* (%)	5 (8)
After ERCP, *n* (%)	1 (2)
Others, *n* (%)	8 (12)
Duration of pain until admission, hours	12 (6–24)*
Duration of hospital stay, days	6 (5–12)*
Severity:	
Mild acute pancreatitis, *n* (%)	46 (71)
Moderately severe acute pancreatitis, *n* (%)	14 (22)
Severe acute pancreatitis, *n* (%)	5 (8)
BISAP score:	
=2 in the first 24 hours, *n* (%)	12 (18)
≥3 in the first 24 hours, *n* (%)	6 (9)
Comorbidities, *n* (%)	50 (80)
SIRS, *n* (%)	8 (12)
Pancreatic or peripancreatic necrosis, *n* (%)	3 (5)
Peripancreatic fluid collections, *n* (%)	5 (8)
Transient organ failure, *n* (%)	6 (9)
Persistent organ failure, *n* (%)	5 (8)
Pleural effusion, *n* (%)	13 (20)
Acute kidney injury (KDIGO), *n* (%)	11 (17)
Renal failure (MMSS), *n* (%)	6 (9)
Antibiotic prophylaxis, *n* (%)	31 (48)
Parenteral nutrition, *n* (%)	3 (5)
Surgery, *n* (%)	3 (5)
Early mortality/late mortality, *n* (%)	0/3 (5)

*Data are presented as median (lower–upper quartile).
n (%): number of patients in each category (percentage of the studied group of 65 patients); BISAP: bedside index for severity in acute pancreatitis; SIRS: systemic inflammatory response syndrome; ERCP: endoscopic retrograde cholangiopancreatography; MMSS: modified Marshall scoring system; KDIGO: Kidney Disease Improving Global Outcomes.

laboratory tests and the measurements of uNGAL, urine albumin, and urine creatinine were performed in the Department of Laboratory Diagnostics, District Hospital in Sucha Beskidzka, Poland. Complete blood counts were analyzed in K_2EDTA-anticoagulated blood with the Sysmex XE 2100 (Sysmex Corp., Japan) automated hematology analyzer. Urine NGAL concentrations were determined with the ARCHITECT Analyzer (Abbott Park, USA) using chemiluminescent microparticle immunoassay. Ang-2 and sNGAL measurements were performed in the Department of Diagnostics, Chair of Biochemistry, Jagiellonian University Medical College in Cracow, Poland. Serum NGAL concentrations were measured with the Human Lipocalin-2/NGAL ELISA kits (BioVendor, Brno, Czech Republic). Serum Ang-2 were measured with the Quantikine ELISA Human Angiopoietin-2 Immunoassay kits (R&D Systems, Minneapolis, USA).

2.2. Statistical Analysis. Data are shown as number of patients (percentage of the group) for categories and median (25th–75th percentile) or mean ± standard deviation for qualitative variables, depending on distribution (as assessed using the Shapiro-Wilk test). As the distributions of Ang-2 concentrations significantly differed from normal, the nonparametric tests were used. In detail, the differences between the groups were studied using the Kruskal-Wallis ANOVA or Mann-Whitney test, and the Spearman coefficient was used to assess correlations. In order to study associations between Ang-2 and the measures of AP severity, simple and multiple logistic regression were used, and the resulting odds ratios (ORs) were reported with 95% confidence intervals (95% CIs). Results were considered statistically significant at $p < 0.05$. The Statistica 10.0 (StatSoft, Tulsa, USA) software package was used for computations.

3. Results

In 71% of the study patients, AP was classified as mild, 22% had MSAP, and 8% had SAP (Table 1). In most patients, comorbidities were observed, most commonly hypertension (22 patients, 34%), ischemic heart disease (18 patients, 28%), and diabetes (10 patients, 15%); seven patients (11%) were diagnosed with lung diseases and 3 (5%) with kidney diseases (chronic kidney disease stages G3 in 2 patients and G4 in 4 patients). During the first 72 hours of AP, 11 patients developed AKI according to the KDIGO criteria, including stage 1 in 10 patients and stage 2 in one patient. According to the modified Marshall scoring system, renal failure was diagnosed in 6 patients. The detailed clinical characteristics of the patients are presented in Table 1.

The median serum concentrations of Ang-2 were about 2 times higher in the whole group of AP patients than in healthy controls (Table 2). However, the median Ang-2 concentrations in MSAP and especially in SAP patients were several times higher; the highest Ang-2 concentrations in those groups were observed in serum samples taken 24 hours after the onset of symptoms of AP (Figure 1). The median concentrations of urea, creatinine, and uNGAL in the whole group of patients were within the reference ranges (Table 2); however, 21 patients had an estimated glomerular filtration rate (eGFR, based on MDRD equation) of less than 60 mL/min/1.73 m^2 at 24 hours. The concentrations of urea and creatinine were the highest at 24 hours and declined with treatment (Table 2).

Ang-2 concentrations in the first 72 hours of AP significantly predicted more severe disease (Table 3). In particular, Ang-2 was a significant predictor of AKI (diagnosed based on the KDIGO criteria) and renal failure (diagnosed according to the modified Marshall scoring system) both in simple analysis (Table 3) and after adjustment for age, sex, and comorbidities, with ORs for AKI of 1.13 (1.01–1.26); 1.40 (1.11–1.77); and 1.66 (1.18–12.32) and ORs for renal failure of 1.14 (1.02–1.28); 1.38 (1.10–1.74); and 1.84 (1.16–2.92) per 1 ng/mL

Table 2: Laboratory data in the whole group of patients with acute pancreatitis ($n = 65$).

Variable	24 hours	48 hours	72 hours	Reference values
Ang-2 [ng/mL]	3.18 (2.10–4.64)	3.33 (2.06–5.65)	3.12 (2.17–5.21)	1.73 ± 0.38 (range: 1.17–2.47)*
CRP [mg/L]	14.00 (2.60–86.70)	119.70 (52.00–237.40)	104.80 (33.80–227.80)	<5.0
WBC [$\times 10^3/\mu$L]	11.21 (9.55–15.05)	9.80 (6.62–12.87)	8.43 (6.17–10.62)	4.00–10.00
HCT [%]	42.8 (39.1–45.5)	39.8 (35.1–42.8)	39.5 (35.4–42.2)	F: 37.0–47.0/M: 40.0–54.0
PLT [$\times 10^3/\mu$L]	234 (188–259)	197 (160–230)	206 (168–246)	150–350
Amylase [U/L]	1069 (618–1844)	162 (114–316)	92 (62–152)	62–220
Glucose [mmol/L]	7.81 (6.43–10.52)	5.39 (4.53–6.20)	5.28 (4.76–6.42)	3.3–5.6
Urea [mmol/L]	6.07 (4.31–7.97)	4.36 (3.35–7.33)	4.39 (3.35–5.44)	2.76–8.07
Creatinine [μmol/L]	76.0 (65.7–99.1)	70.9 (62.4–90.7)	70.5 (57.7–89.1)	45.0–97.0
Fibrinogen [g/L]	2.80 (2.20–3.55)	3.87 (3.11–4.98)	4.49 (3.52–5.36)	1.8–3.5
D-dimer [μg/L]	1683 (982–3293)	2076 (1300–4512)	2039 (975–4670)	<500
sNGAL [μg/L]	117.1 (70.8–208.5)	170.9 (100.8–237.1)	166.7 (102.2–216.6)	F: 21.6–276.0/M: 14.4–169.2
uNGAL [μg/L]	28.5 (15.5–57.0)	38.3 (16.5–95.3)	41.7 (17.0–65.1)	<131.7
uACR [mg/g]	43.3 (21.0–79.4)	35.8 (20.8–89.5)	22.2 (14.2–92.3)	<30

Data are presented as median (lower–upper quartile); *reference values established in 21 healthy controls; Ang-2: angiopoietin-2; CRP: C-reactive protein; WBC: white blood cells; HCT: hematocrit; PLT: platelets; NGAL: neutrophil gelatinase-associated lipocalin in (s) serum and (u) urine; uACR: urine albumin/creatinine ratio.

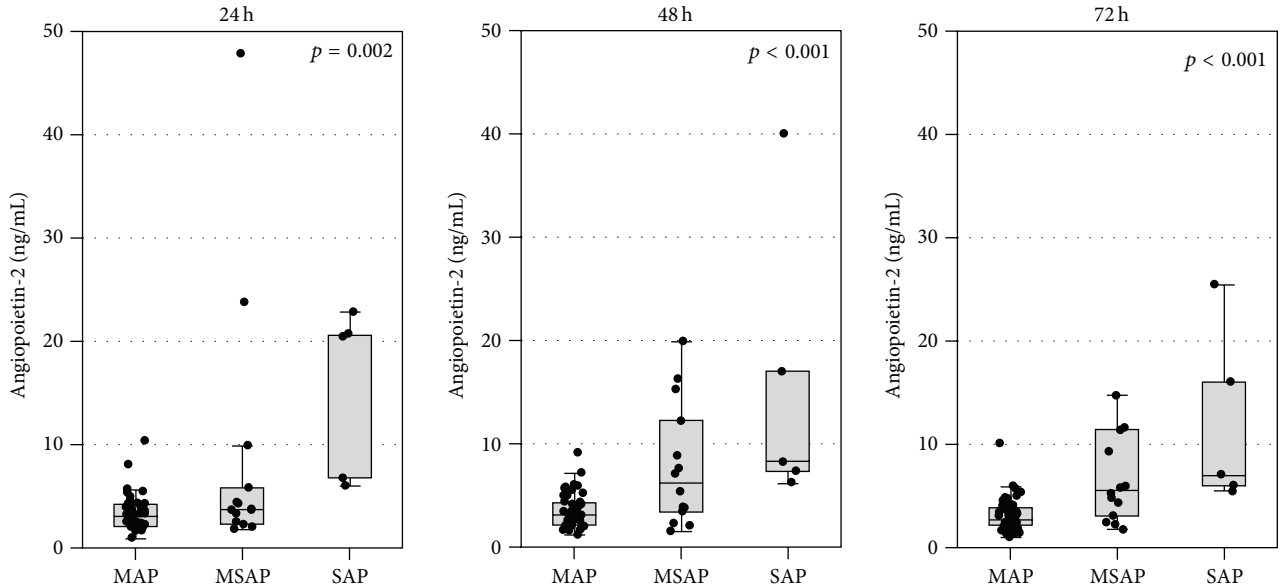

Figure 1: Serum angiopoietin-2 concentrations at 24, 48, and 72 hours after the onset of symptoms in patients with mild (MAP), moderately severe (MSAP), and severe acute pancreatitis (SAP). Data are shown as median, 25th–75th percentile (boxes), and nonoutlier range (whiskers); the points represent raw data.

increase in Ang-2 concentration at 24, 48, and 72 hours, respectively. The concentrations of Ang-2 in patients with a creatinine ≥170 μmol/L (i.e., renal failure according to the modified Marshal scoring system) are shown in Figure 2.

During the first 72 hours of AP, serum Ang-2 concentrations were significantly correlated with several laboratory markers used in routine monitoring of AP patients, that is, hematocrit, CRP, fibrinogen, or D-dimer (Table 4). Also, Ang-2 negatively correlated with albumin and calcium concentrations at 48 and 72 hours (Table 4). Interestingly, Ang-2 concentrations were positively correlated with markers related to kidney function, that is, urea, sNGAL, and uNGAL, during the first 48 hours, as well as creatinine and urine albumin/creatinine ratio (uACR) during the entire study (Table 4).

4. Discussion

Acute pancreatitis is a relatively common disease that in most patients is mild or self-limiting; however, about 20% of patients develop SAP associated with life-threatening complications and a mortality of 20–30% [3, 9]. In older patients (over 60 years of age), being obese (with a body mass index greater than 30) or with chronic comorbidities such as cardiovascular diseases or chronic kidney disease, the mortality can reach 50–80% [4].

Table 3: Angiopoietin-2 serum concentrations as a predictor of the severity of acute pancreatitis—results of simple logistic regression.

Dependent variable	OR (95% CI), per 1 ng/mL increase in Ang-2 concentration		
	24 h	48 h	72 h
SAP	1.11 (1.01–1.22)	1.22 (1.03–1.46)	1.31 (1.07–1.60)
SAP or MSAP	1.29 (1.03–1.62)	1.58 (1.18–2.10)	1.70 (1.21–2.38)
BISAP in the first 24 hours ≥3	1.18 (1.04–1.33)	1.37 (1.12–1.68)	1.68 (1.21–2.20)
SIRS	1.25 (1.08–1.44)	1.49 (1.17–1.90)	1.48 (1.17–1.88)
AKI (KDIGO)	1.12 (1.02–1.24)	1.37 (1.12–1.68)	1.49 (1.17–1.90)
Renal failure (MMSS)	1.10 (1.01–1.21)	1.28 (1.08–1.52)	1.44 (1.15–1.80)
Pleural effusion	NS	1.25 (1.06–1.47)	1.33 (1.10–1.63)
Death	NS	1.27 (1.02–1.59)	1.42 (1.07–1.89)

For abbreviations, see Table 1; AKI: acute kidney injury.

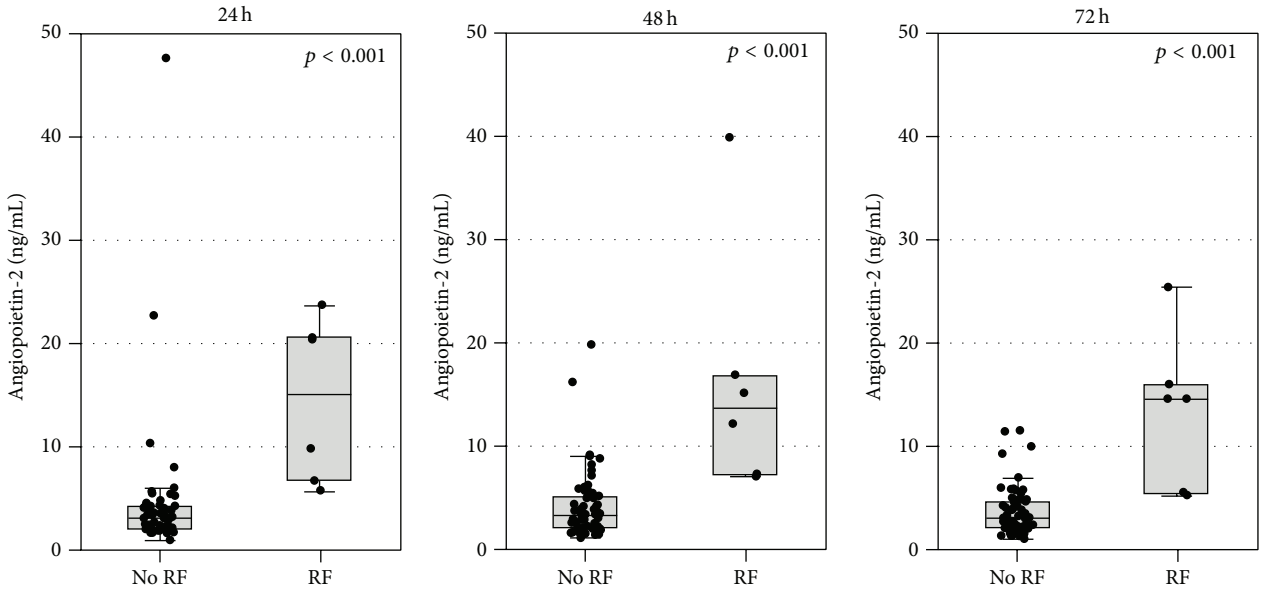

Figure 2: Serum angiopoietin-2 concentrations at 24, 48, and 72 hours after the onset of symptoms in AP patients without renal failure (no RF) and with renal failure (RF) diagnosed according to the modified Marshall scoring system. Data are shown as median, 25th–75th percentile (boxes), and nonoutlier range (whiskers); the points represent raw data.

In the present study, serum Ang-2 concentrations significantly predicted the severity of AP in the early phase of the disease. Also, the correlations were found between Ang-2 and the laboratory markers of organ failure and inflammation. In particular, Ang-2 significantly correlated with the markers of kidney function and predicted both AKI diagnosed according to KDIGO definition and renal failure diagnosed according to modified Marshall scoring system. The most reliable indicator of the severity of AP is organ failure persisting more than 48 hours [17]. The 2012 revision of Atlanta classification recommends the assessment of organ failure in AP with the use of the modified Marshall scoring system [15]. Although the pathogenesis of AKI in SAP is not fully elucidated, AKI significantly contributes to mortality in AP. Especially susceptible are elderly patients with reduced glomerular filtration and patients with history of renal disease [16, 18]. In the revised Atlanta classification, a patient's age over 60 years and the presence of comorbidities are recognized factors that significantly worsen the prognosis and are associated with more severe AP; this should be taken into account in the preliminary assessment of patients [15].

The results of the present study are in concordance with the observations of Buddingh et al. [10] and Whitcomb et al. [11] who reported the association between high serum Ang-2 concentrations and SAP. Also, increased Ang-2 has been recently associated with AKI [19].

Ang-2 is found in endothelial cells at the sites of vascular remodeling, and its autocrine effect weakens the interaction between endothelial cells and the surrounding cells, especially pericytes [20]. Vascular endothelial growth factor (VEGF) participates in the regulation of Ang-2 synthesis [21]. In the initial phase of inflammation and in the absence of VEGF, Ang-2 causes vascular regression by inducing apoptosis of endothelial cells. In the systemic inflammatory process, activated neutrophils and monocytes release a range of enzymes such as phospholipase, elastase, or lipocalin-2,

Table 4: Simple correlations between angiopoietin-2 and selected variables during the first 72 hours of acute pancreatitis.

Variable	24 h		48 h		72 h	
	R	*p*	*R*	*p*	*R*	*p*
Age	0.27	0.033	0.26	0.035	0.26	0.036
Urea	0.36	0.003	0.41	<0.001	0.34	0.004
Creatinine	0.30	0.014	0.34	0.006	0.08	NS
CRP	0.48	<0.001	0.31	0.010	0.35	0.004
Albumin	−0.23	NS	−0.45	<0.001	−0.48	<0.001
Calcium	−0.10	NS	−0.44	<0.001	−0.43	<0.001
HCT	−0.30	0.014	−0.36	0.003	−0.39	0.001
Fibrinogen	0.28	0.024	0.26	0.034	0.46	<0.001
D-dimer	0.40	0.001	0.44	<0.001	0.38	0.001
sNGAL	0.53	<0.001	0.52	<0.001	0.50	<0.001
uNGAL	0.59	<0.001	0.57	<0.001	0.52	<0.001
uACR	0.29	0.022	0.33	0.009	−0.01	NS

Ang-2: angiopoietin-2; HCT: hematocrit; NGAL: neutrophil gelatinase-associated lipocalin in (s) serum and (u) urine; CRP: C-reactive protein; uACR: urine albumin/creatinine ratio.

which intensifies degradation of phospholipids and may lead to pancreatic and peripancreatic necrosis, as well as intensified infiltration of other organs by inflammatory cells [4]. In the present study, the concentrations of Ang-2 in the systemic circulation were higher in patients than in controls and correlated with inflammatory markers, that is, CRP, fibrinogen, and NGAL concentrations in serum.

The renal endothelium is identified as a rich source of Ang-2. Its release from the kidneys may increase due to kidney injury, for example, in the course of AP [2, 14, 22, 23]. The correlations between Ang-2 and urea, creatinine, uNGAL, and albuminuria support the association of Ang-2 with kidney injury. Urine NGAL is a promising biomarker of AKI [24]. As a low molecular weight lipocalin, NGAL is easily filtered by healthy glomeruli and then almost completely absorbed in proximal tubules. The main fraction of uNGAL in AKI is synthesized in the distal tubules of the glomeruli in response to a damaging factor. In turn, increased sNGAL concentrations are observed as a consequence of either decreased glomerular filtration or increased synthesis by activated neutrophils during inflammation. In AP, various factors may lead to AKI, such as the effect of pancreatic amylase on the renal microcirculation, hypoxemia, or a toxic effect of excessively produced pancreatic phospholipase A_2 on the proximal tubules. However, the primary cause of the development and progression of AKI in AP is associated with hypovolemia associated with a decrease in filtration pressure in the kidneys [4]. Hypovolemia is a result of a systemic inflammatory state initiated by AP, associated with vasodilation, escape of fluid into the third space, and increase in intra-abdominal pressure. In patients with SAP, intra-abdominal hypertension is observed, which significantly contributes to the reduced perfusion of organs, including the kidneys [25]. Dehydration and hypovolemia are also intensified by vomiting or fever, often accompanying AP. These factors cause a decrease in renal perfusion and, as a consequence, damage to the renal tubules and the increase in uNGAL concentrations.

In chronic kidney disease, albuminuria is a recognized indicator of renal function, assessed along with eGFR; uACR is recommended as a simple measure of albuminuria [18]. In AKI, albuminuria may be associated with an injury to the endothelium of glomerular vessels, being a part of a filtration barrier. The other mechanism responsible for albuminuria in AKI is an injury to the proximal tubule and reduced albumin reabsorption from primary urine. Recently, albuminuria observed during the first 72 hours of ICU stay has been associated with worse outcomes in patients with sepsis-associated AKI [26]. On the other hand, the meta-analyses performed by CKD prognosis consortium, published in 2015, have recognized albuminuria (uACR) as a risk factor for AKI [27, 28]. In the present study, uACR showed a positive correlation with serum Ang-2 concentrations during the first 48 hours following the onset of AP. Tsai et al. [14] and Chang et al. [29] reported the association between increased Ang-2 and albuminuria in patients with chronic kidney disease.

5. Conclusions

Early diagnosis and quick and accurate determination of the cause are critical for the effective treatment of AKI. The present study shows a correlation between increased serum Ang-2 concentrations in patients with AP and deteriorated renal function in the early phase of the disease. High Ang-2 concentrations correlated with uNGAL and uACR in patients with AP in the "therapeutic window," that is, within the first 48 hours following the onset of AP. Thus, serum Ang-2 may be a relevant predictor of the development of acute pancreatic-renal syndrome and a useful tool for a clinician in the evaluation of disease severity.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Artykuł nr 3

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Serum Soluble Fms-Like Tyrosine Kinase 1 (sFlt-1) Predicts the Severity of Acute Pancreatitis

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Abstract: Organ failure is the most important determinant of the severity of acute pancreatitis (AP). Soluble fms-like tyrosine kinase 1 (sFlt-1) is positively associated with organ failure in sepsis. Our aim was to evaluate the diagnostic utility of automated sFlt-1 measurements for early prediction of AP severity. Adult patients (66) with AP were recruited, including 46 with mild (MAP), 15 with moderately-severe (MSAP) and 5 with severe AP (SAP). Serum and urine samples were collected twice. Serum sFlt-1 was measured with automated electrochemiluminescence immunoassay. Serum concentrations of sFlt-1 were significantly higher in patients with MSAP and SAP as compared to MAP. SAP patients had the highest concentrations. At 24 and 48 h, sFlt-1 positively correlated with inflammatory markers (leukocyte count, C-reactive protein), kidney function (creatinine, urea, cystatin C, serum and urine neutrophil gelatinase-associated lipocalin, urine albumin/creatinine ratio), D-dimer and angiopoietin-2. sFlt-1 positively correlated with the bedside index of severity in AP (BISAP) score and the duration of hospital stay. Serum sFlt-1 above 139 pg/mL predicted more severe AP (MSAP + SAP). In the early phase of AP, sFlt-1 is positively associated with the severity of AP and predicts organ failure, in particular kidney failure. Serum sFlt-1 may be a practical way to improve early assessment of AP severity.

Keywords: vascular endothelial growth factor receptor 1; acute pancreatitis; endothelial dysfunction; angiopoietin-2; diagnostic utility

1. Introduction

Acute pancreatitis (AP) is the leading cause of hospital admissions due to gastrointestinal diseases in developed countries [1,2]. In most patients, the disease is mild; however, up to 20% develop the severe form, associated with persistent organ failure and high mortality [3]. According to current

knowledge, as reflected by the Atlanta classification revised in 2012, the development of organ failure in the course of AP is the main factor determining the severity of the disease and related mortality [3]. Systemic inflammatory response syndrome (SIRS), diffuse endothelial activation and dysfunction and microcirculatory disorders are involved in the pathogenesis of organ failure in acute conditions, including AP [4–6].

Angiopoietin-2 (Ang-2), associated with endothelial dysfunction and vascular leakage in acute states [7], has been recently proposed as a marker of severity in AP [8–10]. Other markers of endothelial activation and dysfunction were shown to be increased in severe AP, including soluble E-selectin, tissue factor or von Willebrand factor and endothelial-specific microRNAs [5,11–13].

Fms-like tyrosine kinase-1 (Flt-1) is a membrane receptor binding vascular endothelial growth factor (VEGF)-A and -B, as well as placental growth factor (PlGF). It is also known as VEGF receptor-1 (VEGFR-1). Alternative splicing of Flt-1 precursor mRNA leads to the production of the soluble form of the receptor (sFlt-1) that acts as a decoy receptor to VEGF and PlGF [14]. VEGF is a potent stimulator of vascular permeability (for this reason, it was first named vascular permeability factor) [15]. Severe endothelial dysfunction observed in sepsis is associated with high concentrations of both VEGF and sFlt-1 in blood, and sFlt-1 is significantly correlated with the severity of organ dysfunction in sepsis patients [16]. VEGF has been implicated in the pathogenesis of experimental AP in rats [17–19], and high VEGF concentrations have been observed in plasma of patients with AP [20,21]; but very little is known about sFlt-1 concentrations in the course of AP.

We hypothesized that sFlt-1 may also be associated with AP severity. A practical advantage of this marker over the previously-mentioned Ang-2 or E-selectin is that it may be rapidly measured using routine automated analyzers. The automated assay to measure sFlt-1 has been developed and positively validated for use in the assessment of preeclampsia in pregnant women [22].

The aim of the study was to assess serum concentrations of sFlt-1 among patients with AP of various severity at the early phase of the disease (first 48 h from the onset of abdominal pain) and to evaluate the diagnostic utility of automated sFlt-1 measurements for the prediction of AP severity.

2. Results

Overall, 66 patients (34 men and 32 women) were included in the study. Among them, mild AP (MAP) was diagnosed in 46, moderately-severe AP (MSAP) in 15 and severe AP (SAP) in 5. Three deaths occurred in the studied group, all in the late phase of the disease (after 13–31 days of hospital stay). Because of the low number of patients with SAP, the data are reported together for MSAP and SAP patients (further referred to as MSAP + SAP). We have verified that the addition of SAP patients did not significantly change the MSAP group.

MAP patients did not differ significantly from those with more severe AP (MSAP + SAP) regarding age, sex, etiology, preexisting comorbid conditions and the duration of abdominal pain before admission (Table 1). As expected, all clinical characteristics related to the severity of the disease were significantly worse in MSAP + SAP group, resulting in more intensive treatment and a longer hospital stay (Table 1).

The MSAP + SAP group was characterized by higher concentrations of C-reactive protein (CRP), glucose, markers of renal function: creatinine, urea, cystatin C, urine albumin/creatinine ratio (uACR), serum and urine neutrophil gelatinase-associated lipocalin (NGAL), and D-dimer (Table 2). Furthermore, on the second day of AP (48 h from the onset of symptoms), leukocyte counts and amylase activity were higher in this group, while albumin and calcium concentrations were lower (Table 2). Serum concentrations of the endothelial markers, Ang-2 and sFlt-1 were higher in patients with MSAP and SAP as compared to MAP, both at 24 and 48 h from the onset of AP (Table 2). In particular, SAP patients had the highest sFlt-1:198 (183–213) pg/mL on the first day ($p = 0.042$ in comparison with the rest of the cohort). However, sFlt-1 significantly decreased after 48 h as compared to the first day of AP, both in patients with MAP ($p = 0.003$) and MSAP + SAP ($p = 0.018$). This was not observed in the case of Ang-2.

Table 1. Clinical characteristics of patients.

Characteristic	MAP (*n* = 46)	MSAP + SAP (*n* = 20)	*p*
Age, years	58 ± 19	66 ± 16	NS
Male sex, N (%)	25 (54)	9 (45)	NS
Etiology	–	–	–
Gallstone, N (%)	27 (59)	8 (40)	NS
Alcohol, N (%)	11 (24)	7 (35)	–
Hypertriglyceridemia, N (%)	3 (7)	2 (10)	–
Other, N (%)	5 (11)	3 (15)	–
Preexisting comorbidities, N (%)	33 (72)	18 (90)	NS
Hypertension, N (%)	14 (30)	9 (45)	–
Ischemic heart disease, N (%)	11 (24)	7 (35)	–
Diabetes, N (%)	5 (11)	5 (25)	–
Lung diseases, N (%)	4 (9)	3 (16)	–
Duration of pain until admission, hours	12 (6–24)	12 (12–24)	NS
Organ failure: transient/persistent, N (%)	0/0	7 (35)/5 (25)	<0.001
BISAP score during first 24 h	0 (0–1)	2 (1–3)	<0.001
≥3 points, N (%)	0	7 (35)	
SIRS, N (%)	0	9 (45)	<0.001
Pancreatic or peripancreatic necrosis, N (%)	0	3 (15)	0.025
Peripancreatic fluid collections, N (%)	0	5 (25)	0.002
Pleural effusion, N (%)	0	14 (70)	<0.001
Antibiotic prophylaxis or treatment, N (%)	15 (33)	17 (85)	<0.001
Parenteral nutrition, N (%)	0	3 (15)	0.025
Surgery, N (%)	0	3 (15)	0.025
Duration of hospital stay, days	6 (5–7)	12 (10–21)	<0.001
Early/late mortality, N (%)	0/0	0/3 (15)	0.025

Abbreviations: MAP, mild acute pancreatitis; MSAP, moderately-severe acute pancreatitis; SAP, severe acute pancreatitis; N, number of patients; BISAP, bedside index of severity in acute pancreatitis; SIRS, systemic inflammatory response syndrome; NS, non-significant.

Table 2. The results of selected laboratory tests within the first 24 and at 48 h from the onset of AP.

Variable	Time Point	MAP (*n* = 46)	MSAP + SAP (*n* = 20)	*p*
Hematocrit, %	24 h	42.5 ± 4.08	42.1 ± 6.91	NS
	48 h	39.6 ± 4.50	37.2 ±6.18	NS
Leukocyte count, $\times 10^3/\mu L$	24 h	11.2 (9.3–14.6)	11.2 (10.3–16.0)	NS
	48 h	8.6 (6.3–11.2)	14.6 (9.7–18.6)	0.001
Platelet count, $\times 10^3/\mu L$	24 h	235 ± 58	212 ± 75	NS
	48 h	209 ± 65	183 ± 80	NS
C-reactive protein, mg/L	24 h	6.5 (2.5–47.9)	74.6 (13.7–133.2)	0.003
	48 h	73.9 (32.1–142.8)	237.5 (161.8–299.0)	<0.001
Albumin, g/L	24 h	40.6 ± 4.23	37.1 ± 6.8	NS
	48 h	38.1 ± 3.23	31.4 ± 7.9	<0.001
Amylase, U/L	24 h	1076 (570–1648)	1031 (733–1917)	NS
	48 h	149 (105–251)	286 (142–478)	0.019
Calcium, mmol/L	24 h	2.33 ± 0.17	2.24 ± 0.29	NS
	48 h	2.28 ± 0.10	1.98 ± 0.26	<0.001
Glucose, mmol/L	24 h	7.70 (6.40–9.79)	9.18 (7.07–12.56)	0.038
	48 h	4.92 (4.53–5.73)	6.20 (5.52–8.07)	0.005
Creatinine, μmol/L	24 h	72.5 (63.1–94.8)	93.4 (72.6–165.3)	0.016
	48 h	69.2 (60.6–84.8)	85.8 (68.0–191.6)	0.020

Table 2. *Cont.*

Urea, mmol/L	24 h	5.37 (4.14–6.70)	7.24 (5.94–13.45)	0.003
	48 h	4.14 (3.33–5.04)	8.80 (3.49–15.78)	0.004
Cystatin C, mg/L	24 h	0.87 (0.65–1.07)	1.37 (0.79–1.78)	0.036
	48 h	0.82 (0.73–1.26)	1.60 (0.81–2.19)	0.008
uNGAL, µg/L	24 h	24.3 (14.6–37.3)	145 (72–670)	<0.001
	48 h	25.6 (15.0–46.0)	118 (71–293)	<0.001
sNGAL, µg/L	24 h	104 (64–139)	199 (116–276)	0.003
	48 h	137 (77–196)	250 (194–416)	<0.001
uACR, mg/g	24 h	28.8 (20.7–67.4)	87.2 (50.1–917.8)	0.011
	48 h	34.0 (19.8–84.6)	68.5 (29.7–94.5)	NS
D-dimer, µg/mL	24 h	1.54 (0.93–2.30)	3.70 (1.47–13.57)	0.001
	48 h	1.70 (1.06–2.17)	5.63 (3.24–11.37)	<0.001
Angiopoietin-2, ng/mL	24 h	2.89 (2.05–4.01)	4.29 (2.40–20.37)	0.006
	48 h	2.78 (1.91–4.24)	7.23 (3.69–15.18)	<0.001
sFlt-1, pg/mL	24 h	128 (104–163)	184 (143–223)	<0.001
	48 h	94 (85–119)	140 (120–179)	0.001

Abbreviations: see Table 1; uNGAL, urine neutrophil gelatinase-associated lipocalin; sNGAL, serum neutrophil gelatinase-associated lipocalin; uACR, urine albumin/creatinine ratio; sFlt-1, soluble fms-like tyrosine kinase 1; NS, non-significant.

During the study, serum sFlt-1 positively correlated with inflammatory markers (leukocyte count, CRP), the markers of kidney function (serum creatinine, urea, cystatin C, serum and urine NGAL, uACR), as well as with the concentrations of D-dimer and Ang-2 (Table 3). Furthermore, sFlt-1 positively correlated with glucose on the first day of AP and negatively with albumin and calcium on the second day (Table 3).

Table 3. Correlations between sFlt-1 and selected laboratory results within the first 24 and at 48 h from the onset of AP.

Variable	24 h		48 h	
	R	***p***	**R**	***p***
Leukocyte count	0.49	<0.001	0.41	0.003
C-reactive protein	0.32	0.021	0.43	0.002
Albumin	−0.16	NS	−0.43	0.002
Glucose	0.34	0.011	0.12	NS
Calcium	−0.01	NS	−0.32	0.021
Creatinine	0.61	<0.001	0.42	0.002
Urea	0.54	<0.001	0.33	0.020
Cystatin C	0.67	<0.001	0.41	0.005
uNGAL	0.41	0.005	0.47	0.001
sNGAL	0.50	<0.001	0.65	<0.001
uACR	0.56	<0.001	0.32	0.022
D-dimer	0.36	0.008	0.36	0.008
Angiopoietin-2	0.38	0.006	0.37	0.008

Abbreviations: see Table 1; uNGAL, urine neutrophil gelatinase-associated lipocalin; sNGAL, serum neutrophil gelatinase-associated lipocalin; uACR, urine albumin/creatinine ratio; sFlt-1, soluble fms-like tyrosine kinase 1; NS, non-significant.

Serum concentrations of sFlt-1 measured within the first 24 h from the onset of AP significantly predicted the severity of the disease, in particular the development of transient or persistent organ failure, both in simple analysis and after adjustment for age and the presence of comorbidities (Table 4; Appendix A). Although sFlt-1 was significantly positively correlated with serum creatinine and

cystatin C, the association between sFlt-1 and more severe AP (MSAP + SAP) was independent of the markers of glomerular filtration (Table 5). CRP and sFlt-1 measured on the first day of AP were independent predictors of MSAP + SAP (Table 5).

Table 4. The results of simple and multiple logistic regression to predict the severity of AP. Multiple models were adjusted for age and the presence of comorbidities.

Dependent Variable	Odds Ratio (95% Confidence Interval) per 10 pg/mL Increase in sFlt-1 Measured within 24 h from the Onset of AP; *p*-Value	
	Simple Analysis	Multiple Analysis [1]
MSAP + SAP	1.28 (1.10–1.50); $p = 0.001$	1.30 (1.09–1.55); $p = 0.003$
BISAP ≥3 in the first 24 h	1.30 (1.07–1.59); $p = 0.007$	1.28 (1.04–1.59); $p = 0.019$
SIRS	1.27 (1.07–1.52); $p = 0.006$	1.30 (1.08–1.57); $p = 0.007$
Transient or persistent organ failure	1.44 (1.16–1.79); $p < 0.001$	1.41 (1.12–1.77); $p = 0.003$
Renal failure	1.31 (1.06–1.63); $p = 0.010$	1.31 (1.03–1.65); $p = 0.022$

[1] The estimated odds ratios and *p*-values for the covariates in multiple models are presented in Appendix A (Tables A1–A5). Abbreviations: see Table 1; sFlt-1, soluble fms-like tyrosine kinase 1.

Table 5. Multiple logistic regression to predict MSAP + SAP. The results of laboratory tests within the first 24 h of AP were used as predictor variables.

Independent Variables	Odds Ratio (95% Confidence Interval); *p*-Value		
	Model 1	Model 2	Model 3
sFlt-1, per 10 pg/mL	1.21 (1.02–1.42); $p = 0.023$	1.20 (1.01–1.44); $p = 0.032$	1.27 (1.08–1.50); $p = 0.004$
Serum creatinine, per 1 μmol/L	1.02 (1.00–1.04); $p = 0.1$	Not included	Not included
Serum cystatin C, per 1 mg/L	Not included	2.49 (0.56–11.01); $p = 0.2$	Not included
CRP, per 10 mg/L	Not included	Not included	1.12 (1.00–1.24); $p = 0.041$

Abbreviations: see Table 1; sFlt-1, soluble fms-like tyrosine kinase 1; CRP, C-reactive protein.

In both measurements, sFlt-1 concentrations were correlated with bedside index of severity in acute pancreatitis (BISAP) score; however, a more clear association of higher sFlt-1 with a higher BISAP score was observed within the first 24 h of AP (Figure 1). Furthermore, sFlt-1 on both days significantly positively correlated with the duration of hospital stay (R = 0.50; $p < 0.001$ on the first day and R = 0.45; $p = 0.001$ on the second day of AP).

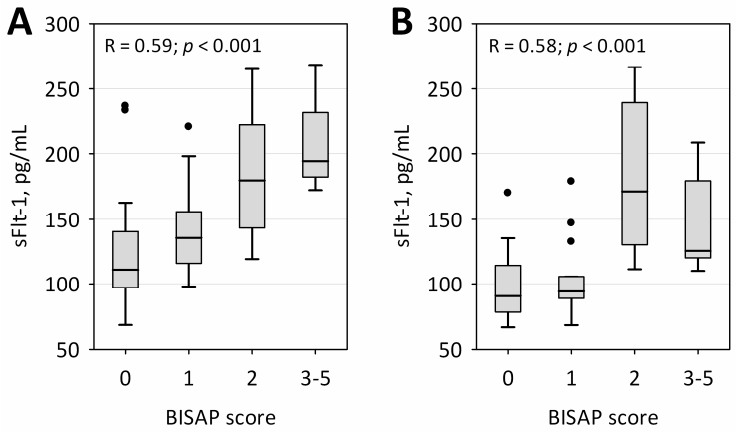

Figure 1. Correlation of Flt-1 serum concentrations with BISAP score during the first 24 (**A**) and at 48 h (**B**) from the onset of AP. Data are shown as the median, interquartile range (boxes), non-outlier range (whiskers) and outliers (points). Spearman correlation coefficients and p-values are shown on the graphs.

On the first day of AP, serum sFlt-1 above 139 pg/mL predicted more severe AP (MSAP + SAP) with sensitivity of 94% and specificity of 63% (Figure 2A). On the second day, sFlt-1 above 120 pg/mL predicted MSAP + SAP with a sensitivity of 78% and a specificity of 77% (Figure 2B).

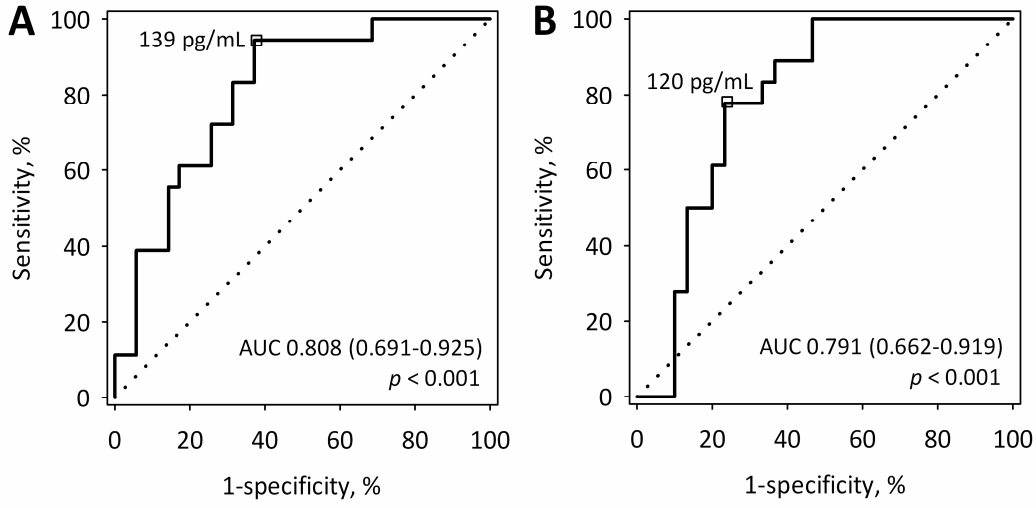

Figure 2. Receiver operating characteristic (ROC) curves for serum sFlt-1 measured within 24 (**A**) and at 48 h (**B**) from the onset of AP in the prediction of more severe acute pancreatitis (MSAP + SAP). The selected cut-off values are highlighted, and the values of area under the ROC curve (AUC) with 95% confidence intervals and *p*-values for the difference of AUC from AUC = 0.5 are shown on the graphs. The diagonal lines are the lines of no-discrimination.

On the first day of AP, the diagnostic utility of sFlt-1 for the prediction of MSAP + SAP was comparable with other single laboratory markers of AP severity, i.e., CRP, D-dimer and Ang-2 (Figure 3). The value of the area under the receiver operating characteristic (ROC) curve (AUC) was highest for sFlt-1, although it did not differ significantly from other markers' AUCs. The combinations of single markers (sFlt-1 + CRP; sFlt-1 + D-dimer; sFlt-1 + Ang-2) did not predict MSAP + SAP significantly better than sFlt-1 alone.

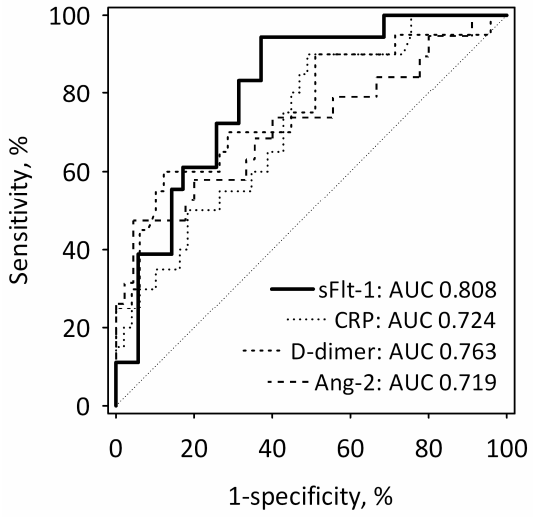

Figure 3. ROC curves for serum sFlt-1 measured within 24 h from the onset of AP in the prediction of more severe acute pancreatitis (MSAP + SAP) in comparison to other laboratory tests associated with AP severity. The values of AUC for each test are shown on the graph. The diagonal line is the line of no-discrimination.

Our intension was to collect the first blood sample within the first 24 h from the onset of pain due to AP at the time points close to the 24-h deadline. However, in 19 patients with MAP and nine patients who subsequently developed more severe AP, the first blood samples were collected between 18 and 21 h from the onset of AP symptoms (in the rest of the patients, samples were collected between 22 and 24 h) (Figure 4A). When we restricted the analysis to the 28 patients with samples drawn at the earliest time points, the estimate of AUC for sFlt-1 in the prediction of MSAP + SAP was even higher (AUC = 0.836; 95% confidence interval 0.680–0.992; $p < 0.001$ versus AUC = 0.5) (Figure 4B).

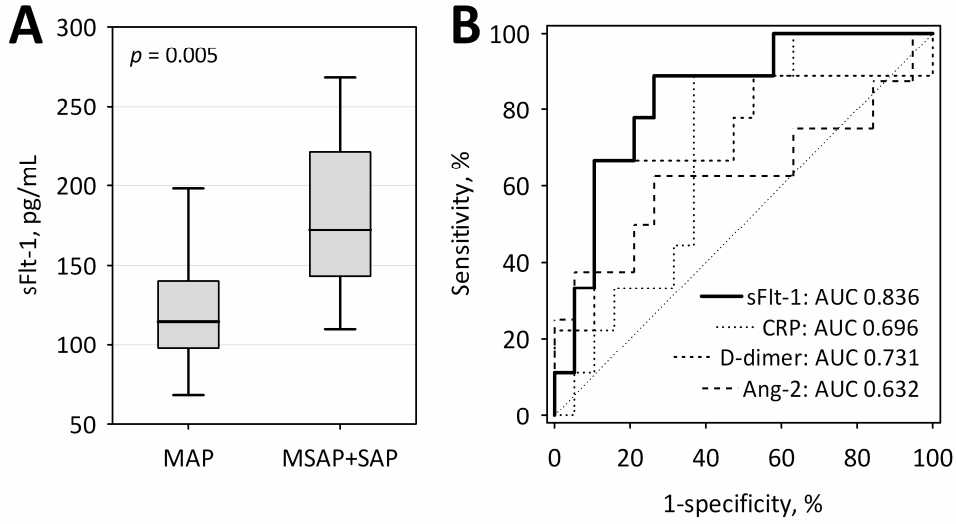

Figure 4. Serum concentrations of sFlt among 28 patients in whom blood samples were drawn at the earliest time point (18–21 h after the onset of AP symptoms), including 19 with MAP and nine who subsequently developed more severe AP (**A**); Data are shown as the median, interquartile range (boxes) and range (whiskers). The ROC curve for serum sFlt-1 measured at 18–21 h from the onset of AP in the prediction of more severe acute pancreatitis (MSAP + SAP) in comparison to other laboratory tests associated with AP severity (**B**). The values of AUC for each test are shown on the graph. The diagonal line is the line of no-discrimination.

3. Discussion

In the report, we show the positive association between sFlt-1 measured in the sera of patients with AP on the first and second day of the disease evolution and the severity of the disease. Within the first 24 h of AP, the concentrations of sFlt-1 were highest in patients with SAP and enabled predicting a more severe course of the disease (MSAP + SAP, the development of organ failure or SIRS). Furthermore, sFlt-1 concentrations correlated with BISAP score, as well as CRP and D-dimer concentrations, recognized as predictors of severity and mortality in AP [23–26]. Moreover, sFlt-1 positively correlated with the length of hospital stay. On the first day of AP, sFlt-1 predicted more severe disease with high sensitivity and reasonable specificity. The diagnostic accuracy of sFlt-1 to predict more severe AP was comparable to other single markers of AP severity, including Ang-2. Recently, high diagnostic utility was reported for early Ang-2 measurements (AUCs of 0.940 and 0.851) in the prediction of SAP [8,9].

To our best knowledge, this is the first report where sFlt-1 was measured with an automated method in patients with AP. We could identify only one previous report on sFlt-1 serum concentrations among patients with AP. In a study of Espinosa et al. [27], including 25 patients with AP, serum sFlt-1 (or VEGFR-1) was measured with the enzyme-linked immunosorbent assay. The authors did not find higher concentrations of VEGFR-1 among seven patients with predicted severe AP, nor among seven patients with unfavorable clinical evolution of AP (defined as kidney, respiratory or cardiovascular failure, or local infectious or necrotic complications). However, they were able to find an association

between Ang-2 serum concentrations and AP severity [27]. The discrepancy between our findings and those of Espinosa et al. may be a result of different methods of measurement or may probably be due to different ways of sample collection and handling (Espinosa et al. only say they used serum samples, with no further details). Moreover, Espinosa et al. collected blood samples at 12 h and five days after the admission of patients, and their patients were included up to 72 h from the onset of symptoms of AP; thus, the time points of blood collection were apparently different than in our study. In our study, the difference in sFlt-1 concentrations between patients with MAP and those with more severe AP was most significant at the earliest time points. Thus, it is possible that at later time points (at 72 h from the onset of AP and later), the association between sFlt-1 concentrations and AP severity becomes weaker. Our study was designed to assess sFlt-1 as an early marker of AP severity, as this is most relevant to clinical practice. Therefore, more studies are needed to explain the discrepancy between the results of Espinosa et al. [27] and ours.

Increased concentrations of sFlt-1 have been shown in sepsis and have been positively associated with more severe sepsis [16,28–31]. In 2010, Shapiro et al. [16] reported a strong association between sFlt-1 plasma concentrations and the severity of sepsis, as well as the development of organ dysfunction in sepsis. High concentrations of sFlt-1 were observed in patients with clinically-diagnosed sepsis and septic shock already at admission to the emergency department and were positively correlated with Acute Physiology and Chronic Health Evaluation II (APACHE II) and Sequential Organ Failure Assessment (SOFA) scores [30,31]. In the study of Skibstead et al. [30], sFlt-1 was the best predictor of organ dysfunction and mortality in sepsis among several markers of endothelial dysfunction. It is disputable whether endothelial dysfunction in patients with a severe course of AP may be comparable to the well-documented severe endothelial impairment observed in sepsis. There are only a few studies directly comparing such patients. In a small study of Hynninen et al. [32], nine patients with severe acute pancreatitis were compared with 11 patients with severe sepsis. In both groups, mortality was about 30%. Furthermore, in both groups, similar plasma concentrations of E-selectin were observed in serial measurements during the first three days following admission. At admission, E-selectin levels were significantly correlated with SOFA scores. E-selectin is expressed on activated endothelial cells; the soluble form is a result of the shedding of this membrane protein. Thus, Hynninen et al. [32] results suggest that similar activation of at least some signaling pathways of endothelial cells is associated with SAP and sepsis. On the other hand, sFlt-1 plasma concentrations were higher in patients with hypotension due to sepsis than in emergency department patients with non-sepsis hypotension of cardiac or hemorrhagic cause (median 227 versus 136 pg/mL); however, this study did not include patients with AP [33].

In the study of Shapiro et al. [16], the concentrations of sFlt-1 in patients with severe sepsis (median concentrations about 200 pg/mL) and septic shock (above 300 pg/mL) were higher than in our patients, including those with SAP (median concentrations 128 pg/mL in MAP, 161 pg/mL in MSAP and 198 pg/mL SAP on the first day). On the other hand, Skibstead et al. [30] reported median concentration of 168 pg/mL in patients with sepsis, comparable with our MSAP and SAP patients. However, we cannot directly compare the measured concentrations, as the measurements were done with different methods. Both Shapiro et al. [16] and Skibstead et al. [30] used a commercially available enzyme immunoassay and EDTA-plasma. The type of sample, as well as the administration of heparin as a part of the patients' treatment have been shown to significantly affect the concentrations of sFlt-1 [34]. We have measured sFlt-1 concentrations in sera obtained from venous blood. Importantly, the assay we used is specifically dedicated to measure sFlt-1 in serum.

Except for sepsis, other acute conditions have also been associated with elevated levels of sFlt-1. Hochholzer et al. [35] measured sFlt-1 in sera of patients with suspected acute myocardial infarction and found increasing concentrations in those with unstable angina, non-ST-segment-elevation myocardial infarction and ST-elevation myocardial infarction. Notably, the study utilized the same method of measurements as ours. In patients without acute coronary syndrome, median sFlt-1 was about 70 pg/mL, while in those with ST-elevation myocardial infarction about 90 pg/mL [35]. In another

study, higher sFlt-1 significantly predicted acute severe heart failure associated with myocardial infarction [34]. Furthermore, higher sFlt-1 was observed in patients who developed acute respiratory distress syndrome in the course of sepsis or trauma and following cardiac arrest [36].

In our study, sFlt-1 positively correlated with Ang-2 (although the correlation is of moderate strength), and as we have previously shown for Ang-2 [10], it was also significantly positively correlated with the markers of kidney function (including creatinine, urea, cystatin C, uACR, uNGAL and sNGAL) and predicted kidney failure. sFlt-1 has been shown to contribute in endothelial dysfunction to chronic kidney disease [37] and to correlate with mortality in patients on maintenance hemodialysis [38]. Furthermore, inhibition of VEGF signaling in renal glomeruli due to increased sFlt-1 has been implicated in the pathophysiology of kidney impairment and proteinuria observed in preeclampsia [39,40]. Interestingly, in our AP patients, sFlt-1 positively correlated with albuminuria (uACR). Kidney failure is among the most common organ complications of AP, observed in 16% of fatal cases [41]. We have previously observed that uNGAL concentrations predict the development of acute kidney injury in the course of AP [42]. Currently, there are possibilities to measure both uNGAL and sFlt-1 using routine automated laboratory methods. Simultaneous use of both markers may allow for early and reliable identification of patients at risk of acute kidney injury complicating AP. Importantly, although serum sFlt-1 concentrations were significantly correlated in our group with the markers of reduced glomerular filtration (serum creatinine and cystatin C), sFlt-1 predicted more severe AP (MSAP + SAP) independently of these markers. We may conclude that, although renal failure might have contributed to the increase in sFlt-1 observed in our patients, it definitely was not the single factor responsible for high sFlt-1 concentrations in more severe disease.

The design of our study does not allow drawing conclusions about the pathophysiological role of increased sFlt-1 in AP. Excerpt for endothelial cells, monocytes seem to be the important source of sFlt-1 in inflammatory conditions [43]. Nonetheless, in sepsis, sFlt-1 correlated significantly with recognized markers of endothelial dysfunction such as E-selectin or PAI-1 [30]. High sFlt-1 concentrations in sepsis and related conditions may reflect a protective response against increased VEGF, an endogenous compensatory anti-inflammatory mechanism [16,44]. In experimental sepsis in mice, endogenous sFlt-1 increased, whereas treatment with exogenous sFlt-1 attenuated the inflammatory response and endothelial dysfunction [44].

The limitation of our study is the low number of patients, especially those with SAP. For this reason, we were not able to reliably assess the diagnostic utility of sFlt-1 for the prediction of SAP. Nonetheless, we were able to show that sFlt-1 measured with the automated assay is positively associated with the severity of the disease and is an early predictor of organ failure, in particular kidney failure. We may hypothesize that the diagnostic utility of sFlt-1 might be better in patients' groups including more SAP patients. If this is confirmed in further studies, serum sFlt-1 measured at admission may become a practical way to improve early assessment of AP severity, considering the availability of automated methods of sFlt-1 measurement. In this aspect, our results are promising, and we believe they ought to be validated in a larger cohort of AP patients, including more patients with SAP.

4. Methods

4.1. Patients and Study Protocol

We used frozen serum samples obtained in a prospective observational study that recruited consecutive patients diagnosed with AP, admitted and treated in the Surgery Department of the District Hospital in Sucha Beskidzka, Poland. AP was diagnosed according to the 2012 revision of the Atlanta Classification, i.e., when at least two of the following features were present: abdominal pain consistent with AP, serum amylase activity above three-times greater than the upper reference limit and characteristic findings of AP on abdominal imaging (contrast-enhanced computer tomography, magnetic resonance imaging or transabdominal ultrasonography) [3]. Only adult patients who gave

written informed consent for the study were included. Patients who were admitted later that 24 h from the onset of pain due to AP were excluded. Furthermore, patients with chronic pancreatitis, chronic liver diseases (cirrhosis or viral hepatitis), diagnosed neoplasms of any origin or those treated with anticoagulants (including heparin in any form) were excluded. During the first 48 h from the onset of pain due to AP (i.e., when the blood samples were collected for the study), none of the patients received heparin or were dialyzed.

Demographic and clinical data were collected from patients at admission (age, sex, history of comorbidities, history of alcohol consumption, duration of pain until admission) and during the hospital stay (data regarding the course of AP, including development and duration of organ failure, development of local or systemic complications, treatment used, duration of hospital stay and outcome). The BISAP score was calculated using data collected during the first 24 h of AP [23]. Organ failure, including kidney failure, was diagnosed according to a modified Marshall scoring system, as cited in the revised Atlanta Classification [3].

Based on clinical evolution of AP, MAP, MSAP or SAP was diagnosed, in concordance with the revised Atlanta Classification [3]. MAP was defined as no organ failure, local or systemic complications during the hospital stay. MSAP was diagnosed when a patient presented transient organ failure (lasting less than 48 h), local (necrosis, acute necrotic collection, walled-off pancreatic necrosis) or systemic complications (exacerbation of preexisting conditions). SAP was diagnosed in patients with persistent organ failure (lasting more than 48 h).

Venous blood and urine samples for laboratory tests were collected from the patients twice, within the first 24 h (first day) and about 48 h (second day) from the onset of pain due to AP.

The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Bioethics Committee of the Jagiellonian University (Approval No. KBET/247/B/2013, permission date 28th November 2013 and 122.6120.242.2015, permission date 22nd November 2015).

4.2. Laboratory Tests

Routine laboratory tests included complete blood counts performed in EDTA-anticoagulated whole blood, as well as the measurements of albumin, calcium, glucose, creatinine, urea and CRP concentrations in serum, amylase activity in serum and D-dimer concentrations in citrated plasma. These tests were done on the day of blood collection, with the use of automated analyzers, in the Department of Laboratory Diagnostics, District Hospital in Sucha Beskidzka, Poland.

Urinary concentrations of NGAL were measured on the day of urine collection, using chemiluminescent microparticle immunoassay and Architect analyzer (Abbott Diagnostics, Lake Forest, IL, USA), in the Department of Laboratory Diagnostics, District Hospital in Sucha Beskidzka, Poland. Aliquots of urine were frozen in −70 °C and further used to measure urinary albumin and creatinine. Urinary albumin was measured by immunonephelometry and urinary creatinine by the Jaffe method on automated analyzers in the Diagnostic Department, University Hospital, Kraków, Poland. The results of these measurements were expressed as the urine albumin to creatinine ratio (uACR).

Serum samples for measurements of serum NGAL, cystatin C, Ang-2 and sFlt-1 were processed according to standard procedure, i.e., blood was collected from antecubital vein into standard serum tubes, allowed to fully clot for 30 min and centrifuged (10 min, 2000× *g*); serum was aliquoted and frozen in −70 °C (the whole procedure was completed within 1 hour from blood collection). The procedure was consistent with the instructions of the manufacturers of the laboratory assays used, including the sFlt-1 assay. Cystatin C was measured by immunonephelometry using the Nephelometer BN II analyzer (Siemens Healthcare, Erlangen, Germany), and sFlt-1 was measured by electrochemiluminescence immunoassay using the Cobas 8000 analyzer (Roche Diagnostics, Mannheim, Germany) in the Diagnostic Department, University Hospital, Kraków, Poland. The enzyme immunoassays were used to measure sNGAL and Ang-2, i.e., Human Lipocalin-2/NGAL ELISA (BioVendor, Brno, Czech Republic) and Quantikine ELISA Human

Angiopoietin-2 (R&D Systems, Minneapolis, MN, USA), respectively. Enzyme immunoassays were performed in the Department of Diagnostics, Chair of Clinical Biochemistry, Jagiellonian University Medical College, Kraków, Poland.

4.3. Statistical Analysis

Data were shown as the number (percentage) for categories, the median (lower-upper quartile) for non-normally distributed quantitative variables and the mean ± standard deviation for normally-distributed quantitative variables. Distributions were tested for normality with the Shapiro–Wilk test. The chi-squared test, Mann–Whitney test and unpaired t-test were used to study the differences between groups, respectively. The Wilcoxon signed rank test was used to analyze differences in repeated measurements. The correlations of sFlt-1 were assessed using the Spearman rank correlation coefficient, as the distribution of sFlt-1 differed significantly from normal. Simple and multiple logistic regression adjusted for age and the presence of comorbidities (i.e., the variables recognized as important predictors of AP severity [45]) were calculated to evaluate sFlt-1 as a predictor of severity of AP. Furthermore, separate multiple logistic regression models were calculated in order to check whether sFlt-1 predicts AP severity independently of renal function (serum creatinine and cystatin C concentrations) and inflammatory marker (CRP). Receiver operating characteristic (ROC) curves were used to assess the diagnostic accuracy of sFlt-1. The tests were two-tailed, and the results were considered significant at $p \leq 0.05$. The Statistica 12 software package (StatSoft, Tulsa, OK, USA) was used for computations.

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

Abbreviations

AP	acute pancreatitis
SIRS	systemic inflammatory response syndrome
Ang-2	angiopoietin-2
sFlt-1	soluble fms-like tyrosine kinase-1
VEGF	vascular endothelial growth factor
PlGF	placental growth factor
VEGFR-1	vascular endothelial growth factor receptor-1
BISAP	bedside index of severity in acute pancreatitis
MAP	mild acute pancreatitis
MSAP	moderately severe acute pancreatitis
SAP	severe acute pancreatitis
CRP	C-reactive protein
uNGAL	urinary neutrophil gelatinase-associated lipocalin
uACR	urine albumin to creatinine ratio
sNGAL	serum neutrophil gelatinase-associated lipocalin
ROC	receiver operating characteristic
AUC	area under receiver operating characteristic curve

Appendix

Multiple logistic regression models to predict the severity of acute pancreatitis are presented in Tables A1–A5. Serum concentrations of sFlt-1 on Day 1 (within the first 24 h from the onset of pain), age and comorbidities were assessed as independent variables to predict the development of MSAP or

SAP (Table A1), BISAP $\geq$ 3 (Table A2), SIRS (Table A3), transient or persistent organ failure (Table A4) and renal failure (Table A5).

Table A1. Multiple logistic regression model to predict MSAP + SAP.

Independent Variable	Odds Ratio (95% Confidence Interval)	*p*
sFlt-1 on Day 1, per 10 pg/mL	1.30 (1.09–1.55)	0.003
Age, per year	0.99 (0.95–1.03)	0.6
Presence of comorbidities	7.99 (0.69–92.80)	0.09
Whole model	not applicable	<0.001

Table A2. Multiple logistic regression model to predict BISAP $\geq$ 3 in the first 24 h.

Independent Variable	Odds Ratio (95% Confidence Interval)	*p*
sFlt-1 on Day 1, per 10 pg/mL	1.28 (1.04–1.59)	0.019
Age, per year	1.01 (0.94–1.08)	0.8
Presence of comorbidities	2.47 (0.30–20.70)	0.4
Whole model	not applicable	0.014

Table A3. Multiple logistic regression model to predict SIRS.

Independent Variable	Odds ratio (95% Confidence Interval)	*p*
sFlt-1 on Day 1, per 10 pg/mL	1.30 (1.08–1.57)	0.007
Age, per year	0.98 (0.93–1.03)	0.5
Presence of comorbidities	2.06 (0.17–24.71)	0.6
Whole model	not applicable	0.016

Table A4. Multiple logistic regression model to predict transient or persistent organ failure.

Independent Variable	Odds Ratio (95% Confidence Interval)	*p*
sFlt-1 on Day 1, per 10 pg/mL	1.41 (1.12–1.77)	0.003
Age, per year	1.04 (0.97–1.12)	0.2
Presence of comorbidities	1.04 (0.14–7.81)	0.9
Whole model	not applicable	<0.001

Table A5. Multiple logistic regression model to predict renal failure.

Independent Variable	Odds Ratio (95% Confidence Interval)	*p*
sFlt-1 on Day 1, per 10 pg/mL	1.31 (1.03–1.65)	0.022
Age, per year	1.03 (0.96–1.11)	0.4
Presence of comorbidities	0.39 (0.04–4.19)	0.4
Whole model	not applicable	0.018

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Artykuł nr 4

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Serum Concentrations of Angiopoietin-2 and Soluble fms-Like Tyrosine Kinase 1 (sFlt-1) Are Associated with Coagulopathy among Patients with Acute Pancreatitis

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Abstract: In severe acute pancreatitis (SAP), systemic inflammation leads to endothelial dysfunction and activation of coagulation. Thrombotic disorders in acute pancreatitis (AP) include disseminated intravascular coagulation (DIC). Recently, angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) were proposed as markers of endothelial dysfunction in acute states. Our aim was to assess the frequency of coagulation abnormalities in the early phase of AP and evaluate the relationships between serum angiopoietin-2 and sFlt-1 and severity of coagulopathy. Sixty-nine adult patients with AP were recruited: five with SAP, 15 with moderately severe AP (MSAP) and 49 with mild AP. Six patients were diagnosed with DIC according to International Society on Thrombosis and Haemostasis (ISTH) score. All patients had at least one abnormal result of routine tests of hemostasis (low platelet count, prolonged clotting times, decreased fibrinogen, and increased D-dimer). The severity of coagulopathy correlated with AP severity according to 2012 Atlanta criteria, bedside index of severity in AP and duration of hospital stay. D-dimers correlated independently with C-reactive protein and studied markers of endothelial dysfunction. Angiopoietin-2, D-dimer, and ISTH score were best predictors of SAP, while sFlt-1 was good predictor of MSAP plus SAP. In clinical practice, routine tests of hemostasis may assist prognosis of AP.

Keywords: acute pancreatitis; disseminated intravascular coagulation; D-dimer; angiopoietin-2; soluble fms-like tyrosine kinase 1

1. Introduction

Acute pancreatitis (AP) is an inflammatory disorder, characterized by a spectrum of severity, ranging from mild disease in most patients, to severe life-threatening condition [1,2]. Current guidelines [3] classify the disease course as mild (MAP), moderately-severe (MSAP) and severe AP (SAP) on the basis of organ failure (transient or persistent), and local and systemic complications. Organ failure (including cardiovascular, pulmonary and renal) occurring in the early phase of AP is the most important determinant of severity and the main cause of early deaths [3]. Severe course of AP is associated with excessive systemic inflammation, involving systemic activation and dysfunction of endothelial cells, leading to vascular leak syndrome and organ failure [4].

Angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) have been proposed as novel predictors of severity in acute states, such as sepsis or AP [5–11]. Angiopoietin-2 is an angiogenic growth factor, binding and inhibiting Tie-2 receptor on endothelial cells, which has been associated with destabilization of endothelium and increased vascular leakage [12,13]. Flt-1 is a receptor for vascular endothelial growth factor and placental growth factor, its soluble form (sFlt-1) acts as a decoy receptor [14]. Both angiopoietin-2 and sFlt-1 may be considered markers of endothelial dysfunction in acute states. We have previously reported the associations between serum concentrations of angiopoietin-2 and the development of acute kidney injury and renal failure in the course of AP as well as the severity of AP [7]. In addition, we have reported the association between serum sFlt-1 concentrations in the early phase of AP and the severity of the disease [8].

Endothelial dysfunction may result in activation of platelets and coagulation. Clinically significant hemorrhagic and thrombotic disorders were observed, respectively, among 6% and 7% of patients who died due to AP [15]. Several coagulopathies were reported as the complications of AP, including among others disseminated intravascular coagulation (DIC) and thrombotic thrombocytopenic purpura [16–19]. The results of laboratory tests used to assess hemostasis were shown to predict severity of AP and related mortality with reasonable diagnostic utility, in some reports exceeding those observed for C-reactive protein [16,20]. In particular, high diagnostic accuracy to predict SAP was reported for D-dimer plasma concentrations [16,20–23].

The aim of the study was to assess the frequency of coagulopathy in a cohort of consecutive patients with AP in the early phase of the disease. Moreover, we studied the associations between the serum concentrations of angiopoietin-2 and sFlt-1 and the presence as well as the severity of abnormalities of coagulation in the early phase of AP.

2. Results

Sixty-nine patients (35 men and 34 women; mean age 69 $\pm$ 18) were included in the study, among them 49 were diagnosed with MAP, 15 with MSAP, and 5 with SAP.

During the first 48 h of AP, six patients (7% of the total cohort) were assigned six points or more in the International Society on Thrombosis and Haemostasis (ISTH) score for overt DIC [24], thus fulfilling the criteria of overt DIC. The diagnosis of DIC was significantly associated with more severe AP, higher bedside index of severity in AP (BISAP) [25] and Glasgow [26] severity scores, longer hospital stays, and higher mortality (Table 1). Patients subsequently diagnosed with DIC had longer prothrombin times and higher plasma concentrations of D-dimer at admission, as well as higher serum urea (Table 1).

Twenty-one healthy volunteers (12 women and nine men; mean age 51 $\pm$ 10 years) provided serum samples in order to compare angiopoietin-2 and sFlt-1 concentrations between healthy controls and AP patients. In the control group, angiopoietin-2 concentrations were in the range of 1.17–2.41 ng/mL with

median (lower-upper quartile) of 1.69 (1.44–2.08) ng/mL and sFlt-1 concentrations were in the range of 63–108 pg/mL with median (lower-upper quartile) of 89 (79–94) pg/mL. Both angiopoietin-2 ($p < 0.001$ at 24 h and $p < 0.001$ at 48 h) and sFlt-1 ($p < 0.001$ at 24 h and $p < 0.001$ at 48 h) were significantly higher in AP patients than in controls (Figure 1). In multiple logistic regression, these differences between AP patients and controls were independent of age. The concentrations of angiopoietin-2 and sFlt-1 were also significantly higher among AP patients with DIC comparing to patients without DIC, both on admission and on the second day of hospital stay (Table 1, Figure 1).

Table 1. Clinical characteristics of patients and the results of laboratory tests performed at 24 h from the onset of acute pancreatitis (AP) symptoms.

Variables	Patients without DIC (*N* = 63)	Patients with DIC (*N* = 6)	*p*
Age, years	59 ± 18	71 ± 16	0.1
Male sex, *N* (%)	31 (49)	4 (67)	0.4
Body mass index >30 kg/m^2	2 (3)	0	0.7
Etiology:			
Gallstone, *N* (%)	32 (51)	3 (50)	0.9
Alcohol, *N* (%)	17 (27)	1 (17)	
Hypertriglyceridemia, *N* (%)	6 (10)	1 (17)	
Other/idiopathic, *N* (%)	8 (13)	1 (17)	
Severity:			
MAP, *N* (%)	48 (76)	1 (17)	0.003
MSAP, *N* (%)	12 (19)	3 (50)	
SAP, *N* (%)	3 (5)	2 (33)	
Pre-existing comorbidities, *N* (%)	48 (76)	6 (100)	0.2
Hypertension, *N* (%)	21 (33)	2 (33)	1.0
Diabetes, *N* (%)	9 (14)	1 (17)	0.9
Ischemic heart disease, *N* (%)	15 (24)	3 (50)	0.2
Lung diseases, *N* (%)	6 (10)	2 (33)	0.08
BISAP score, points	1 (0–1)	2 (1–2)	0.019
Glasgow severity score, points	0 (0–1)	2 (1–3)	0.035
SIRS, *N* (%)	8 (13)	1 (17)	0.8
Length of hospital stay, days	6 (5–9)	10 (8–31)	0.013
Early/late death, *N* (%)	0/1 (2)	0/2 (33)	0.018
C-reactive protein, mg/L	13.6 (2.6–86.7)	36.3 (13.7–84.5)	0.3
Leukocyte count, $\times 10^3/\mu L$	11.4 (9.6–15.4)	10.8 (9.8–11.2)	0.3
Hematocrit, %	42 ± 5	42 ± 5	1.0
Amylase, U/L	1076 (588–1844)	976 (925–1917)	0.9
Albumin, g/L	40 (37–43)	41 (34–42)	0.7
Calcium, mmol/L	2.35 (2.20–2.43)	2.29 (2.18–2.38)	0.8
Glucose, mmol/L	7.76 (6.43–10.29)	10.15 (7.79–12.25)	0.2
Creatinine, µmol/L	74.2 (63.4–97.6)	101.7 (76.0–113.4)	0.1
Urea, mmol/L	5.94 (4.26–7.08)	8.04 (6.72–12.73)	0.036
Bilirubin, µmol/L	36.0 (21.1–71.4)	36.8 (32.5–59.9)	0.9
Platelet count, $\times 10^3/\mu L$	229 ± 60	227 ± 102	0.9
Prothrombin time, s	14.4 ± 1.6	16.1 ± 1.2	0.015
APTT, s	29.5 ± 4.8	29.9 ± 6.1	0.8
Fibrinogen, g/L	2.78 (2.15–3.58)	3.18 (2.75–4.46)	0.4
D-dimer, µg/mL	1.61 (0.98–3.09)	3.99 (2.10–13.89)	0.019
Angiopoietin-2, ng/mL	3.06 (2.05–4.29)	4.97 (3.59–5.92)	0.037
sFlt-1, pg/mL	182 (163–221)	139 (110–179)	0.041

Abbreviations: DIC, diffuse intravascular coagulation; *N*, number of patients; MAP, mild acute pancreatitis; MSAP, moderately severe acute pancreatitis; SAP, severe acute pancreatitis; BISAP, bedside index of severity in acute pancreatitis; SIRS, systemic inflammatory response syndrome; APTT, activated partial thromboplastin time; sFlt-1, soluble fms-like tyrosine kinase 1.

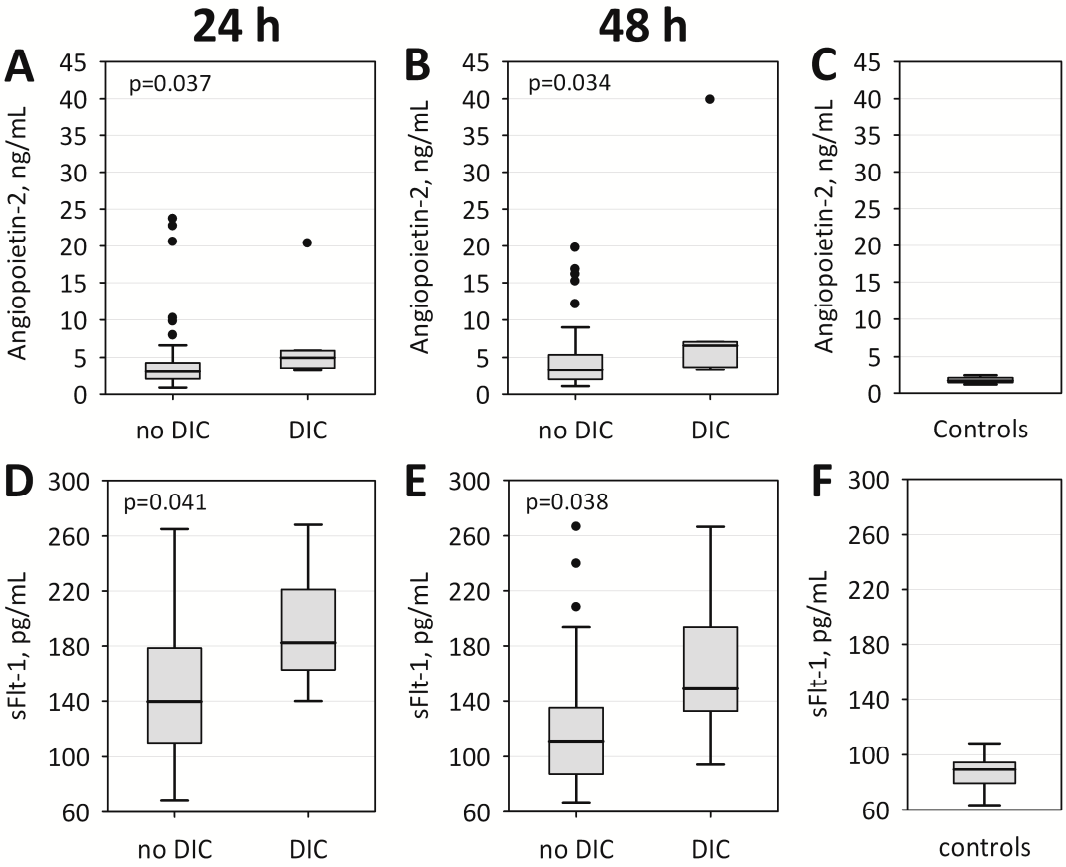

Figure 1. Serum angiopoietin-2 and sFlt-1 concentrations on: admission (**A**,**D**); and on Day 2 of hospital stay (**B**,**E**), among acute pancreatitis (AP) patients with ISTH score for overt DIC below 5 (no DIC) and ≥5 points (DIC). Serum: angiopoietin-2 (**C**); and sFlt-1 (**F**) concentrations observed among 21 healthy controls are presented for comparison. Data are shown as median, interquartile range (box), non-outlier range (whiskers), and outliers (points). Abbreviations: sFlt-1, soluble fms-like tyrosine kinase 1; DIC, diffuse intravascular coagulation; ISTH, International Society on Thrombosis and Haemostasis.

The abnormal results of routine tests of hemostasis, suggestive of consumptive coagulopathy, were significantly associated with AP severity. On admission, low platelet counts were observed in 60% of patients with SAP, 13% with MSAP and 6% with MAP; prothrombin time was prolonged in 80% of patients with SAP, 13% with MSAP and 22% with MAP; and activated partial thromboplastin time (APTT) was prolonged in 60% of those with SAP, 20% with MSAP and none patients with MAP (Figure 2A,C,E, respectively). Following treatment, these differences became non-significant already on Day 2 of hospital stay (Figure 2B,D,F). No significant associations were detected between AP severity and fibrinogen concentrations, however, patients with SAP tended towards high concentrations (Figure 3A,B). High concentrations of D-dimer were associated with more severe AP both at 24 and 48 h after the onset of AP symptoms. Severely increased D-dimer levels (>5 μg/mL) were observed in 80% of patients with SAP, 13% of those with MSAP and 6% with MAP on Day 1, and 80% of patients with SAP, 47% with MSAP, and 10% with MAP on Day 2, respectively (Figure 2C,D). Consequently, significantly higher results in ISTH score for overt DIC were observed in SAP and MSAP patients versus those with MAP (Figure 2E,F). On Day 2 of AP, 40% of patients with SAP, 20% of those with MSAP and 2% with MAP were assigned ≥5 points in ISTH score, supporting the diagnosis of overt DIC. Consistent with the fact that D-dimer concentrations were increased above the reference limit (0.5 μg/mL) in all the studied patients (except for one lower result achieved on admission), the lowest ISTH score among studied patients was 2 points.

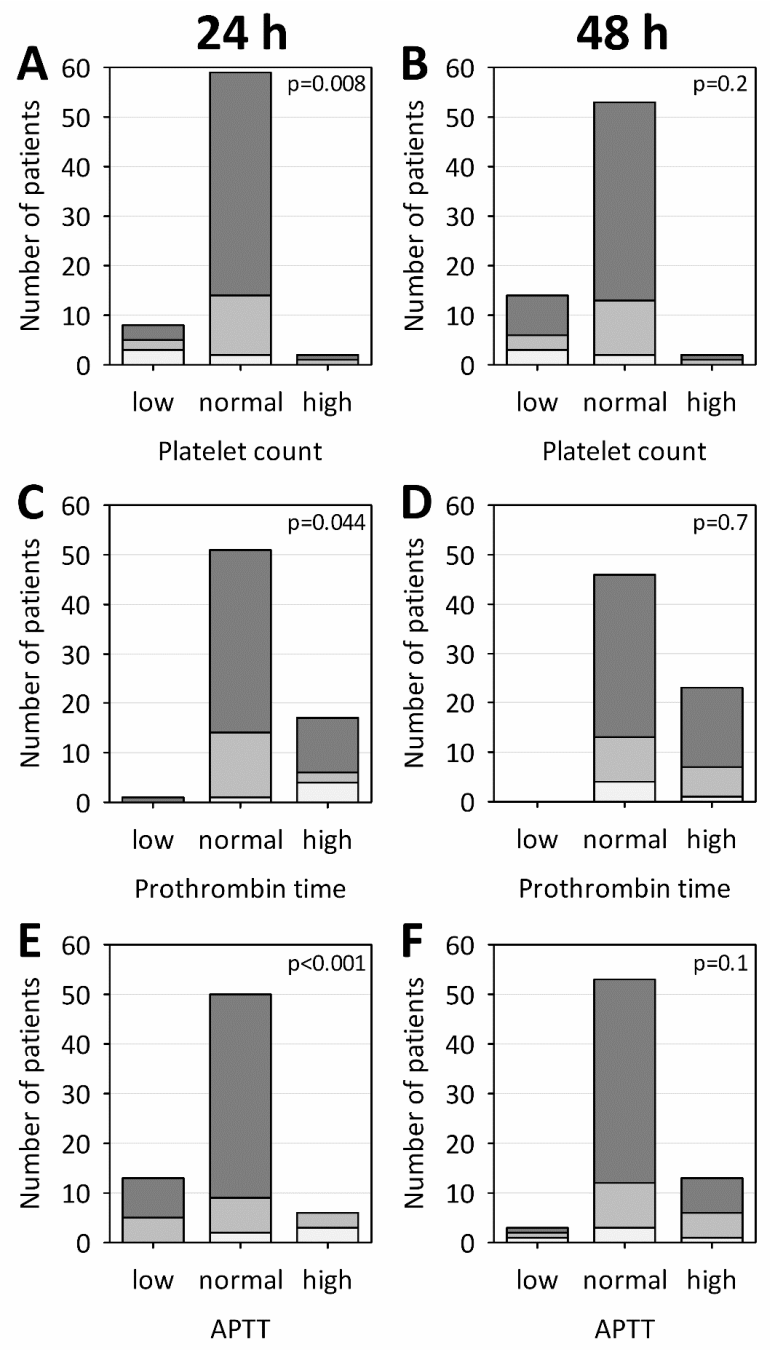

Figure 2. Numbers of patients with platelet counts (**A**,**B**); prothrombin times (**C**,**D**); and activated partial thromboplastin times (APTT) (**E**,**F**) below (low), within (normal) and above (high) the reference intervals. Panels **A**, **C** and **E** show the results obtained at 24 h and Panels **B**, **D** and **F** at 48 h from the onset of AP symptoms. Numbers of patients with mild AP (MAP), moderately severe AP (MSAP) and severe AP (SAP) are represented by dark, medium and light boxes, respectively; p-values for the chi-squared tests are shown on the graphs.

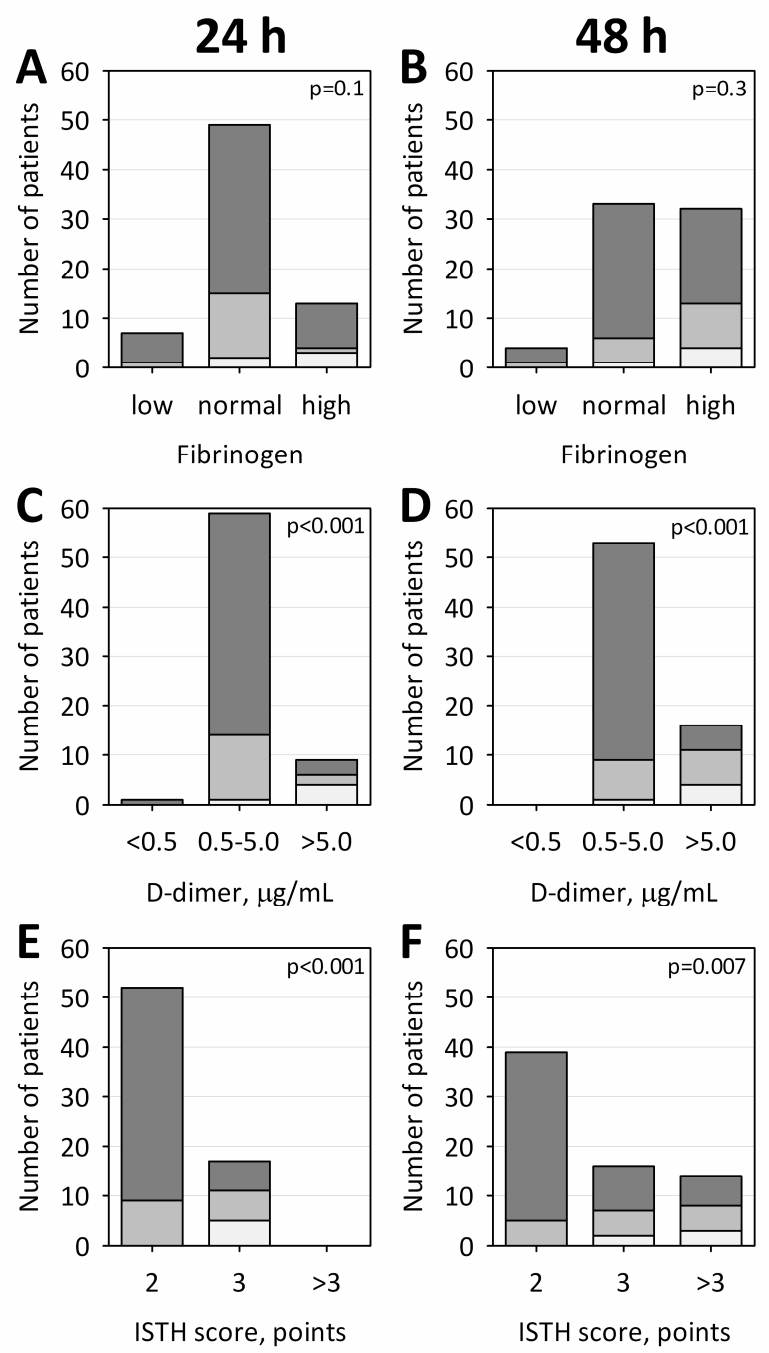

Figure 3. Numbers of patients with fibrinogen concentrations below (low), within (normal) and above (high) the reference interval (**A**,**B**); with D-dimer concentrations below reference limit (<0.5 μg/mL), increased up to 10 times (0.5–5.0 μg/mL) and increased more than 10 times (> 5. 0 μg/mL) (**C**,**D**); and with ISTH score for overt DIC of 2, 3, and more than 3 points (**E**,**F**). Panels **A**, **C** and **E** show the results obtained at 24 h and Panels **B**, **D** and **F** at 48 h from the onset of AP symptoms. Numbers of patients with MAP, MSAP and SAP are represented by dark, medium and light boxes, respectively; *p*-values for the chi-squared tests are shown on the graphs.

Positive correlations were observed between log-transformed C-reactive protein and fibrinogen concentrations ($R = 0.54$; $p < 0.001$ on Day 1 and $R = 0.27$; $p = 0.025$ on Day 2), D-dimer ($R = 0.45$; $p < 0.001$ on Day 1 and $R = 0.64$; $p < 0.001$ on Day 2), APTT ($R = 0.33$; $p = 0.007$ on Day 1), and ISTH score ($R = 0.29$; $p = 0.017$ on Day 1 and $R = 0.49$; $p < 0.001$ on Day 2). Only fibrinogen concentrations

correlated with amylase activity (for log-transformed variables: $R = -0.32$; $p = 0.010$ on Day 1). On admission, log-transformed D-dimer ($R = 0.51$; $p < 0.001$), APTT ($R = 0.34$; $p = 0.005$), platelet count ($R = -0.29$; $p = 0.017$) and ISTH score ($R = 0.43$; $p < 0.001$) significantly correlated with BISAP score, and log (D-dimer) ($R = 0.34$; $p = 0.004$), platelet count ($R = -0.33$; $p = 0.006$) and ISTH score ($R = 0.24$; $p = 0.049$) significantly correlated with Glasgow score. Moreover, log (fibrinogen) ($R = 0.27$; $p = 0.028$ on admission), log (D-dimer) ($R = 0.46$; $p < 0.001$ on Day 1 and $R = 0.59$; $p < 0.001$ on Day 2), and ISTH score ($R = 0.38$; $p = 0.001$ on Day 1 and $R = 0.44$; $p < 0.001$ on Day 2) positively correlated with the duration of hospital stay.

Serum angiopoietin-2 and sFlt-1 concentrations positively correlated with D-dimer and ISTH score (Table 2). Additionally, angiopoietin-2 significantly correlated with APTT and fibrinogen on Day 1 as well as with platelets and prothrombin time on Day 2, while sFlt-1 significantly correlated with prothrombin time on Day 2 (Table 2). The correlations between angiopoietin-2 concentrations and D-dimer were independent of C-reactive protein concentrations ($\beta = 0.29 \pm 0.13$; $p = 0.029$ on Day 1, and $\beta = 0.32 \pm 0.09$; $p = 0.001$ on Day 2). In addition, sFlt-1 correlated with D-dimer independently of C-reactive protein ($\beta = 0.27 \pm 0.12$; $p = 0.029$ on Day 1 and $\beta = 0.25 \pm 0.12$; $p = 0.042$ on Day 2).

Table 2. Correlations between angiopoietin-2 and sFlt-1 concentrations (log-transformed) and the results of the tests of hemostasis at 24 and 48 h from the onset of AP symptoms.

Variables	24 h				48 h			
	log (Ang-2)		log (sFlt-1)		log (Ang-2)		log (sFlt-1)	
	R	*p*	*R*	*p*	*R*	*p*	*R*	*p*
Platelet count	−0.18	0.2	−0.16	0.3	−0.30	0.015	−0.21	0.1
Prothrombin time	0.22	0.08	0.06	0.7	0.25	0.042	0.38	0.003
APTT	0.53	<0.001	0.19	0.2	−0.01	0.9	0.23	0.06
log (fibrinogen)	0.33	0.008	0.09	0.5	0.18	0.2	−0.04	0.8
log (D-dimer)	0.45	<0.001	0.40	<0.001	0.48	<0.001	0.27	0.028
ISTH score for overt DIC	0.33	0.008	0.40	<0.001	0.37	0.003	0.50	<0.001

Abbreviations: Ang-2, angiopoietin-2; sFlt-1, soluble fms-like tyrosine kinase 1; APTT, activated partial thromboplastin time; DIC, diffuse intravascular coagulation; ISTH, International Society on Thrombosis and Haemostasis.

On admission, the results of all the studied tests of hemostasis and both the endothelial markers enabled the prognosis of SAP, although the diagnostic utility differed between the tests (Table 3). Among the routine tests of hemostasis, D-dimer revealed the best diagnostic utility for the prediction of severity of AP (Table 3). However, the highest values of area under the receiver operating characteristic (ROC) curves were achieved for angiopoietin-2 as a predictor of SAP, and for sFlt-1 as a predictor of MSAP+SAP (Table 3). At 24 h from the onset of AP symptoms, angiopoietin-2 at a cut-off value of 5.92 ng/mL predicted SAP with a diagnostic sensitivity of 100% and specificity of 92%; ISTH score at a cut-off value of 3 points predicted SAP with a diagnostic sensitivity of 100% and specificity of 81%; and D-dimer at a cut-off value of 6.55 μg/mL predicted SAP with a diagnostic specificity of 80% and sensitivity of 94% (Figure 4).

Table 3. The values of area under the ROC curve for the studied laboratory tests of hemostasis and markers of endothelial dysfunction measured on admission (within 24 h from the onset of AP symptoms) to predict the severity of AP (diagnosis of SAP or MSAP+SAP versus less severe AP). The data for C-reactive protein are shown for comparison.

Diagnostic Tests	SAP	MSAP+SAP
Platelet count	0.803 ± 0.126	NS
Prothrombin time	0.830 ± 0.068	NS
APTT	0.768 ± 0.126	NS
Fibrinogen	0.788 ± 0.105	NS
D-dimer	0.902 ± 0.062	0.753 ± 0.070
ISTH score for overt DIC	0.913 ± 0.037	0.702 ± 0.077
Angiopoietin-2	0.946 ± 0.029	0.721 ± 0.077
sFlt-1	0.744 ± 0.064	0.810 ± 0.059
C-reactive protein	0.886 ± 0.064	0.733 ± 0.066

NS, no significant difference between the area under the ROC curve calculated for the particular test and the value of 0.5. Abbreviations: AP, acute pancreatitis; SAP, severe acute pancreatitis; MSAP, moderately severe acute pancreatitis; ROC, receiver operating characteristic; APTT, activated partial thromboplastin time; ISTH, International Society on Thrombosis and Haemostasis; DIC, diffuse intravascular coagulation; sFlt-1, soluble fms-like tyrosine kinase 1; BISAP, bedside index of severity in acute pancreatitis.

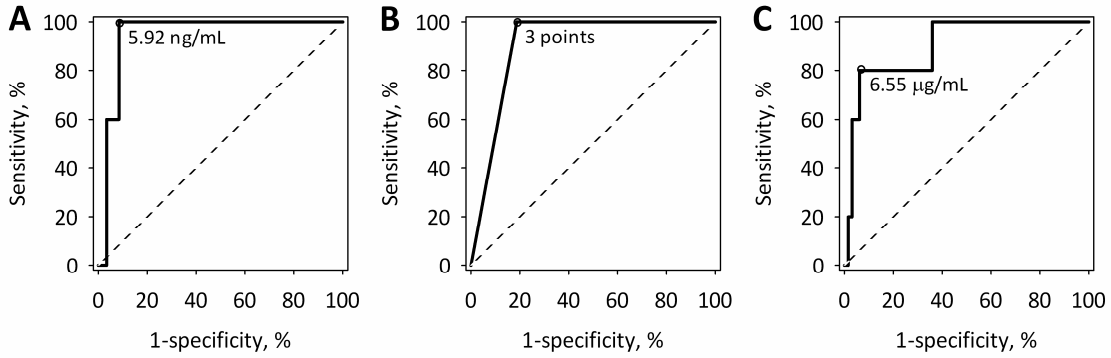

Figure 4. ROC curves for: angiopoietin-2 (**A**); ISTH score for overt DIC (**B**); and D-dimer (**C**) assessed on admission (within 24 h from the onset of AP symptoms) showing diagnostic utility to predict SAP. The selected cut-off values are shown on the graphs. The diagonal dashed lines are the lines of no-discrimination.

3. Discussion

In the present study, we have shown that AP is associated with abnormalities of coagulation leading to abnormal results of routinely used tests of hemostasis. Of importance, we have excluded patients with preexisting abnormalities of coagulation and none of our patients were treated with anticoagulants before or during the study. Low platelet counts, prolonged prothrombin times, prolonged APTT, and increased D-dimer concentrations observed in our patients suggest consumptive coagulopathy. Six patients (7% of the group) fulfilled the ISTH criteria for overt DIC [24]. These abnormalities correlated with both inflammation (as reflected by C-reactive protein concentrations) and endothelial dysfunction (as reflected by the concentrations of angiopoietin-2 and sFlt-1). Abnormal results of coagulation tests on admission were significantly associated with severity of AP, in particular, high plasma concentrations of D-dimer as well as ISTH score for DIC predicted more severe AP (SAP or MSAP+SAP) with reliable diagnostic accuracy.

These observations are consistent with the results of former studies. In 2006, Maeda et al. [16] reported significant associations between the results of laboratory tests of hemostasis (including platelet counts, antithrombin activity, and the concentrations of D-dimer, fibrin/fibrinogen degradation products E, and thrombin–antithrombin complexes) and severity of AP assessed in 5-stage Japanese

staging system for AP. In that study, low platelet count, low antithrombin activity, and high concentrations of thrombin–antithrombin complexes and fibrin formation markers (fibrin/fibrinogen degradation product E and D-dimers) predicted death from AP with reliable diagnostic accuracy (the areas under the ROC curves between 0.768 for thrombin–antithrombin complexes and 0.926 for antithrombin activity) [16]. In addition, Radenković et al. [20] reported high diagnostic accuracy (areas under the ROC curves of 0.908 and 0.915, respectively) for D-dimer measured at admission and 24 h thereafter in the prognosis of organ failure complicating AP (pulmonary, renal failure or shock). More recently, Ke et al. [22] studied a prospective cohort of 173 patients with AP of whom 47 were diagnosed with critical AP (persistent organ failure plus infected pancreatic necrosis). D-dimer was found to be a significant predictor of critical AP (area under the ROC curve was 0.810), in contrast to C-reactive protein. Several smaller studies reported D-dimer to be a marker of severe complications, i.e., multiorgan failure or pancreatic infection or death in the course of AP [21,27,28]. On the other hand, D-dimer was also reported to significantly predict MSAP diagnosed in accordance with revised 2012 Atlanta classification [29]. Consequently, a recent position paper following the international meeting of the American Pancreatic Association and the Japanese Pancreas Society includes D-dimer in a proposed AP activity index [4]. However, from practical point of view, the lack of standardization of D-dimer measurements leads to difficulties in the interpretation of results [30]. This is reflected by discrepant cut-off values reported in the studies cited above, from 0.4 to about 1.2 μg/mL in most studies [20–22,27,28] and even as high as 6.1 μg/mL in the study of Maeda et al. [16]. In fact, our results confirm the data of Maeda et al. [16].

Of interest, in our study, the results of functional coagulation tests, i.e., prothrombin time, APTT and fibrinogen were associated with AP severity on admission and the associations became weaker or non-significant a day thereafter, while the highly significant association between D-dimer and AP severity persisted at 48 h from the onset of AP. One explanation may be that the functional tests are more influenced by the treatment of AP. In in the early phase of AP, intensive fluid resuscitation is introduced [2,4]. On the other hand, all patients in our study exhibited at least one abnormal result of coagulation tests, and the severity of coagulopathy as detected by the laboratory tests progressed from 24 to 48 h of AP. Thus, it seems that, in patients with more severe AP, coagulopathy develops earlier and is detectable already on admission while those with mild AP also develop some coagulation abnormalities but they became evident later.

The diagnosis of DIC is not always straightforward. Several scores are used that include the results of routine and, in some cases, also of less accessible laboratory tests [31]. In our study, we decided to use ISTH score for overt DIC, the one based on the results of routine and easily available tests [24]. Organ destruction such as pancreatitis is listed among the conditions associated with DIC, allowing the use of the clinical/laboratory scores for the diagnosis of DIC [24,31]. Indeed, several authors reported clinically significant DIC as a complication of AP [16,18,32]. However, other coagulopathies were also observed in the course of AP, such a localized thrombosis and pulmonary embolism [33–37] or thrombotic thrombocytopenic purpura [17,38]. The diagnosis of DIC in AP patients is further complicated by the fact that acute inflammation is a known factor leading to an increase in platelet counts [39], fibrinogen, and D-dimer concentrations [30]. Thus, in the early phase of AP, platelet counts and fibrinogen concentrations may increase due to inflammation and decrease due to consumptive coagulopathy; single laboratory result would be influenced by both processes. This is reflected by increased, rather than deceased fibrinogen observed in our cohort, including patients with SAP. As a positive acute phase protein, fibrinogen is highly influenced by inflammation, being the least sensitive marker of DIC in acute inflammatory conditions.

The pathomechanisms of activation of coagulation in AP are complex. Microvascular involvement is an important part of early pathogenesis of AP. SAP is characterized by significantly decreased blood flow in the capillaries of the pancreas [40,41], however, decreased blood flow velocity, increased vascular permeability and microvascular thrombosis are also observed in distant organs, including lungs, kidney and liver [42,43]. In 1970s, the release of proteolytic enzymes from damaged pancreas

has been suggested as a causative factor for coagulopathy in AP [44]. At present, it is rather thought, that the activation or dysfunction of vascular endothelium in consequence of acute inflammation leads to procoagulant changes, including (but not confined to) the expression of tissue factor on endothelial cells, constituting the main trigger for activation of coagulation (extensively reviewed in [23]). Although indirectly, our results seem to support this assumption: in our cohort, significant correlations were found between D-dimer and inflammatory marker (C-reactive protein) as well as the markers of endothelial dysfunction (angiopoietin-2 and sFlt-1), but not between D-dimer and amylase activity. Of note, the associations between inflammation, coagulation and endothelial dysfunction are complex and reciprocal. The results of experimental studies in AP raise hope that these detrimental interactions may be in part inhibited with anticoagulant treatment [23,45–48].

The present report supplements our earlier reports regarding angiopoietin-2 [7] and sFlt-1 [8] as the markers of severity in AP; hereby we show (pathophysiologically relevant) associations of these endothelial markers with coagulopathy in AP. There is a body of evidence showing that high concentrations of angiopoietin-2 predicts more severe AP [5,6] and our results are consistent with these reports. To the contrary, according to our knowledge, our reports are the only ones showing the association between high sFlt-1 concentrations and AP severity. In 2011, Espinosa et al. [49] evaluated the relationships between soluble angiogenesis-related markers including soluble vascular endothelial growth factor receptor-1 (or sFlt-1) and AP severity and did not found the significant association. However, the discrepancy between the results of Espinosa et al. and ours may be explained by the different time-points of sample collection (Espinosa et al. took the blood samples considerably later after onset of AP symptoms than we did) and by the differences in laboratory methods. On the other hand, sFlt-1 proved useful in prediction of the severity of sepsis [9]. Still, further studies are definitely necessary to evaluate the usefulness of sFlt-1 in prediction of AP severity; such studies are especially awaited as the availability of an automated sFlt-1 assay would make it easier to introduce the marker into clinical practice.

Several limitations of our study must be admitted. First, low number of patients with SAP makes it impossible to draw definitive conclusions regarding diagnostic utility of studied tests to predict SAP. These results must be treated as hypothesis-generating and needs further evaluation in larger sample. Second, we have not measured other markers of endothelial activation or dysfunction. The degranulation of Weibel-Palade bodies of activated endothelial cells leads to the increase in plasma/serum concentrations of numerous bioactive substances besides angiopoietin-2, e.g., von Willebrand factor, interleukin-8, endothelin-1, or P-selectin [23]. The associations we have observed among angiopoietin-2, sFlt-1, coagulation abnormalities and more severe course of AP might, in fact, be mediated by other bioactive compounds. Still, this fact does not exclude the possibility for the use of the studied markers in clinical practice. Third, although we have investigated and presented the most important demographic and clinical characteristics of patients, including data on etiology of AP, and comorbidities, there are numerous unmeasured confounders that may influence our results, including diet, lifestyle and genetic factors that may influence the development of pancreatitis [50], as well as the inflammatory response [51] and the severity of coagulopathy [52].

In conclusion, our study shows that the abnormalities of coagulation are present already in the very early phase of AP (first 24 h from the onset of symptoms). The coagulopathy is associated with the severity of inflammation and endothelial dysfunction. Thus, the results of routine tests of hemostasis, and in particular, the measurement of D-dimer, may assist the prognosis of AP severity and should be taken into account in clinical practice. Although our results regarding the diagnostic utility of studied tests for the diagnosis of SAP must be treated with caution due to low number of patients with SAP, our data support the promising reports on angiopoietin-2 as an early marker of SAP. In contrast, concentrations of sFlt-1 in early phase of AP seem to predict both transient and persistent organ failure, as reflected by reasonable diagnostic accuracy for the diagnosis of MSAP plus SAP. There is a need for novel markers with high diagnostic accuracy to predict severity of AP, and the studied markers of vascular leakage and endothelial dysfunction seem promising in this setting, however, especially in

case of sFlt-1, more studies are needed to verify our results. In the meantime, our results underscore the value of laboratory assessment of coagulation in clinical practice: coagulopathy detected by routine tests may be viewed as a surrogate marker of endothelial dysfunction among patients with AP.

4. Materials and Methods

4.1. Study Protocol

The prospective observational study recruited a cohort of consecutive adult patients admitted with the diagnosis of AP and treated in the Surgery Department of the District Hospital in Sucha Beskidzka, Poland. AP was diagnosed in concordance with 2012 revision of the Atlanta Classification [3], i.e., when two of the three following criteria were fulfilled: abdominal pain consistent with AP, serum amylase activity above three times greater than the upper reference limit, and characteristic findings of AP on abdominal imaging (contrast-enhanced computer tomography, magnetic resonance imaging or transabdominal ultrasonography). In addition, the inclusion criteria were admission to hospital within first 24 h from the onset of pain due to AP, and the written informed consent for the study. Patients treated with anticoagulants due to pre-existing conditions, those with pre-existing coagulopathies, as well as those with chronic pancreatitis, neoplasms, and chronic liver diseases (cirrhosis or viral hepatitis) were excluded.

The blood samples for laboratory tests were collected twice: on admission, i.e., within first 24 h from the onset of pain, and on Day 2 of hospital stay, i.e., at 48 h from the onset of pain as a symptom of AP.

DIC was diagnosed according to ISTH score for overt DIC [24]. D-dimer was used as a marker of fibrin formation. Moderately increased D-dimer concentrations were defined as 0.5–5 μg/mL (i.e., elevated up to 10-times above the reference limit), and severely increased as above 5 μg/mL.

The severity of AP was defined following 2012 revision of the Atlanta Classification [3]. MAP was diagnosed when no organ failure, local or systemic complications occurred. MSAP was diagnosed when a patient presented transient organ failure (resolving within 48 h), local (necrosis, acute necrotic collection, walled-off pancreatic necrosis) or systemic complications (exacerbation of preexisting conditions). SAP was diagnosed in patients with persistent (lasting longer than 48 h) organ failure. The diagnosis of MAP, MSAP or SAP was based on the clinical evolution of AP during the hospital stay of a patient. BISAP [25] and Glasgow [26] severity scores were calculated based on the assessment of patients during first 24 h of AP.

Additional group of healthy volunteers were recruited in order to obtain reference values for the non-routine laboratory tests, i.e., angiopoietin-2 and sFlt-1 in serum. The volunteers were men and women without any known pancreatic, renal, or liver diseases, malignancies or autoimmune diseases, without pregnancy in women, without thrombotic disorders in anamnesis, and with serum C-reactive protein concentrations below 3 mg/L. They provided written informed consent for the study. Single venous blood samples were collected from healthy volunteers into serum tubes.

The study protocol was approved by the Bioethics Committee of the Jagiellonian University (approval no. KBET/247/B/2013, permission date 28 November 2013, and 122.6120.242.2015, permission date 22 November 2015).

4.2. Laboratory Tests

Plasma was prepared by centrifugation of sodium citrate–anticoagulated venous blood (1 volume of citrate per 9 volumes of blood), within 20 min from blood collection. The concentrations of D-dimer, fibrinogen, as well as prothrombin time and APTT were measured on the day of blood collection, using Coatron A4 automated hemostasis analyzer (TECO Medical Instruments, Neufahrn, Germany). The reference ranges for the tests, as provided by the laboratory, were as following: <0.5 μg/mL for D-dimer, 2–4 g/L for fibrinogen, 11.4–15.5 s for prothrombin time and 26–39 s for APTT.

EDTA–anticoagulated full venous blood was used to perform complete blood counts, including platelet counts. Blood counts were performed on the day of blood collection, with the use of Sysmex XE 2100 analyzer (Sysmex Corporation, Kobe, Japan). The laboratory reference range for platelet count was $150 \times 10^3/\mu L$–$350 \times 10^3/\mu L$.

The concentrations of C-reactive protein, glucose, creatinine, urea, bilirubin, albumin, calcium, and amylase activity were measured in serum on the day of blood collection, using automated analyzer Cobas 6000 (Roche Diagnostics, Basel, Switzerland).

All the above tests were performed in the Department of Laboratory Diagnostics, District Hospital in Sucha Beskidzka, Poland.

Serum for angiopoietin-2 and sFlt-1 measurements was obtained by centrifugation of venous blood. The blood samples collected from AP patients and from healthy volunteers were proceeded in the same way. Serum samples were aliquoted and kept frozen at -70 °C until assayed (no longer than three months). Angiopoietin-2 was measured with the Quantikine ELISA Human Angiopoietin-2 immunoassay (R&D Systems, McKinley Place, MN, USA) in the Department of Diagnostics, Chair of Clinical Biochemistry, Jagiellonian University Medical College, Kraków, Poland. The concentrations of sFlt-1 were measured by electrochemiluminescence immunoassay using Cobas 8000 analyzer (Roche Diagnostics, Mannheim, Germany) in the Department of Diagnostics, University Hospital, Kraków, Poland.

4.3. Statistical Analysis

Data were reported as numbers (percentage) for categories, median (lower-upper quartile) for non-normally distributed quantitative variables, and mean $\pm$ standard deviation for normally distributed quantitative variables (as tested with Shapiro–Wilk test). Chi-squared test, Mann–Whitney test, and unpaired *t*-test were used to study differences between groups, respectively. Logistic regression was used to check whether the differences between patients and controls were independent of age. Right-skewed variables were log-transformed before calculating Pearson's correlation coefficients and before including in multiple linear regression. ROC curves were used to assess diagnostic utility of studied tests, and the results are expressed as area under the ROC curve $\pm$ standard error. Results were considered significant at p-value below 0.05. The calculations were made with the use of Statistica 12 software package (StatSoft, Tulsa, OK, USA).

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Jako współautor prac pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.
2. Dumnicka P., Sporek M., Mazur-Laskowska M., Ceranowicz P., Kuźniewski M., Drożdż R., Ambroży T., Olszanecki R., Kuśnierz-Cabala B.: Serum soluble Fms-like tyrosine kinase 1 (sFlt-1) predicts the severity of acute pancreatitis. Int. J Mol. Sciences. 2016; 17, 2038: Doi: 10.3390/ijms17122038.
3. Dumnicka P., Maduzia D., Ceranowicz P., Olszanecki R., Drożdż R., Kuśnierz-Cabala B.: The interplay between inflammation, coagulation and endothelial injury in the early phase of acute pancreatitis: clinical implications. Int. J Mol. Sciences. 2017; 18, 354; Doi: 10.3390/ijms18020354.
4. Dumnicka P., Kuśnierz-Cabala B., Sporek M., Mazur-Laskowska M., Gil K., Ceranowicz P., Warzecha Z., Dembinski A., Bonior J., Drożdż R.: Serum concentrations of angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) are associated with coagulopathy among patients with acute pancreatitis. Int. J Mol. Sciences. 2017; 18, 753: Doi: 102290/ijms18040753.

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie prac w formie publikacji to:
udział w opracowaniu manuskryptu, korekta opracowania, przygotowanie piśmiennictwa oraz udział w procesie publikacji i korespondencji autorskiej

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

Piotr Ceranowicz

.....
podpis wnioskodawcy

Prof. dr hab. n. med. Artur Dembinski
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 03.04.2017 r.

OŚWIADCZENIE

Jako współautor prac pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.
2. Dumnicka P., Kuśnierz-Cabala B., Sporek M., Mazur-Laskowska M., Gil K., Ceranowicz P., Warzecha Z., Dembinski A., Bonior J., Drożdż R.: Serum concentrations of angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) are associated with coagulopathy among patients with acute pancreatitis. Int. J Mol. Sciences. 2017; 18, 753: Doi: 10.3390/ijms18040753.

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie prac w formie publikacji to:
udział w przygotowaniu manuskryptu oraz dobór piśmiennictwa i korekta opracowania

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

.....

podpis wnioskodawcy

Dr hab. n. med. Ryszard Drożdż
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 04.04.2017 r.

OŚWIADCZENIE

Jako współautor prac pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.
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3. Dumnicka P., Maduzia D., Ceranowicz P., Olszanecki R., Drożdż R., Kuśnierz-Cabala B.: The interplay between inflammation, coagulation and endothelial injury in the early phase of acute pancreatitis: clinical implications. Int. J Mol. Sciences. 2017; 18, 354; Doi: 10.3390/ijms18020354.
4. Dumnicka P., Kuśnierz-Cabala B., Sporek M., Mazur-Laskowska M., Gil K., Ceranowicz P., Warzecha Z., Dembinski A., Bonior J., Drożdż R.: Serum concentrations of angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) are associated with coagulopathy among patients with acute pancreatitis. Int. J Mol. Sciences. 2017; 18, 753: Doi: 10.3390/ijms18040753.

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie prac w formie publikacji to:
udział w opracowaniu koncepcji manuskryptu, opracowaniu statystycznym oraz interpretacji wyników badań, pomoc w przygotowaniu piśmiennictwa

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

.....
podpis wnioskodawcy

Dr med. Agnieszka Gala-Błądzińska
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 23.03.2017 r.

OŚWIADCZENIE

Jako współautor pracy pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:
udział w opracowaniu manuskryptu oraz dyskusji części klinicznej dotyczącej zagadnień nefrologicznych

Jednocześnie wyrażam zgodę na uznanie, że w/w praca przedłożona przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowi jej indywidualny wkład w rozwój medycyny.

Agnieszka Gala-Błądzińska
.....
podpis wnioskodawcy

Dr hab. n. med. Krzysztof Gil
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 03. 04. 2017 r.

OŚWIADCZENIE

Jako współautor pracy pt.:

Dumnicka P., Kuśnierz-Cabala B., Sporek M., Mazur-Laskowska M., Gil K., Ceranowicz P., Warzecha Z., Dembinski A., Bonior J., Drożdż R.: Serum concentrations of angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) are associated with coagulopathy among patients with acute pancreatitis. Int. J Mol. Sciences. 2017; 18, 753: Doi: 10.3390/ijms18040753.

oświadczam, że mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

udział w przygotowaniu koncepcji manuskryptu

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

Katedra Patofizjologii UJ CM
.....dr hab. n. med. Krzysztof Gil.....
p.o. Kierownik

(podpis wnioskodawcy)

Dr hab. n. med. Beata Kuśnierz-Cabala, prof. UJ
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 03.04.2017 r.

OŚWIADCZENIE

Jako współautor prac pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.
2. Dumnicka P., Sporek M., Mazur-Laskowska M., Ceranowicz P., Kuźniewski M., Drożdż R., Ambroży T., Olszanecki R., Kuśnierz-Cabala B.: Serum soluble Fms-like tyrosine kinase 1 (sFlt-1) predicts the severity of acute pancreatitis. Int. J Mol. Sciences. 2016; 17, 2038: Doi: 10.3390/ijms17122038.
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oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie prac w formie publikacji to:
udział w opracowaniu manuskryptu, wykonanie badań laboratoryjnych, opracowanie bazy danych i dyskusja wyników

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

B. Kuśnierz-Cabala

.....

podpis wnioskodawcy

Prof. dr hab. n. med. Marek Kuźniewski
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 03.04.2017 r.

OŚWIADCZENIE

Jako współautor prac pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.
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oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie prac w formie publikacji to:
udział w opracowaniu koncepcji manuskryptu, opracowaniu statystycznym oraz interpretacji uzyskanych wyników badań, pomoc w przygotowaniu piśmiennictwa

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

M. Kuźniewski

.....

podpis wnioskodawcy

Lek. Dawid Maduzia
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 03.04.2017 r.

OŚWIADCZENIE

Jako współautor pracy pt.:

Dumnicka P., Maduzia D., Ceranowicz P., Olszanecki R., Drożdż R., Kuśnierz-Cabala B.: The interplay between inflammation, coagulation and endothelial injury in the early phase of acute pancreatitis: clinical implications. Int. J Mol. Sciences. 2017; 18, 354; Doi: 10.3390/ijms18020354.

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udział w przygotowaniu pracy przeglądowej oraz pomoc w doborze piśmiennictwa

Jednocześnie wyrażam zgodę na uznanie, że w/w praca przedłożona przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowi jej indywidualny wkład w rozwój medycyny.

Dawid Maduzia
LEKARZ
2899361

.....
podpis wnioskodawcy

Dr n. med. Małgorzata Mazur-Laskowska
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 03.04.2017 r.

OŚWIADCZENIE

Jako współautor prac pt.:

1. Dumnicka P., Sporek M., Mazur-Laskowska M., Ceranowicz P., Kuźniewski M., Drożdż R., Ambroży T., Olszanecki R., Kuśnierz-Cabala B.: Serum soluble Fms-like tyrosine kinase 1 (sFlt-1) predicts the severity of acute pancreatitis. Int. J Mol. Sciences. 2016; 17, 2038: Doi: 10.3390/ijms17122038.
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oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie prac w formie publikacji to:

udział w przeprowadzaniu badań laboratoryjnych i przygotowaniu bazy danych

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

Małgorzata Mazur-Laskowska

(podpis wnioskodawcy)

Prof. dr hab. n. med. Rafał Olszanecki
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 04. 04. 2017 r.

OŚWIADCZENIE

Jako współautor prac pt.:

1. Dumnicka P., Sporek M., Mazur-Laskowska M., Ceranowicz P., Kuźniewski M., Drożdż R., Ambroży T., Olszanecki R., Kuśnierz-Cabala B.: Serum soluble Fms-like tyrosine kinase 1 (sFlt-1) predicts the severity of acute pancreatitis. Int. J Mol. Sciences. 2016; 17, 2038: Doi: 10.3390/ijms17122038.
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oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie prac w formie publikacji to:
udział w przygotowaniu manuskryptu oraz doborze piśmiennictwa i interpretacji danych klinicznych

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

.....
podpis wnioskodawcy

Dr n. med. Mateusz Sporek
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 03.04.2017 r.

OŚWIADCZENIE

Jako współautor prac pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.
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3. Dumnicka P., Kuśnierz-Cabala B., Sporek M., Mazur-Laskowska M., Gil K., Ceranowicz P., Warzecha Z., Dembinski A., Bonior J., Drożdż R.: Serum concentrations of angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) are associated with coagulopathy among patients with acute pancreatitis. Int. J Mol. Sciences. 2017; 18, 753: Doi: 10.3390/ijms18040753.

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie prac w formie publikacji to:
udział w zebraniu materiału od pacjentów, opracowaniu bazy danych i przygotowanie wyników

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

Mateusz Sporek
.....
podpis wnioskodawcy

Dr hab. n. med. Ewa Stępień, prof. UJ
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 14.03.2017 r.

OŚWIADCZENIE

Jako współautor pracy pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:
udział w omawianiu i dyskusji wyników

Jednocześnie wyrażam zgodę na uznanie, że w/w praca przedłożona przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowi jej indywidualny wkład w rozwój medycyny.

Ewa Stępień
.....
podpis wnioskodawcy

Prof. dr hab. med. Jerzy Walocha
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 06.03.2017 r.

OŚWIADCZENIE

Jako współautor pracy pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.

oświadczam, że mój wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:
udział w opracowaniu manuskryptu oraz dyskusji wyników

Jednocześnie wyrażam zgodę na uznanie, że w/w praca przedłożona przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowi jej indywidualny wkład w rozwój medycyny.

Jerzy Walocha
.....
podpis wnioskodawcy

dr hab. n. med. Zygmunt Warzecha, prof. UJ
(tytuł naukowy, imię i nazwisko)

Kraków, dn. 03.04. 2017 r.

OŚWIADCZENIE

Jako współautor prac pt.:

1. Sporek M., Dumnicka P., Gala-Błądzińska A., Ceranowicz P., Warzecha Z., Dembinski A., Stepien E., Walocha J., Drozdz R., Kuzniewski M., Kusnierz-Cabala B.: Angiopoietin-2 is an early indicator of acute pancreatic-renal syndrome in patients with acute pancreatitis. Mediators Inflammation 2016; Doi: 10.1155/2016/5789903.
2. Dumnicka P., Kuśnierz-Cabala B., Sporek M., Mazur-Laskowska M., Gil K., Ceranowicz P., Warzecha Z., Dembinski A., Bonior J., Drożdż R.: Serum concentrations of angiopoietin-2 and soluble fms-like tyrosine kinase 1 (sFlt-1) are associated with coagulopathy among patients with acute pancreatitis. Int. J Mol. Sciences. 2017; 18, 753: doi: 10.3390/ijms18040753.

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udział w przygotowaniu manuskryptu oraz dobór piśmiennictwa i korekta opracowania

Jednocześnie wyrażam zgodę na uznanie, że w/w prace przedłożone przez lek. Paulinę Dumnicką jako część jej rozprawy doktorskiej, stanowią jej indywidualny wkład w rozwój medycyny.

.....

podpis wnioskodawcy

Spis skrótów

Skrót	Nazwa angielska	Nazwa polska
ADAMTS-13	a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13	-
AKI	acute kidney injury	ostre uszkodzenie nerek
Ang-2	angiopoietin-2	angiopoetyna-2
ANOVA	analysis of variance	analiza wariancji
AP	acute pancreatitis	ostre zapalenia trzustki
APACHE	Acute Physiology and Chronic Health Evaluation	-
APC	activated protein C	aktywowane białko C
APTT	activated partial thromboplastin time	czas aktywowanej częściowej tromboplastyny
AUC	area under curve	pole pod krzywą
BISAP	Bedside Index of Severity in Acute Pancreatitis	-
CI	confidence interval	przedział ufności
CRP	C-reactive protein	białko C-reaktywne
CT	computed tomography	tomografia komputerowa
DIC	disseminated intravascular coagulation	zespół rozsianego wykrzepiania wewnątrznaczyniowego
eGFR	estimated glomerular filtration rate	szacowany wskaźnik filtracji kłębuszkowej
ERCP	endoscopic retrograde cholangiopancreatography	endoskopowa wsteczna cholangiopankreatografia
Flt-1	fms-like tyrosine kinase-1	fms-podobna kinaza tyrozynowa-1
HCT	hematocrit	hematokryt
ICAM-1	intercellular adhesion molecule-1	cząsteczka adhezji międzykomórkowej-1
IL	interleukin	interleukina
ISTH	International Society on Thrombosis and Haemostasis	Międzynarodowe Stowarzyszenie Zakrzepicy i Hemostazy
KDIGO	Kidney Disease: Improving Global Outcomes	-
KIM-1	kidney injury molecule-1	cząsteczka uszkodzenia nerek-1
LMWH	low molecular weight heparin	heparyna drobnocząsteczkowa
MAP	mild acute pancreatitis	łagodne ostre zapalenie trzustki
MDRD	Modification of Diet in Renal Disease	modyfikacja diety w chorobie nerek
MSAP	moderately-severe acute pancreatitis	średnio-ciężkie ostre zapalenie trzustki
MMSS	Modified Marshall Scoring System	zmodyfikowana skala Marshalla
NGAL	neutrophil gelatinase-associated lipocalin	lipokalina związana z żelatynazą neutrofili

OR	odds ratio	iloraz szans
NFκB	nuclear factor κB	czynnik jądrowy κB
NS	non-significant	nieistotny statystycznie
PAF	platelet activating factor	czynnik aktywujący płytki
PAI-1	plasminogen activator inhibitor-1	aktywator inhibitora plazminogenu-1
PAR	protease activated receptor	receptor aktywowany przez proteazy
PLT	platelet count	liczba płytek
RIFLE	risk, injury, failure, loss, end stage	ryzyko, uszkodzenie, niewydolność, utrata funkcji, schyłkowa niewydolność
ROC	receiver operating characteristic	charakterystyka operacyjna odbiornika
rTM	recombinant human soluble thrombomodulin	rekombinowana ludzka rozpuszczalna trombomodulina
SAP	severe acute pancreatitis	ciężkie ostre zapalenie trzustki
sCD40L	soluble CD40 ligand	rozpuszczalny ligand dla CD40
sFlt-1	soluble fms-like tyrosine kinase-1	rozpuszczalna fms-podobna kinaza tyrozynowa-1
SIRS	systemic inflammatory response syndrome	zespół uogólnionej odpowiedzi zapalnej
sNGAL	serum neutrophil gelatinase-associated lipocalin	lipokalina związana z żelatynazą neutrofili w surowicy
SOFA	Sequential Organ Failure Assessment	-
TF	tissue factor	czynnik tkankowy
TFPI	tissue factor pathway inhibitor	inhibitor szlaku czynnika tkankowego
TNF	tumor necrosis factor	czynnik martwicy nowotworów
tPA	tissue plasminogen activator	tkankowy aktywator plazminogenu
uACR	urine albumin/creatinine ratio	wskaźnik albumina/kreatynina w moczu
uNGAL	urine neutrophil gelatinase-associated lipocalin	lipokalina związana z żelatynazą neutrofili w moczu
VCAM-1	vascular cell adhesion molecule-1	cząsteczka adhezji komórkowej naczyń-1
VEGF	vascular endothelial growth factor	czynnik wzrostu śródbłonka naczyniowego
VEGFR-1	vascular endothelial growth factor receptor-1	receptor-1 dla czynnika wzrostu śródbłonka naczyniowego
vWF	von Willebrand factor	czynnik von Willebranda
WBC	white blood count	liczba leukocytów

Załącznik nr 1. Kryteria diagnostyczne i skale kliniczne wykorzystane w pracy doktorskiej

Kryteria rozpoznania i klasyfikacja ciężkości ostrego zapalenia trzustki (zmodyfikowana klasyfikacja Atlanta z 2012 roku)

Banks P.A., Bollen T.L., Dervenis C., Gooszen H.G., Johnson C.D., Sarr M.G. i wsp. Classification of acute pancreatitis - 2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; 62: 102–11.

Definicja ostrego zapalenia trzustki

Rozpoznanie ostrego zapalenia trzustki wymaga spełnienia dwóch z trzech kryteriów:

1. ból brzucha charakterystyczny dla ostrego zapalenia trzustki (utrzymujący się, nasilony ból o ostrym początku, zlokalizowany w nadbrzuszu, często promieniujący do pleców);
2. aktywność lipazy (lub amylazy) w surowicy co najmniej trzykrotnie przekraczająca górną granicę wartości prawidłowych;
3. zmiany charakterystyczne dla ostrego zapalenia trzustki w badaniu tomografii komputerowej z kontrastem, rzadziej w tomografii rezonansu magnetycznego lub w przezbrzusznym badaniu ultrasonograficznym.

Stopnie ciężkości ostrego zapalenia trzustki

Łagodne ostre zapalenie trzustki (*mild acute pancreatitis*, MAP):

- bez niewydolności narządowej
- bez powikłań miejscowych i ogólnoustrojowych

Średnio-ciężkie ostre zapalenie trzustki (*moderately-severe acute pancreatitis*, MSAP):

- niewydolność narządowa ustępująca w ciągu 48 godzin (przemijająca) i/lub
- powikłania miejscowe lub ogólnoustrojowe bez przetrwałej niewydolności narządowej

Ciężkie ostre zapalenie trzustki (*severe acute pancreatitis*, SAP):

- przetrwała niewydolność narządowa (> 48 godzin) dotycząca jednego lub wielu narządów

Niewydolność narządowa powinna być oceniana zgodnie ze zmodyfikowaną skalą Marshalla.

Powikłania ogólnoustrojowe definiowane są jako zaostrzenie chorób współistniejących (takich jak choroba niedokrwienna serca lub przewlekła obturacyjna choroba płuc) spowodowane ostrym zapaleniem trzustki.

Do powikłań miejscowych zaliczono:

- ostry okołotrzustkowy zbiornik płynowy,
- torbiel rzekomą,
- ostry zbiornik martwiczy,
- odizolowaną martwicę.

Początek ostrego zapalenia trzustki zdefiniowano jako moment wystąpienia objawów klinicznych (w tym bólu brzucha).

Kryteria niewydolności narządowej według zmodyfikowanej skali Marshalla

Banks P.A., Bollen T.L., Dervenis C., Gooszen H.G., Johnson C.D., Sarr M.G. i wsp. Classification of acute pancreatitis - 2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; 62: 102–11.

Niewydolność narządową należy rozpoznać przy wynikach ≥ 2 punktów dla dowolnego narządu lub układu.

Układ lub narząd; oceniany wskaźnik	Punktacja				
	0	1	2	3	4
układ oddechowy; PaO_2/FiO_2, mmHg	> 400	301-400	201-300	101-200	≤ 100
nerki; kreatynina w surowicy, µmol/l	≤ 134	134-169	170-310	311-439	> 439
układ sercowo-naczyniowy; ciśnienie skurczowe, mmHg	> 90	< 90, odpowiedź na płyny	<90, brak odpowiedzi na płyny	< 90, pH < 7,3	< 90, pH < 7,2

Kryteria rozpoznania uogólnionego zespołu odpowiedzi zapalnej (systemic inflammatory response syndrome, SIRS)

Banks P.A., Bollen T.L., Dervenis C., Gooszen H.G., Johnson C.D., Sarr M.G. i wsp. Classification of acute pancreatitis - 2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013; 62: 102–11.

SIRS definiowany jest jako obecność co najmniej dwóch z wymienionych kryteriów:

- akcja serca > 90 uderzeń na minutę,
- temperatura centralna < 36 °C lub > 38 °C,
- liczba krwinek białych we krwi obwodowej < 4 x 10^3/µl lub > 12 x 10^3/µl,
- liczba oddechów > 20 na minutę lub PCO_2 < 32 mmHg.

Skala Bedside Index of Severity in Acute Pancreatitis (BISAP)

Wu B.U., Johannes R.S., Sun X., Tabak Y., Conwell D.L., Banks P. The early prediction of mortality in acute pancreatitis: A large population-based study. *Gut* 2008; 57: 1698–703.

Za spełnienie każdego z poniższych kryteriów przyznawany jest 1 punkt:

- stężenie azotu mocznika w surowicy > 25 mg/dl (8,9 mmol/l),
- zaburzenia świadomości (wynik < 15 punktów w skali śpiączki Glasgow),
- SIRS,
- wiek > 60 lat,
- płyn w jamie opłucnej.

Suma punktów ≥ 3 wiąże się z wyższym prawdopodobieństwem zgonu.

Skala Glasgow

Blamey S.L., Imrie C.W., O'Neill J., Gilmour W.H., Carter D.C. Prognostic factors in acute pancreatitis. *Gut* 1984; 25: 1340–6.

Za spełnienie każdego z poniższych kryteriów przyznawany jest 1 punkt:

- stężenie wapnia w surowicy < 2 mmol/l,
- stężenie mocznika w surowicy > 16 mmol/l,
- aktywność dehydrogenazy mleczanowej w surowicy > 600 U/l,
- stężenie glukozy w surowicy > 10 mmol/l,
- PaO_2 < 60 mmHg,
- liczba leukocytów we krwi obwodowej > 15 x 10^3/µl,
- stężenie albuminy w surowicy < 32 g/l,
- wiek > 55 lat.

Suma punktów > 3 wskazuje na wysokie prawdopodobieństwo ciężkiego ostrego zapalenia trzustki.

Kryteria rozpoznania ostrego uszkodzenia nerek (acute kidney injury, AKI) według Kidney Disease: Improving Global Outcomes (KDIGO)

Kellum J.A., Lameire N., Aspelin P., Barsoum R.S., Burdmann E.A., Goldstein S.L. I wsp. KDIGO clinical practice guideline for acute kidney injury. *Kidney Int. Suppl.* 2012; 2: 1–138.

AKI należy rozpoznać przy spełnieniu któregokolwiek z poniższych kryteriów:

- wzrost stężenia kreatyniny w surowicy o ≥ 0,3 mg/dl (≥ 26,5 µmol/l) w ciągu 48 godzin;
- wzrost stężenia kreatyniny w surowicy ≥ 1,5-krotny wobec wartości wyjściowych, który nastąpił lub prawdopodobnie nastąpił w ciągu 7 dni;
- diureza < 0,5 ml/kg/godz. przez 6 godzin.

Ocena ciężkości AKI:

Stopień	Stężenie kreatyniny w surowicy	Diureza
1	1,5–1,9-krotny wzrost wobec wartości wyjściowych lub wzrost o > 0,3 mg/dl (>26,5 µmol/l)	< 0,5 ml/kg/h przez 6-12 godzin
2	2,0–2,9-krotny wzrost wobec wartości wyjściowych	< 0,5 ml/kg/h przez ≥ 12 godzin
3	≥ 3,0-krotny wzrost wobec wartości wyjściowych lub wzrost do wartości ≥ 4,0 mg/dl (≥ 353,6 µmol/l) lub rozpoczęcie leczenia nerkozastępczego	< 0,3 ml/kg/h przez ≥ 24 godziny lub anuria przez ≥ 12 godzin

Kryteria rozpoznania zespołu rozsianego wykrzepiania wewnątrznaczyniowego (disseminated intravascular coagulation, DIC) według International Society on Thrombosis and Haemostasis (ISTH)

Levi M., Toh C.H., Thachil J., Watson H.G. Guidelines for the diagnosis and management of disseminated intravascular coagulation. *Br. J. Haematol.* 2009; 145: 24–33.

Poniższe laboratoryjne kryteria rozpoznania jawnego (ostrego) DIC należy stosować u pacjentów z rozpoznaniem schorzenia, które wiąże się z ryzykiem wystąpienia DIC (w tym z rozpoznaniem ostrego zapalenia trzustki).

Punktacja:

- liczba płytek we krwi obwodowej:
  > 100 x 10^3/µl 0 punktów
  50-100 x 10^3/µl 1 punkt
  < 50 x 10^3/µl 2 punkty
- markery fibrynolizy (np. D-dimery, fragmenty degradacji fibryny):
  niepodwyższone 0 punktów
  umiarkowanie podwyższone 2 punkty
  znacznie podwyższone 3 punkty
- czas protrombinowy:
  przedłużony o < 3 s 0 punktów
  przedłużony o 3-6 s 1 punkt
  przedłużony o > 6 s 2 punkty
- stężenie fibrynogenu w osoczu
  > 1 g/l 0 punktów
  < 1 g/l 1 punkt

Suma punktów ≥ 5 pozwala na rozpoznanie jawnego (ostrego) DIC.

Załącznik nr 2. Przedziały referencyjne dla badań laboratoryjnych wykorzystanych w pracy doktorskiej

Nazwa badania	Przedział referencyjny (K – kobiety / M – mężczyźni)
Morfologia krwi obwodowej:	
liczba erytrocytów, x 10^6/µl	K: 3,5 – 5,5 / M: 4,5 – 6,5
hemoglobina, g/dl	K: 11,0 – 15,0 / M: 12,0 – 17,0
hematokryt, %	K: 37,0 – 47,0 / M: 40,0 – 54,0
liczba leukocytów, x 10^3/µl	4,0 – 10,0
liczba płytek, x 10^3/µl	150 – 350
Badania biochemiczne w surowicy:	
amylaza, U/l	62 – 220
albumina, g/l	35,0 – 52,0
wapń całkowity, mmol/l	2,02 – 2,61
białko C-reaktywne, mg/l	< 5,0
glukoza, mmol/l	3,3 – 5,6
bilirubina całkowita, µmol/l	0 – 21,0
kreatynina, µmol/L	45,0 – 97,0
mocznik, mmol/l	2,76 – 8,07
cystatyna C, mg/l	0,59 – 1,04
NGAL, µg/l	K: 21,6–276,0 / M: 14,4–169,2
angiopoetyna-2, ng/ml	1,17–2,47*
sFlt-1, pg/ml	63 – 108*
Badania układu krzepnięcia:	
czas protrombinowy, s	11,4 – 15,5
APTT, s	26 – 39
fibrynogen, g/l	2 – 4
D-dimery, µg/ml	<0,5
Badania biochemiczne w moczu:	
NGAL, µg/l	<131,7
wskaźnik albumina/kreatynina, mg/g	<30

* przedział wartości uzyskanych w grupie 21 zdrowych ochotników

NGAL, lipokalina związana z żelatynazą neutrofili
APTT, czas aktywowanej częściowej tromboplastyny

Streszczenie

Ostre zapalenie trzustki charakteryzuje się zróżnicowanym przebiegiem klinicznym, od postaci łagodnej, ustępującej samoistnie, do ciężkiej, obarczonej śmiertelnością sięgającą 50%. Obecnie stosowana zmodyfikowana klasyfikacja Atlanta z 2012 roku wyróżnia łagodne (MAP), średnio-ciężkie (MSAP) i ciężkie (SAP) ostre zapalenie trzustki. Wczesne rozpoznanie ciężkiego przebiegu choroby jest ważne, ponieważ pacjenci z ciężkim ostrym zapaleniem trzustki odnoszą korzyści z wczesnego wdrożenia intensywnego nadzoru medycznego i intensywnej terapii. Jednocześnie wczesne rozpoznanie SAP w praktyce klinicznej jest wciąż wyzwaniem. W przebiegu SAP uogólniony stan zapalny prowadzi do aktywacji i uszkodzenia komórek śródbłonka, zwiększenia przepuszczalności naczyń i aktywacji układu hemostazy. Te zaburzenia są przyczyną rozwoju niewydolności narządowej, w tym niewydolności nerek.

Celem niniejszej pracy była ocena stężeń wybranych białek związanych z aktywacją i uszkodzeniem komórek śródbłonka: angiopoetyny-2 i rozpuszczalnej fms-podobnej kinazy tyrozynowej-1 (sFlt-1) w surowicy pacjentów we wczesnej fazie ostrego zapalenia trzustki. Oceniono związek badanych wskaźników dysfunkcji śródbłonka z ciężkością ostrego zapalenia trzustki, z rozwojem ostrego uszkodzenia i niewydolności nerek oraz z występowaniem zaburzeń hemostazy w przebiegu choroby.

Do badania zakwalifikowano łącznie 70 dorosłych chorych z ostrym zapaleniem trzustki leczonych w Oddziale Chirurgicznym Szpitala Powiatowego w Suchej Beskidzkiej, przyjętych w ciągu 24 godzin od wystąpienia objawów choroby. Krew do badań laboratoryjnych, w tym oznaczeń stężeń angiopoetyny-2 i sFlt-1, pobierano trzykrotnie: przy przyjęciu oraz po 48 i 72 godzinach od wystąpienia objawów ostrego zapalenia trzustki. MAP (u 50 chorych), MSAP (u 15 chorych) i SAP (u 5 chorych) rozpoznawano zgodnie z klasyfikacją Atlanta z 2012 roku. Ostre uszkodzenie nerek diagnozowano zgodnie z kryteriami *Kidney Disease: Improving Global Outcomes* (KDIGO), a niewydolność nerek według zmodyfikowanej skali Marshalla. Zespół rozsianego wykrzepiania wewnątrznaczyniowego diagnozowano zgodnie z zaleceniami *International Society on Thrombosis and Haemostasis* (ISTH).

Stężenia angiopoetyny-2 i sFlt-1 korelowały z ciężkością ostrego zapalenia trzustki i były najwyższe u chorych z SAP. Stwierdzono dodatnie korelacje badanych wskaźników uszkodzenia śródbłonka z nasileniem stanu zapalnego oraz wyższe stężenia u pacjentów, u których wystąpił zespół uogólnionej odpowiedzi zapalnej. Stężenia angiopoetyny-2 i sFlt-1 korelowały dodatnio z laboratoryjnymi wskaźnikami uszkodzenia nerek: kreatyniną, mocznikiem, cystatyną C, lipokaliną związaną z żelatynazą neutrofili (NGAL) w surowicy oraz wskaźnikiem albumina/kreatynina i stężeniem NGAL w moczu. Angiopoetyna-2 była istotnym czynnikiem prognostycznym wystąpienia ostrego uszkodzenia nerek i niewydolności nerek. Stężenia angiopoetyny-2 i sFlt-1 korelowały z wynikami rutynowych badań układu hemostazy, najsilniejsze były dodatnie korelacje ze stężeniem D-dimerów. W ciągu pierwszych 24 godzin od wystąpienia objawów ostrego zapalenia trzustki oznaczenia angiopoetyny-2 i D-dimerów wykazywały najlepszą użyteczność diagnostyczną w prognozowaniu SAP, zaś oznaczenia sFlt-1 w prognozowaniu MSAP i SAP.

Przeprowadzone badania potwierdzają użyteczność oznaczeń angiopoetyny-2 w surowicy we wczesnym rozpoznaniu ciężkiego ostrego zapalenia trzustki. Po raz pierwszy wykazano użyteczność oznaczeń sFlt-1 w surowicy w prognozowaniu ciężkości ostrego zapalenia trzustki. Zautomatyzowana metoda oznaczania sFlt-1 stwarza możliwość wykonywania tego badania w praktyce klinicznej, jeśli wyniki niniejszej pracy zostaną potwierdzone w badaniu większej grupy chorych.

Abstract

Acute pancreatitis (AP) is an inflammatory disease of various severity, from mild and self-limiting, to severe, associated with mortality reaching 50%. According to the Atlanta classification revised in 2012, AP severity is categorized as mild (MAP), moderately severe (MSAP) and severe (SAP). Early recognition of severe course of AP is important, because patients with SAP benefit from early initiation of intensive care and intensive therapy. Nonetheless, early diagnosis of SAP remains challenging in clinical practice. In SAP, systemic inflammation leads to diffuse activation and injury of endothelial cells, increased vascular leak and activation of hemostasis. The disturbances cause organ failure, including renal failure.

The aim of the present study was to assess serum concentrations of selected proteins associated with endothelial activation and injury, namely angiopoietin-2 and soluble fms-like tyrosine kinase-1 (sFlt-1) among patients in early phase of AP. The associations were studied between the selected markers of endothelial dysfunction and AP severity, the development of acute kidney injury (AKI) and renal failure, as well as coagulopathy in the course of AP.

The study included 70 adult patients with AP treated in the Surgery Department of the District Hospital in Sucha Beskidzka, Poland, admitted within the first 24 hours from the onset of symptoms. Blood samples for laboratory tests, including measurements of angiopoietin-2 and sFlt-1 concentrations, were collected trice: on admission, and after 48 and 72 hours from the onset of symptoms of AP. MAP (in 50 patients), MSAP (in 15 patients) and SAP (in 5 patients) were diagnosed in accordance with 2012 Atlanta classification. AKI was diagnosed following Kidney Disease: Improving Global Outcomes (KDIGO) guidelines and renal failure was diagnosed according to modified Marshall scoring system. Diffuse intravascular coagulation was diagnosed following International Society on Thrombosis and Haemostasis (ISTH) guidelines.

Serum concentrations of angiopoietin-2 and sFlt-1 positively correlated with AP severity and were the highest among patients with SAP. Positive correlations were observed between the markers of endothelial dysfunction and the intensity of inflammation. Patients with systemic inflammatory response syndrome presented higher angiopoietin-2 and sFlt-1. Both studied endothelial markers correlated positively with laboratory markers of renal injury: serum creatinine, urea, cystatin C, neutrophil gelatinase-associated lipocalin (NGAL) as well as urine albumin/creatinine ratio and urine NGAL. Angiopoietin-2 was significant predictor of AKI and renal failure. Both angiopoietin-2 and sFlt-1 correlated with the results of routine tests of hemostasis: the strongest were positive correlations with D-dimer concentrations and ISTH scores. During first 24 hours from the onset of AP symptoms, angiopoietin-2 and D-dimer achieved the best diagnostic usefulness for the prediction of SAP, while sFlt-1 was the best predictor of MSAP plus SAP.

The present results confirm usefulness of serum angiopoietin-2 in the early prediction of severe course of AP. The usefulness of serum sFlt-1 in the prognosis of severe AP was shown for the first time. The automated laboratory method of sFlt-1 measurements allows to use this test in clinical practice, however, the present results should be confirmed in larger group of patients.