THE JOURNAL OF THE CZECH AND SLOVAK ONCOLOGICAL SOCIETIES

KLINICKÁ ONKOLOGIE

Monoclonal Gammopathies – Scientific Progress in the Czech Republic

Monoklonální gamapatie – vědecký pokrok v České republice

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Vydává ČLS JEP. ISSN 0862-495X. ISSN 1802-5307 on-line přístup
Indexed in MEDLINE/PubMed, EMBASE/Excerpta Medica, EBSCO, SCOPUS, Bibliographia medica čechoslovaca, Index Copernicus

ročník 30 | 2017 | Supplementum 2

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Před zahájením nového cyklu léčby absolutní počet neutrofilů má být ≥ 1 000/mm³, počet trombocytů má být ≥ 75 000/mm³ a nehematologické toxicity se obvykle mají vrátit na hodnoty, jaké měl pacient při zahájení léčby, nebo na hodnotu ≤ stupeň 1. *Zpoždění nebo vynechání dávky:* V případě, že dojde ke zpoždění nebo vynechání dávky přípravku NINLARO, má se tato dávka užít jen tehdy, pokud je časový odstup od následující plánované dávky ≥ 72 hodin. Nemá se užívat dvojnásobná dávka, aby se nahradila vynechaná dávka. Pokud pacient po užití dávky zvrací, nemá se dávka opakovat, ale má se pokračovat v užívání v době další plánované dávky. *Úprava dávkování:* Při výskytu středně těžké nebo těžké poruchy funkce jater, těžké poruchy funkce ledvin nebo konečného stadia onemocnění ledvin (ESRD) vyžadujícího dialýzu je doporučena snížená dávka 3 mg. V případě překrývajících se toxicit, jako jsou trombocytopenie, neutropenie a vyrážka, se u přípravku NINLARO a lenalidomidu doporučuje postup střídavé úpravy dávky. U těchto toxicit je prvním krokem při úpravě dávky vysazení/snížení dávky lenalidomidu. Postup při snižování dávky u těchto toxicit naleznete v Souhrnu údajů o přípravku pro lenalidomid. Postup při snižování dávky přípravku NINLARO je uveden v Tabulce 1 a pokyny pro úpravu dávky jsou uvedeny v tabulce 2 v Souhrnu údajů o přípravku pro Ninlaro. *Souběžně podávané léčivé přípravky:* U pacientů léčených přípravkem NINLARO je třeba zvážit antivirovou profylaxi ke snížení rizika reaktivace herpes zoster. U pacientů léčených přípravkem NINLARO v kombinaci s lenalidomidem a dexamethasonem se doporučuje tromboprofylaxe, která má vycházet z vyhodnocení rizik a klinického stavu pacienta. *Starší pacienti:* U pacientů starších 65 let není nutná úprava dávky přípravku NINLARO. *Porucha funkce jater:* U pacientů s lehkou poruchou funkce jater není nutná žádná úprava dávky přípravku NINLARO. U pacientů se středně těžkou nebo těžkou poruchou funkce jater se doporučuje snížená dávka 3 mg. *Porucha funkce ledvin:* U pacientů s lehkou nebo středně těžkou poruchou funkce ledvin (clearance kreatininu ≥ 30 ml/min) není nutná úprava dávky přípravku NINLARO. Snížená dávka 3 mg se doporučuje u pacientů s těžkou poruchou funkce ledvin (clearance kreatininu < 30 ml/min) nebo v konečném stadiu onemocnění ledvin (ESRD) vyžadujícím dialýzu. Přípravek NINLARO není dialyzovatelný. *Způsob podání:* Přípravek NINLARO je určen k perorálnímu podání. **Kontraindikace:** Hypersenzitivita na léčivou látku nebo na kteroukoli pomocnou látku. **Zvláštní upozornění*:** Vzhledem k tomu, že se přípravek NINLARO podává v kombinaci s lenalidomidem a dexamethasonem, je třeba si přečíst další zvláštní upozornění a opatření pro použití v Souhrnu údajů o přípravku těchto léčivých přípravků. Při léčbě přípravkem NINLARO byla hlášena trombocytopenie, přičemž k největšímu poklesu počtu trombocytů došlo obvykle mezi 14.–21. dnem každého 28denního cyklu a k obnovení výchozích hodnot došlo před zahájením dalšího cyklu. V případě závažných příznaků gastrointestinální toxicity (stupeň 3–4) se má provést úprava dávky. V případě závažných gastrointestinálních příhod se doporučuje sledovat hladinu draslíku v séru. U pacienta má být sledován případný výskyt příznaků periferní neuropatie. Pacienti s nově vzniklou nebo zhoršující se periferní neuropatií mohou vyžadovat úpravu dávky. Při léčbě přípravkem NINLARO byl hlášen výskyt periferního otoku. U pacienta mají být vyhodnoceny základní příčiny a dle potřeby mu má být poskytnuta podpůrná péče. Dávka dexamethasonu má být upravena. Při léčbě přípravkem NINLARO byla hlášena vyrážka. Vyrážka má být léčena podpůrnou péčí nebo úpravou dávky v případě stupně 2 či vyššího. Při léčbě přípravkem NINLARO byla méně často hlášena poškození jater vyvolaná lékem. Jaterní enzymy mají být pravidelně sledovány a dávka má být upravena pro příznaky stupně 3 nebo 4. Ženy léčené přípravkem NINLARO se mají vyhnout otěhotnění. U pacientů užívajících přípravek NINLARO se vyskytl syndrom posteriorní reverzibilní encefalopatie (PRES). U pacientů, u kterých se PRES vyskytl, NINLARO vysaďte. Silné induktory mohou snižovat účinnost přípravku NINLARO, a proto je třeba se vyhnout souběžnému podávání silných induktorů CYP3A, jako např. karbamazepin, fenytoin, rifampicin a třezalka tečkovaná. **Lékové interakce:** *Inhibitory CYP:* Souběžné podávání ixazomibu a klarithromycinu (silný inhibitor CYP3A) nebo souběžné podávání ixazomibu se silnými inhibitory CYP1A2 nevedlo ke klinicky významné změně v systémové expozici ixazomibu. *Induktory CYP:* Souběžné podávání ixazomibu s rifampicinem vedlo ke snížení hodnoty C_{max} ixazomibu o 54 % a hodnoty AUC o 74 %. Proto se současné podávání silných induktorů CYP3A s ixazomibem nedoporučuje. *Účinek ixazomibu na jiné léčivé přípravky:* Nepředpokládá se, že u ixazomibu dochází prostřednictvím inhibice nebo indukce CYP k interakci s jinými léčivy. *Transportní interakce:* Nepředpokládá se, že u ixazomibu dochází k transportní interakci s jinými léčivy. *Perorální kontraceptiva:* Pokud se přípravek NINLARO podává společně s dexamethasonem (slabý až střední induktor CYP3A4 i jiných enzymů a transportérů), je třeba vzít v úvahu riziko snížené účinnosti perorálních kontraceptiv. Ženy, které používají hormonální antikoncepci, mají navíc používat i některou z metod bariérové antikoncepce. **Fertilita, těhotenství a kojení:** Pacientky a pacienti ve fertilním věku musí během léčby a 90 dní po jejím ukončení používat účinné antikoncepční metody. *Těhotenství:* Podávání přípravku NINLARO se v těhotenství nedoporučuje, protože je-li podán těhotné ženě, může způsobit poškození plodu. Údaje o podávání přípravku NINLARO těhotným ženám nejsou k dispozici. Studie na zvířatech prokázaly reprodukční toxicitu. *Kojení:* Není známo, zda se přípravek NINLARO nebo jeho metabolity vylučují do lidského mateřského mléka. Nejsou dostupné žádné údaje získané u zvířat. Riziko pro novorozence / kojence nelze vyloučit, a proto má být kojení přerušeno. *Fertilita:* U přípravku NINLARO nebyly provedeny žádné studie fertility. **Nežádoucí účinky*:** Přípravek NINLARO se podává v kombinaci s lenalidomidem a dexamethasonem, proto další nežádoucí účinky naleznete v Souhrnu údajů o přípravku pro tyto léčivé přípravky. Velmi časté: infekce horních cest dýchacích, trombocytopenie, neutropenie, periferní neuropatie, průjem, nauzea, zvracení, zácpa, vyrážka, bolest zad, periferní otok. Časté: Herpes zoster. *Ukončení léčby:* U každého nežádoucího účinku došlo k ukončení léčby jedním nebo několika ze tří léčivých přípravků u ≤ 1 % pacientů v režimu NINLARO. *Trombocytopenie:* U tří procent pacientů v režimu NINLARO a u 1 % pacientů v režimu placebo byl během léčby počet krevních destiček ≤ 10 000/mm³. Méně než 1 % pacientů v obou režimech mělo během léčby počet krevních destiček ≤ 5 000/mm³. K ukončení léčby jedním nebo několika ze tří léčivých přípravků došlo kvůli trombocytopenii u < 1 % pacientů v režimu NINLARO a u 1 % pacientů v režimu placebo. Trombocytopenie nevedla k nárůstu hemoragických příhod ani transfuzí krevních destiček. *Gastrointestinální toxicity:* Průjem vedl k ukončení léčby jedním nebo několika ze tří přípravků u 1 % pacientů v režimu NINLARO a u < 1 % pacientů v režimu placebo. Ostatní nežádoucí účinky viz úplné znění SPC. Hlášení podezření na nežádoucí účinky: Žádáme zdravotnické pracovníky, aby hlásili podezření na nežádoucí účinky na adresu: Státní ústav pro kontrolu léčiv, Šrobárova 48, 100 41 Praha 10, webové stránky: www.sukl.cz/nahlasit-nezadouci-ucinek. **Doba použitelnosti:** 3 roky. **Zvláštní opatření pro uchovávání:** Neuchovávejte při teplotě nad 30 °C. Chraňte před mrazem. Uchovávejte v původním obalu, aby byl přípravek chráněn před vlhkostí. **Druh obalu a obsah balení:** PVC-Al/Al blistr zatavený do pouzdra, které obsahuje jednu tobolku. Tři pouzdra s jedním blistrem jsou zabalena do jedné krabičky. **Zvláštní opatření pro likvidaci přípravku a pro zacházení s ním:** Přípravek NINLARO je cytotoxický. Tobolku vyjměte až těsně před užitím. Tobolky se nesmějí otvírat ani drtit. Je třeba zamezit přímému kontaktu s obsahem tobolky. **Držitel rozhodnutí o registraci:** Takeda Pharma A/S, Dybendal Alle 10, 2630 Taastrup, Dánsko. **Registrační číslo:** EU/1/16/1094/001, EU/1/16/1094/003, EU/1/16/1094/003. **Datum poslední revize:** 06. 07. 2017

Přípravek není hrazen z prostředků veřejného zdravotního pojištění a je vydáván pouze na lékařský předpis. Před předepsáním se seznamte s úplným zněním Souhrnu údajů o přípravku.

*Všimněte si, prosím, změn v informacích o léčivém přípravku

Reference: 1. SPC Ninlaro

CZ/IXA/1708/0013

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Takeda

Editorial

Dear colleagues,

six years passed, and I would like to present our new supplement which was prepared by the researchers of the Myeloma Section of the Czech Hematology Association and the Czech Myeloma Group in cooperation with the team of the Institute of Biostatistics and Analyses at Masaryk University (IBA MU). This supplement is focused on the research in the field of monoclonal gammopathies and multiple myeloma. In the introduction for the previous supplement, I wrote: "Multiple myeloma research progression in the first decade of this century can be described as rapid as development of therapeutic modalities. It is extraordinary how rapidly knowledge from the area of applied research has gone into clinical practice, the so-called bench to bedside strategy. On the contrary, development in the field of basic research is gradual, which stems from the very nature of basic research and complicated oncogenesis of multiple myeloma." It is easy to say that development in the second decade of the 21st century is similar, but applied research progression became even more intensive and development of the therapeutic modalities is "super rapid". It is so intensive that it would be enough for another supplement exclusively drug-oriented. Introduction of new drugs into clinical settings is dramatic and creates a lot of pressure on research primarily in the field of standardization of minimal residual disease (MRD) with the highest sensitivity. This supplement of Klinicka onkologie 2017 is focused on the most important research problems and activities in which the Czech Republic is involved and plays a significant role.

Of course, this includes the linking of individual biobanks into one biobank with more than 40,000 archived samples and an extension program enabling researchers to identify easily requested samples serving all researchers in the Czech Republic, as described by Almasi et al. in the introductory article. At the same time, a high world-class standard is reached in the preliminary phase of cell sorting for single cell analysis.

MRD for multiple myeloma can be described as an area of rapid development. In 2011, it was topic for 4-color flowcytometry and a very lab-intensive testing using the ASO PCR method. The efficacy of treatment by which we can achieve MRD negativity with a sensitivity of 10^{-6} in 80% of patients has greatly accelerated demands in this area. Rihova et al. describes in her publication the development of flowcytometry from the 4-color approach to the 8-color method to the so-called Next Generation Flow. Similar progress has been noted even in genomic methods using next-generation sequencing at the level of individual cells, which is described by Zatopkova et al. The issue is enhanced by the researchers' efforts to overcome limitations of existing methods requiring bone marrow sampling to assess residual disease by finding a sufficiently sensitive method using a peripheral blood sample, the so-called liquid biopsy, which is especially a major challenge and hope for the future in multiple myeloma. Researchers from Brno are intensively involved in this research, as evidenced by several national and international grants and, in particular, by a summary of the issue in two articles by this group (Kubaczkova et al. and Bezdekova et al.).

Further articles of this supplement are focused on selected innovations in the field of flowcytometry and biomarkers in rare monoclonal gammopathies, AL amyloidosis and Waldenström macroglobulinemia. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) method can be considered as one of the major methodological discoveries in the area of manipulation of the genome of recent years. Simicek et al. describe the use of the CRISPR method for specific projects in the field of monoclonal gammopathies in the Czech Republic.

This supplement is concluded with several methodological and result articles focused on the Czech Myeloma Group's Registry of Monoclonal Gammopathies, which was founded exactly 10 years ago. In the past 10 years, the benefit of continuous registries has gained importance as it reflects benefits of drugs in real-life better than clinical trials. It should be emphasized that a lot of expert working groups in the Czech Republic reach the world standard in this area, also thanks to the extraordinary conditions related to the existence of the Institute of Biostatistics and Analyses at Masaryk University. Our myeloma registry is one of the largest and highest quality in the world, and I would like to thank very much to the physicians and datamanagers of all participating centers, as well as the analysts and statisticians from IBA MU, mainly Jarkovsky and Brozova, for their excellent systematic long-term work.

I would like to thank all authors, who participated in this supplement. I would like to especially thank Assoc. Prof. Sevcikova for her role in preparation of this supplement. I hope that our articles will be of interest to you.

prof. MUDr. Roman Hajek, CSc.
Head of the Czech Myeloma Group and Myeloma Section
of the Czech Hematology Association Czech Medical Association
of J. E. Purkyne

Editorial

Milé kolegyně a vážení kolegové,

po šesti letech si vám „znovu" dovolujeme nabídnout k přečtení supplementum připravené výzkumníky myelomové sekce České hematologické společnosti ČLS JEP a České myelomové skupiny ve spolupráci s týmem Institutu biostatistických analýz Masarykovy univerzity (IBA MU). Je zaměřeno na výzkum v oblasti monoklonálních gamapatií a mnohočetného myelomu. V prvním supplementu jsem v úvodním slovu napsal: „Vývoj ve výzkumu mnohočetného myelomu v první dekádě tohoto století lze označit za překotný, podobně jako vývoj léčebných modalit, které používáme. Je mimořádné, jak rychlým způsobem došlo k urychlení znalostí především z oblasti aplikovaného výzkumu do klinické praxe, tzv. strategie from bench to bedside. V oblasti základního výzkumu je vývoj naopak pozvolný, což souvisí s vlastní podstatou základního výzkumu a komplikovanou onkogenezí mnohočetného myelomu."

Lze jednoduše říct, že podobný vývoj platí i pro druhou dekádu 21. století, jen vývoj v aplikovaném výzkumu se stal ještě intenzivnějším a vývoj léčebných modalit lze označit za „super překotný". Je natolik intenzivní, že by vystačil na další supplementum výhradně lékově orientované. Zavádění nových účinných léků do klinické praxe vytváří velký tlak na výzkumné zázemí, především v reálné praxi dnes stále důležitější standardizaci hodnocení minimálního zbytkového onemocnění (minimal residual disease – MRD) s co nejvyšší citlivostí. V supplementu Klinické onkologie 2017 jsme se zaměřili jen na výzkumnou problematiku a z ní především na aktivity, ve kterých má Česká republika významnou roli.

Zcela jistě sem patří propojení jednotlivých biobank do jedné biobanky sloužící všem výzkumníkům v České republice s více než 40 000 archivovanými vzorky a s nádstavbovým programem umožňujícím výzkumníkům snadno identifikovat žádané vzorky, což popisují Almáši et al v úvodním článku. Rovněž v přípravné fázi třídění buněk pro „single cell" analýzy dosahujeme vysokého světového standardu.

Téma MRD u mnohočetného myelomu lze označit za oblast překotného rozvoje. Ještě v roce 2011 šlo o téma pro čtyřbarevnou flowcytometrii a velmi laboratorně náročné vyšetření pomocí ASO PCR metody. Efektivní léčba, po které lze dosáhnout MRD negativity s citlivostí 10^{-6} u 80 % nemocných, zásadně urychlila nároky v této oblasti. Vývoj v oblasti flowcytometrie od čtyřbarevné přes osmibarevnou metodu k tzv. next generation flow popisují ve svém článku Říhová et al. Podobně je tomu u metod genomických s využitím sekvenování nové generace na úrovni jednotlivých buněk, které ve svém článku popisují Zátopková et al. Celou problematiku umocňuje snaha výzkumníků překonat limitace stávajících metod vyžadujících vždy odběr kostní dřeně pro hodnocení zbytkové choroby nalezením dostatečně citlivé metody využívající vzorek periferní krve, tzv. tekuté biopsie, které jsou zejména u mnohočetného myelomu velkou výzvou a nadějí pro budoucnost. Výzkumníci z Brna se intenzivně účastní tohoto výzkumu, čehož dokladem je několik národních a mezinárodních grantů a především shrnutí problematiky ve dvou článcích této skupiny (Kubaczková et al a Bezděková et al).

Další články supplementa jsou zaměřeny na vybrané novinky z oblasti flowcytometrie a oblasti biomarkerů u raritních monoklonálních gamapatií, AL amyloidózy a Waldenströmovy makroglobulinemie. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) metodu lze považovat za jeden ze zásadních metodických objevů v oblasti manipulace s genomem posledních let. Šimíček et al na závěr popisují využití CRISPR metody u konkrétních projektů v oblasti monoklonálních gamapatií v České republice.

Supplementum je zakončeno několika metodickými a výsledkovými články zaměřenými na Registr monoklonálních gamapatií České myelomové skupiny, který slaví v tomto roce 10. výročí od založení. Za 10 let nabyl přínos kontinuálních registrů nebývale na významu, neboť lépe než klinické studie odráží přínos léků v reálném životě. Je třeba zdůraznit, že v této oblasti dosahuje řada odborných pracovních skupin v České republice světové úrovně, a to rovněž díky mimořádným podmínkám souvisejících s existencí IBA MU. Náš myelomový registr patří k největším a nejkvalitnějším na světě a velký dík patří lékařům a datamanažerům jednotlivých center, rovněž tak i analytikům a statistikům z IBA MU za kvalitně odvedenou systematickou dlouhodobou práci.

Děkuji všem autorům připravených publikací, zvláštní poděkování patří doc. Ševčíkové za významnou roli při přípravě supplementa. Věřím, že vás připravené publikace zaujmou.

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KRd i Kd kombinace

SCHVÁLENO

PRO LÉČBU V EU

Důrazná[§]

ODPOVĚĎ

na relaps mnohočetného myelomu

„HEAD TO HEAD" STUDIE POTVRDILA SUPERIORITU PFS LÉČEBNÉ KOMBINACE KYPROLIS® + DEX VS. BORTEZOMIB + DEX[1]

Hluboká odpověď – Téměř každý 3. pacient s kombinací KRd[2] a 2× více pacientů s kombinací Kd vs. Vd[1] dosáhlo kompletní či lepší léčebné odpovědi.

Setrvalá účinnost – Superiorita v parametru doby do zhoršení onemocnění nebo úmrtí – s KRd déle než 2 roky[2] a dvojnásobně s Kd vs. Vd.[1]

Dobrá snášenlivost[#] – Významně nižší výskyt periferních neuropatií s Kd vs. Vd (st. ≥ 2)[1] a příznivý poměr přínos/riziko během léčby založené na přípravku Kyprolis®.[1,2]

V léčbě přípravkem Kyprolis® lze pokračovat až do progrese onemocnění či výskytu nepřijatelné toxicity.[&,3]

Kyprolis® (karfilzomib) Prášek pro infuzní roztok

Léčba založená na přípravku Kyprolis® není v současné době hrazena z prostředků veřejného zdravotního pojištění.

Zkratky: Kd – Kyprolis® (karfilzomib), dexamethason; **Vd** – Velcade® (bortezomib), dexamethason; **KRd** – Kyprolis® (karfilzomib), Revlimid® (lenalidomid), dexamethason; **Rd** – Revlimid® (lenalidomid), dexamethason; **PFS** – přežití bez progrese onemocnění (progression free survival).

§ Významné prodloužení mediánu PFS díky kombinační léčbě založené na přípravku Kyprolis® – KRd i Kd.[1,2]

V klinické studii fáze 3 – ASPIRE – bylo téměř u každého třetího pacienta, léčeného kombinací karfilzomib, lenalidomid a dexamethason (KRd), dosaženo kompletní odpovědi (31,8 % vs. 9,3 % v případě léčebné kombinace lenalidomidu s dexamethasonem (Rd); deskriptivní p < 0,001). Léčebná kombinace KRd rovněž prokázala významné prodloužení mediánu PFS na více než 2 roky (26,3 měsíce vs. 17,6 měsíce pro Rd; p=0,0001). Léčba kombinací KRd byla také spojena s příznivým poměrem: přínos / riziko.[2]

& Detailní informace k profilu bezpečnosti a snášenlivosti (všechny stupně závažnosti, ve více klinických studiích, různé dávkování) lze nalézt v Souhrnu údajů o přípravku.[3] Léčba karfilzomibem v kombinaci s lenalidomidem a dexamethasonem po dobu delší než 18 cyklů má být založena na individuálním vyhodnocení přínosů a rizik, protože údaje o snášenlivosti a toxicitě karfilzomibu pro léčbu delší než 18 cyklů jsou omezené.[3]

Odkazy: 1. Dimopoulos MA *et al. Lancet Oncol* 2016;17:27–38. **2.** Stewart AK *et al. N Engl J Med* 2015;372:142–152. **3.** Kyprolis® (karfilzomib), Souhrn údajů o přípravku.

Zkrácená informace o léčivém přípravku Kyprolis®:
▼ Tento léčivý přípravek podléhá dalšímu sledování. To umožní rychlé získání nových informací o bezpečnosti. Žádáme zdravotnické pracovníky, aby hlásili jakákoli podezření na nežádoucí účinky. **Název přípravku*:** Kyprolis 10 mg, 30 mg, 60 mg prášek pro infuzní roztok. **Kvalitativní a kvantitativní složení**: Jedna injekční lahvička obsahuje carfilzomibum 10 mg, 30 mg nebo 60 mg. Po rekonstituci 1 ml roztoku obsahuje 2 mg karfilzomibu. **Léková forma**: Prášek pro infuzní roztok. **Terapeutické indikace***: Kyprolis v kombinaci s lenalidomidem a dexamethasonem nebo pouze s dexamethasonem je indikován k léčbě dospělých pacientů s mnohočetným myelomem, kteří již prodělali nejméně jednu předchozí léčbu. **Dávkování a způsob podání***: Léčba přípravkem Kyprolis má probíhat pod dohledem lékaře se zkušenostmi s léčbou rakoviny. V kombinaci s dexamethasonem a lenalidomidem se Kyprolis podává intravenózně jako 10minutová infuze dva po sobě jdoucí dny, každý týden po dobu tří týdnů (1., 2., 8., 9., 15. a 16. den), poté následuje období bez léčby trvající 12 dní (17. až 28. den). Každé 28denní období se považuje za jeden léčebný cyklus. Zahajovací dávka je 20 mg/m² (maximální dávka je 44 mg) v prvním cyklu 1. a 2. den. V případě snášenlivosti se má dávka zvýšit na 27 mg/m² (maximální dávka je 60 mg) 8. den prvního cyklu. Od 13. cyklu se dávky přípravku Kyprolis v 8. a 9. dni vynechají. V kombinaci s dexamethasonem* se Kyprolis podává jako 30minutová infuze dva po sobě jdoucí dny, každý týden po dobu tří týdnů, poté následuje období bez léčby trvající 12 dní, v průběhu 28denního léčebného cyklu. Zahajovací dávka je 20 mg/m² (maximální dávka je 44 mg) v prvním cyklu 1. a 2. den. V případě snášenlivosti se má dávka zvýšit na 56 mg/m² (maximální dávka je 123 mg) 8. den prvního cyklu. Léčba může pokračovat až do progrese onemocnění nebo do vzniku neakceptovatelné toxicity. Kyprolis se nesmí míchat nebo podávat jako infuze s jinými léčivými přípravky. Kyprolis se nesmí podat jako bolus. Před podáním dávky přípravku Kyprolis v 1. cyklu je nutná přiměřená hydratace. Dávkování se má upravovat podle toxicity přípravku Kyprolis. Funkce ledvin má být posouzena při zahájení léčby. Na základě dostupných farmakokinetických údajů není doporučena úprava počáteční dávky u pacientů s lehkou nebo středně těžkou poruchou funkce jater. **Kontraindikace:** Hypersenzitivita na léčivou látku nebo na kteroukoli pomocnou látku. Kojící ženy. Protože se přípravek Kyprolis podává v kombinaci s jinými léčivými přípravky, odkazujeme na jejich Souhrn údajů o přípravku pro další kontraindikace. **Zvláštní upozornění a opatření pro použití:** Jelikož se lenalidomid může použít v kombinaci s přípravkem Kyprolis, je nutné při léčbě lenalidomidem věnovat mimořádnou pozornost provádění těhotenského testu a provádění preventivních opatření. Srdeční poruchy: Po podání přípravku Kyprolis se objevily nové případy nebo zhoršení stávajícího srdečního selhání (např. městnavé srdeční selhání, plicní edém, snížená ejekční frakce), ischemie myokardu a infarktu myokardu. Vyskytlo se úmrtí na srdeční zástavu v průběhu jednoho dne po podání přípravku Kyprolis a byly hlášeny smrtelné následky srdečního selhání a infarktu myokardu. Všichni pacienti mají být sledováni z hlediska známek objemového přetížení organismu, zejména pacienti s rizikem srdečního selhání. Změny EKG: V klinických studiích byly hlášeny případy prodloužení QT intervalu. Plicní toxicita: Vyskytly se případy syndromu akutní dechové tísně, akutního respiračního selhání a akutní difuzní infiltrativní plicní nemoci, jako je pneumonitida a intersticiální plicní nemoc. Plicní hypertenze: Byly zaznamenány případy plicní hypertenze. Dušnost: Často byla hlášena dušnost. Vyhodnoťte dušnost z důvodu vyloučení kardiopulmonální nemoci včetně srdečního selhání a plicních syndromů. Hypertenze: Byly pozorovány případy hypertenze, včetně hypertenzní krize a urgentních hypertenzních stavů. Všichni pacienti mají být standardně vyšetřeni na přítomnost hypertenze a podle potřeby léčeni. Akutní selhání ledvin: Byly hlášeny případy akutního selhání ledvin. Funkce ledvin se má monitorovat alespoň měsíčně anebo v souladu se schválenými doporučeními pro klinickou praxi, zejména u pacientů s nižšími výchozími hodnotami clearance kreatininu. Syndrom nádorového rozpadu: Byly popsány případy syndromu nádorového rozpadu (TLS). Pacienti s velkou zátěží nádorovým onemocněním mají být považováni za pacienty s vyšším rizikem TLS. Infuzní reakce: Byly hlášeny případy infuzních reakcí, včetně život ohrožujících. Ke snížení výskytu a závažnosti reakcí se má před podáním přípravku Kyprolis aplikovat dexamethason. Krvácení a trombocytopenie*: Byly hlášeny případy krvácení (např. gastrointestinální, pulmonální a intrakraniální), některé byly fatální. Žilní trombóza*: Byly hlášeny případy žilních tromboembolických příhod včetně hluboké žilní trombózy a plicní embolie s fatálními následky Na základě individuálního posouzení rizika a přínosu se má zvážit tromboprofylaxe. Jaterní toxicita: Byly hlášeny případy jaterního selhání, a to i smrtelné. Kyprolis může způsobit zvýšení hladin sérových transamináz. Jaterní enzymy a bilirubin se během léčby karfilzomibem mají monitorovat při zahájení léčby, a poté v měsíčních intervalech bez ohledu na hodnoty na začátku léčby. Trombotická mikroangiopatie: Byly hlášeny případy trombotické mikroangiopatie, včetně trombotické trombocytopenické purpury (TTP) a hemolyticko-uremického syndromu (HUS). Při podezření na tuto diagnózu ukončete léčbu přípravkem Kyprolis a vyšetřete pacienty z hlediska možného TTP/HUS. Syndrom posteriorní reverzibilní encefalopatie: Byly zaznamenány případy syndromu posteriorní reverzibilní encefalopatie (PRES) projevující se jako křeče, bolest hlavy, letargie, zmatenost, slepota, porucha vědomí a další zrakové a neurologické poruchy spolu s hypertenzí. Při podezření na PRES se má Kyprolis vysadit. **Interakce s jinými léčivými přípravky a jiné formy interakce:** Opatrnosti je třeba při podávání karfilzomibu v kombinaci s perorálními antikoncepčními přípravky. Jestliže pacientka užívá perorální antikoncepci, měla by používat alternativní účinnou antikoncepční metodu. Při podávání karfilzomibu v kombinaci se substráty P-gp (např. digoxinem a kolchicinem) je nutná opatrnost. **Fertilita, těhotenství a kojení:** Je zapotřebí provést účinná opatření k zabránění těhotenství. Ženy, které mohou otěhotnět (a/nebo jejich partneři) musí používat účinné antikoncepční metody během léčby a jeden měsíc po léčbě. Pacienti (muži) musí používat účinné antikoncepční metody během léčby a 3 měsíce po ukončení léčby, je-li jejich partnerka těhotná, nebo může otěhotnět a nepoužívá účinnou antikoncepci. Údaje o podávání karfilzomibu těhotným ženám nejsou k dispozici. Není známo, zda se karfilzomib nebo jeho metabolity vylučují do lidského mateřského mléka. Proto jako preventivní opatření je kojení kontraindikováno během léčby a nejméně 2 dny po léčbě přípravkem Kyprolis. **Nežádoucí účinky:** K nejzávažnějším nežádoucím účinkům patří: kardiální toxicita, plicní toxicity, plicní hypertenze, dušnost, hypertenze včetně hypertenzní krize, akutní selhání ledvin, syndrom rozpadu nádoru, infuzní reakce, trombocytopenie, jaterní toxicita, PRES a TTP/HUS. Nejčastější nežádoucí účinky (vyskytující se u > 20 % subjektů) byly: anémie, únava, průjem, trombocytopenie, nauzea, pyrexie, dušnost, infekce dýchacích cest, kašel a periferní otoky. **Zvláštní opatření pro uchovávání:** Uchovávejte v chladničce (2 °C – 8 °C). Chraňte před mrazem. Uchovávejte v původním obalu, aby byl přípravek chráněn před světlem. Chemická a fyzikální stabilita rekonstituovaných roztoků v injekční lahvičce, stříkačce nebo intravenózním vaku byla prokázána po dobu 24 hodin při teplotě 2 °C – 8 °C nebo po dobu 4 hodin při teplotě 25 °C. Doba mezi rekonstitucí a podáním nemá překročit 24 hodin. **Držitel rozhodnutí o registraci:** Amgen Europe B.V., Minervum 7061, NL-4817 ZK Breda, Nizozemsko. **Registrační číslo** EU/1/15/1060/001, 002, 003. **Datum revize textu:** prosinec 2016. Před předepsáním přípravku se, prosím, seznamte s úplným zněním Souhrnu údajů o přípravku. Výdej léčivého přípravku je vázán na lékařský předpis. Přípravek není hrazen z prostředků veřejného zdravotního pojištění.
* Všimněte si, prosím, změn v informacích o léčivém přípravku.
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Contents | Obsah

Editorial 2S3

Biobanking – the First Step to Successful Liquid Biopsy Experiments 2S9
Biobankování – první krok k úspěšným tekutým biopsiím
Almasi M., Sevcikova S., Penka M., Krejci M., Adam Z., Vrublova P., Jelinek T., Hajek R.

Liquid Biopsies – the Clinics and the Molecules 2S13
Tekuté biopsie – klinika a molekuly
Kubaczkova V., Sedlarikova L., Bollova B., Sandecka V., Stork M., Pour L., Sevcikova S.

Minimal Residual Disease Assessment in Multiple Myeloma by Multiparametric Flow Cytometry 2S21
Analýza minimální reziduální nemoci u mnohočetného myelomu pomocí multiparametrické průtokové cytometrie
Rihova L., Vsianska P., Bezdekova R., Kralova R., Penka M., Krejci M., Pour L., Hajek R.

Circulating Plasma Cells in Monoclonal Gammopathies 2S29
Cirkulující plazmocyty u monoklonálních gamapatií
Bezdekova R., Penka M., Hajek R., Rihova L.

Epidemiology of Multiple Myeloma in the Czech Republic 2S35
Epidemiologie mnohočetného myelomu v České republice
Maluskova D., Svobodova I., Kucerova M., Brozova L., Muzik J., Jarkovsky J., Hajek R., Maisnar V., Dusek L.

Czech Registry of Monoclonal Gammopathies – Technical Solution, Data Collection and Visualisation 2S43
Český Registr monoklonálních gamapatií – technické řešení, sběr dat a jejich vizualizace
Brozova L., Schwarz D., Snabl I., Kalina J., Pavlickova B., Komenda M., Jarkovsky J., Nemec P., Horinek D., Stefanikova Z., Pour L., Hajek R., Maisnar V.

Asymptomatic and Treatment-requiring Multiple Myeloma – Data from the Czech Registry of Monoclonal Gammopathies 2S51
Asymptomatický a léčbu vyžadující mnohočetný myelom – data z českého Registru monoklonálních gamapatií
Brozova L., Jarkovsky J., Pour L., Minarik J., Jungova A., Gregora E., Spicka I., Maisnar V., Hajek R.

Biomarkers in Immunoglobulin Light Chain Amyloidosis 2S60
Biomarkery v amyloidóze lehkého řetězce imunoglobulinů
Kufova Z., Sevcikova T., Growkova K., Vojta P., Filipova J., Adam Z., Pour L., Penka M., Rysava R., Nemec P., Brozova L., Vychytilova P., Jurczyszyn A., Grosicki S., Barchnicka A., Hajduch M., Simicek M., Hajek R.

CRISPR in Research and Treatment of Multiple Myeloma 2S68
CRISPR ve výzkumu a léčbě mnohočetného myelomu
Simicek M., Growkova K., Hajek R.

Whole Exome Sequencing of Aberrant Plasma Cells in a Patient with Multiple Myeloma Minimal Residual Disease **2S75**
Celoexomové sekvenování aberantních plazmatických buněk pacienta s minimální reziduální chorobou u mnohočetného myelomu
Zatopkova M., Filipova J., Jelinek T., Vojta P., Sevcikova T., Simicek M., Rihova L., Bezdekova R., Growkova K, Kufova Z., Smejkalova J., Hajduch M., Pour L., Minarik J., Jungova A., Maisnar V., Kryukov F., Hajek R.

Diagnostic Tools of Waldenström's Macroglobulinemia – Best Possibilities for Non-invasive and Long-term Disease Monitoring **2S81**
Diagnostické přístupy u Waldenströmovy makroglobulinemie – nejvhodnější dostupné možnosti neinvazivního a dlouhodobého monitorování nemoci
Growkova K., Kufova Z., Sevcikova T., Filipova J., Kascak M., Jelinek T., Grosicki S., Barchnicka A., Roziakova L., Mistrik M., Simicek M., Hajek R.

FARYDAK®

(panobinostat) tobolky

10mg/15mg/20mg

JE V KOMBINACI S BORTEZOMIBEM A DEXAMETHAZONEM URČEN K LÉČBĚ DOSPĚLÝCH PACIENTŮ S RELABUJÍCÍM A/NEBO REFRAKTERNÍM MNOHOČETNÝM MYELOMEM, KTEŘÍ DOSTÁVALI NEJMÉNĚ DVA PŘEDCHOZÍ REŽIMY VČETNĚ BORTEZOMIBU A IMUNOMODULAČNÍHO LÉKU.[1]

SCHVÁLENO

EMA/SÚKL - 8/2015

1. SPC Farydak 06/2017; www.sukl.cz

Zkrácená informace

▼ Tento léčivý přípravek podléhá dalšímu sledování. To umožní rychlé získání nových informací o bezpečnosti. Žádáme zdravotnické pracovníky, aby hlásili jakákoli podezření na nežádoucí účinky. Podrobnosti o hlášení nežádoucích účinků viz bod 4.8.

FARYDAK 10 mg tvrdé tobolky **FARYDAK 15 mg tvrdé tobolky** **FARYDAK 20 mg tvrdé tobolky**

Složení: *Léčivá látka:* Panobinostatum 10 mg, 15 mg nebo 20 mg. Indikace: Farydak je v kombinaci s bortezomibem a dexamethazonem určen k léčbě dospělých pacientů s relabujícím a/nebo refrakterním mnohočetným myelomem, kteří dostávali nejméně dva předchozí režimy včetně bortezomibu a imunomodulačního léku. Dávkování: Doporučená zahajovací dávka panobinostatu je 20 mg, podávaná perorálně jednou denně ve dnech 1, 3, 5, 8, 10 a 12 během 21denního cyklu. Pacienti by měli být nejdříve léčeni osmi cykly. Doporučuje se, aby pacienti profitující z léčby pokračovali v léčbě dalšími osmi cykly. Celkové trvání léčby je až 16 cyklů (48 týdnů). Panobinostat se podává v kombinaci s bortezomibem a dexamethazonem podle popisu v Tabulce 1 a 2 (viz Souhrn údajů o přípravku). Příbalové informace bortezomibu a dexamethazonu mají být konzultovány před zahájením kombinační léčby za účelem posouzení možnosti snížení dávky. Doporučená dávka bortezomibu je 1,3 mg/m2 podávaných injekcí. Doporučená dávka dexamethazonu je 20 mg užívaných perorálně na plný žaludek. Na základě individuální tolerability může být vyžadována úprava dávky a/nebo rozvrhu léčby. Pokud u pacienta dojde ke vzniku nežádoucích účinků, je nutné klinické rozhodnutí, jak pokračovat v léčbě. Pokud je vyžadováno snížení dávky, má být dávka panobinostatu snížena o 5 mg. Dávka nemá být snížena pod 10 mg a má být udržován stejný léčebný rozvrh (3týdenní léčebný cyklus). Kontraindikace: Hypersenzitivita na léčivou látku nebo na kteroukoli pomocnou látku. Kojení. Zvláštní upozornění/opatření: Panobinostat se používá při kombinované léčbě, proto musí být před zahájením léčby panobinostatem konzultovány preskripční informace bortezomibu a dexamethazonu. U pacientů léčených panobinostatem byly hlášené hematologické nežádoucí účinky, proto musí být před zahájením léčby provedeno vyšetření krevního obrazu, s častým sledováním během léčby (zejména před každou injekcí bortezomibu, jak uvádí SPC bortezomibu). Existuje zvýšené riziko trombocytopenie a možnost krvácení, zejména u pacientů s poruchou koagulace nebo u pacientů, kteří užívají chronickou antikoagulační terapii. U pacientů užívajících panobinostat byly hlášené i lokalizované a systémové infekce, včetně pneumonie, dalších bakteriálních onemocnění, invazivních plísňových infekcí jako je aspergilóza nebo kandidóza a virových infekcí včetně virové hepatitidy B a herpes simplex. Léčba přípravkem Farydak nemá být zahájena u pacientů s aktivními infekcemi. Byly hlášené i nežádoucí účinky jako závažná nauzea, průjem, zácpa a zvracení, a proto během léčby má být periodicky sledováno množství tekutin a hladiny elektrolytů v krvi, zejména draslíku, hořčíku a fosfátu a upraveno dle klinické potřeby aby se zamezilo potenciální dehydrataci a poruše rovnováhy elektrolytů. Antiemetika se známým rizikem prodloužení QT intervalu jako je dolasetron, granisetron, ondansetron a tropisetron mají být užívány s opatrností. Panobinostat může prodloužit srdeční ventrikulární repolarizaci (QT interval). Přípravek Farydak má být užíván s opatrností u pacientů, kteří již měli nebo kteří jsou ve významném riziku vzniku prodloužení QTc. Před léčbou a pravidelně během léčby mají být sledovány i jaterní funkce. Doporučuje se sledovat pacienty starší 65 let častěji, především trombocytopenii a gastrointestinální toxicitu. Měla by být sledována i funkce hypofýzy a štítné žlázy měřením hladiny hormonů (např. volného T4 a TSH). Interakce: U pacientů, kteří užívají konkomitantní léčivé přípravky, které jsou silnými inhibitory CYP3A a/nebo Pgp, které zahrnují například ketokonazol, itrakonazol, vorikonazol, ritonavir, sachuinavir, telithromycin, posakonazol a nefazodon, má být dávka panobinostatu snížena. Pacienti by měli být poučeni o tom, aby se vyhýbali konzumaci karamboly, grapefruitu, grapefruitové šťávy, granátového jablka a šťávy z granátových jablek, protože je u nich prokázaná inhibice enzymů cytochromu P450 3A a mohou zvýšit biologickou dostupnost panobinostatu. Mělo by být vyloučeno konkomitantní užívání silných induktorů CYP3A4 včetně například karbamazepinu, fenobarbitalu, fenytoinu, rifabutinu, rifampicinu a třezalky tečkované (Hypericum perforatum). Vyhněte se užívání panobinostatu u pacientů, kteří užívají substráty CYP2D6 s úzkým terapeutickým indexem (jde například o pimozid). Při souběžném podávání přípravku Farydak s citlivými substráty CYP2D6 (např. atomoxetin, dextromethorfan, metoprolol, nebivolol, perfenazin, a pimozid) titrujte dávky jednotlivých substrátů CYP2D6 na základě tolerability a častého sledování pacientů z důvodu výskytu nežádoucích účinků. Souběžné užívání antiarytmických léčivých přípravků (včetně například amiodaronu, disopyramidu, prokainamidu, chinidinu a sotalolu) a dalších látek, o nichž je známo, že prodlužují QT interval (včetně například chlorochinu, halofantrinu, klarithromycinu, metadonu, moxifloxacinu, bepridilu a pimozidu) není doporučeno. Těhotenství a kojení: Ženám ve fertilním věku má být před zahájením léčby přípravkem Farydak proveden těhotenský test a ženy musí během léčby a tři měsíce po podání poslední dávky přípravku Farydak používat vysoce účinnou metodu antikoncepce. Ženy užívající hormonální antikoncepci by měly doplňkově používat bariérovou metodu antikoncepce. Na základě cytostatického/cytotoxického mechanismu účinku je kojení během léčby přípravkem Farydak kontraindikováno. Vliv na řízení vozidel a obsluhu strojů: Přípravek Farydak může mít malý vliv na schopnost řídit a obsluhovat stroje. Po podání přípravku Farydak se může objevit závrať. Nežádoucí účinky: *Velmi časté:* infekce horních cest dýchacích, pneumonie, pancytopenie, trombocytopenie, anemie, leukopenie, neutropenie, lymfopenie, snížená chuť k jídlu, hypofosfatémie, hyponatrémie, hypokalémie, insomnie, závrať, bolest hlavy, hypotenze, kašel, dušnost, průjem, nauzea, zvracení, bolest břicha, dyspepsie, únava, periferní edém, pyrexie, astenie, snížení tělesné hmotnosti. *Časté:* septický šok, infekce močových cest, virové infekce, ústní herpes, kolitida způsobená *Clostridium difficile*, zánět středního ucha, celulitida, sepse, gastroenteritida, infekce dolních cest dýchacích, kandidóza, hypotyroidismus, hyperglykémie, dehydratace, hypoalbuminémie, zadržování tekutin, hyperurikémie, hypokalcémie, hypomagnezémie, intrakraniální krvácení, synkopa, třes, dysgeuzie, krvácení do spojivek, bradykardie, fibrilace síní, sinusová tachykardie, tachykardie, palpitace, hypertenze, hematom, ortostatická hypotenze, respirační selhání, chrůpky, sípot, epistaxe, gastrointestinální krvácení, hematochezie, gastritida, cheilitida, břišní distenze, sucho v ústech, plynatost, neobvyklé výsledky jaterních funkcí, hyperbilirubinémie, kožní léze, vyrážka, erytém, otoky kloubů, selhání ledvin, hematurie, inkontinence moči, třesavka, malátnost, zvýšená hladina močoviny v krvi, snížená rychlost glomerulární filtrace, zvýšená hladina alkalické fosfatázy v krvi, prodloužený elektrokardiogram QT, zvýšená hladina kreatininu v krvi, zvýšená SGPT alanintransaminázy (ALT), zvýšená SGOT aspartáttransaminázy (AST). *Další nežádoucí účinky - viz úplná informace o přípravku.* Podmínky uchovávání: Neuchovávejte při teplotě nad 30°C. Uchovávejte v původním obalu, aby byl přípravek chráněn před vlhkostí. Dostupné lékové formy/velikosti balení: 6 tobolek. *Poznámka: Dříve než lék předepíšete, přečtěte si pečlivě úplnou informaci o přípravku.* Reg. číslo: Farydak 10 mg – EU/1/15/1023/001, Farydak 15 mg – EU/1/15/1023/004, Farydak 20 mg – EU/1/15/1023/007. Datum registrace: 28.08.2015. Datum poslední revize textu SPC: 23.6.2017. Držitel rozhodnutí o registraci: Novartis Europharm Limited, Frimley Business Park, Camberley GU16 7SR, Velká Británie. *Výdej přípravku je vázán na lékařský předpis, úhrada přípravku dosud nebyla stanovena.*

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CZ1707673992-08/2017

Biobanking – the First Step to Successful Liquid Biopsy Experiments

Biobankování – první krok k úspěšným tekutým biopsiím

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Summary

Background: Archiving of biological materials in biobanks is considered to be the initial crucial part of research activities. Most often, biobanks are founded for research purposes since they allow collection of sufficient material for analysis of new or testing of previously identified biomarkers. Biobanking needs to quickly react to current needs of researchers as well as clinicians, it is not a rigid system. Laboratory analyses of monoclonal gammopathies are based on separation of plasma cells from bone marrow of patients. A specific problem is usually a lack of tumor cell fraction, which is due to location of tumor cell in bone marrow in combination with low infiltration. One of the challenges in clinical research is the necessity of changes in biobanking for samples allowing detection of minimal residual disease in the bone marrow but also from peripheral blood by the so-called liquid biopsies. ***Aim:*** The aim of this review is to show the importance of archiving biological material in the Czech Republic and to show concrete examples of its usage in hematooncology. ***Conclusion:*** A general problem in solving many research questions is the availability of a critical amount of specimens for statistical analysis. Obtaining critical amount of specimens of biological material can be quickly archived by cooperation of biobanks sharing both methodological standards and informations about the availability of samples for research projects.

Key words

archiving – biological material – informed consent – multiple myeloma – plasma cells

Souhrn

Východiska: Archivace biologického materiálu v biobankách je v současné době zásadním krokem vedoucím k úspěchu výzkumných aktivit. Biobanky jsou zřizovány většinou k výzkumným účelům, protože umožňují shromáždění dostatečného množství materiálu pro analýzy nových nebo otestování již identifikovaných biomarkerů. Biobankování musí reagovat vždy na aktuální potřeby výzkumníků i kliniků, nejde o rigidní systém. Laboratorní analýzy monoklonálních gamapatií jsou založeny na separaci plazmatických buněk z kostní dřeně nemocných. Specifickým problémem je zpravidla nedostatek nádorové frakce buněk, což je dáno umístěním nádorových buněk v kostní dřeni v kombinaci s nízkou infiltrací. Jednou z výzev klinického výzkumu vyžadující změny nastavení v biobance je detekce zbytkového nádorového onemocnění v kostní dřeni, ale i z periferní krve tzv. tekutými biopsiemi. ***Cíl:*** Cílem této práce je přiblížit stávající stav v České republice a konkrétní případy jeho využití v hematoonkologii. ***Závěr:*** Obecným problémem při řešení řady výzkumných otázek je dostupnost kritického množství vzorků pro statistickou analýzu. Získání kritického množství vzorků biologického materiálu může být rychleji dosaženo spoluprácí biobank sdílejících jak metodické standardy, tak informace o dostupnosti vzorků pro výzkumné projekty.

Klíčová slova

archivace – biologický materiál – informovaný souhlas – mnohočetný myelom – plazmatické buňky

This work was supported by grant of Ministry of Health, Czech Republic – Conceptual development of research organization (FNBr, 65269705) and by MH CZ – DRO – FNOs/2017.

Tato práce byla podpořena MZ ČR – RVO (FNBr, 65269705) a MZ ČR – RVO – FNOs/2017.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 25. 6. 2017
Accepted/Přijato: 28. 6. 2017

doi: 10.14735/amko20172S9

Introduction

Biobanks are well-organized resources containing biological specimens and related information available for scientific research [1]. Biobanking is the first key step to successful clinical research. In the research of monoclonal gammopathies (MG), separated pathological populations of plasma cells (PC), plasma of the bone marrow (BM), serum and plasma of peripheral blood (PB) are most commonly obtained. All methods used for processing of these materials need to be standardized, optimized and performed well. For successful research and quick application of its results into clinics, it is important to process thousands of samples of clinical material that need to be archived in a biobank and available for various analyses of research teams [2].

Tab. 1. Share (%) of individual categories of archived material.

Archived material	Share (%)
serum and plasma of PB	60
plasma of BM	15
negative fraction CD138$^-$	10
positive fraction CD138$^+$	5
mononuclear cells (BM)	5
mononuclear cells (PB)	5

PB – peripheral blood
BM – bone marrow

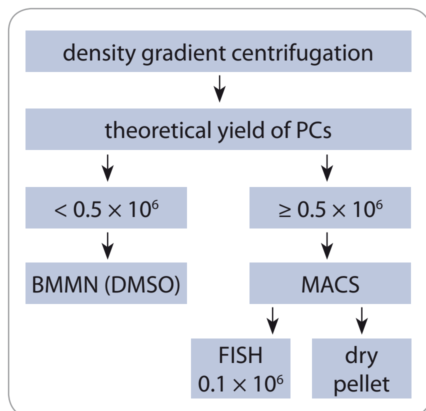


Fig. 1. CD138$^+$ cells separation strategy.
PCs – plasma cells, BMMC – bone marrow mononuclear cells, DMSO – dimethyl sulfoxide, MACS – magnetic-activated cell sorting, FISH – fluorescence *in situ* hybridization

Processing and archiving of biological material

Since 1999, initial processing of biological materials and biobanking for research in the area of hematology, especially in monoclonal gammopathies, have been carried out at the Laboratory of Experimental Hematology and Cellular Immunotherapy at the Department of Clinical Hematology, University Hospital Brno (LEHABI OKH FN Brno). Currently, about 200 samples of BM are processed in our laboratory every year. The laboratory cooperates with the flow cytometry laboratory at the OKH FN Brno, which determines PC immunophenotype. LEHABI is focused on initial processing and archiving of biological material from PB (serum and plasma collection) and from BM (plasma of BM and immunomagnetic PC separation).

Since 2013, there is a new biobank in FN Ostrava that has been created based on the methods established in Brno. Besides the locality of Northern Moravia, it also collects samples of rare monoclonal gammopathies (amyloidosis, Waldenstrom macroblobulinemia) from the Vysegrad region. Since early 2017, both biobanks have been connected into a functional interface due to collaboration with the Institute of Biostatistics and analysis, Ltd. (IBA) using the electronic data collection system (EDC) and webdesign CLADE-IS (Clinical Data Warehousing Information System). Thanks to this new approach, available samples for a predefined research project may be found. The next step is connection to the Registry of Monoclonal Gammopathies that will help the research teams to set up research projects and will allow faster access to clinical data for publications. The list of samples are listed in Graph 1 and Tab. 1.

While BM biopsies are still the golden standard for diagnostics of MG, it is important to look for new markers available from PB that would decrease discomfort of the patients while giving the same or better information than BM biopsies. Our research team has been involved in the research of such molecules for quite some time [3–7].

BM samples

In MG, most analyses are still based on separated PC from the BM of patients. There are several possible ways of separation, and our team has been working on this consistently [8–13]. To separate cells based on their different weight and volume, the BM sample is overlaid on density gradient (Ficoll, Histopaque). After centrifugation, a mix of monocytes and lymphocytes (the so-called mononuclear cells – MC) is separated. The principles of separation methods, which are followed for sample processing have been previously published [14]. Briefly, in BM mononuclear cells (BMMC) fraction, percentage of PC is measured by flow cytometry (using CD38 and CD138 markers). We have modified our previously published algorithm (Fig. 1) [14]. Instead of using infiltration of CD38$^+$138$^+$ cells in BMMC (> 5% for MACS separation – Magnetic-Activated Cell Sorting; < 5% for FACS – Fluorescence-Activated Cell Sorting), we are currently using a calculation of theoretical yield of CD138$^+$. Theoretical yield of PC $\times 10^6$ = % PC in BMMC/100× total number of cells in BMMC.

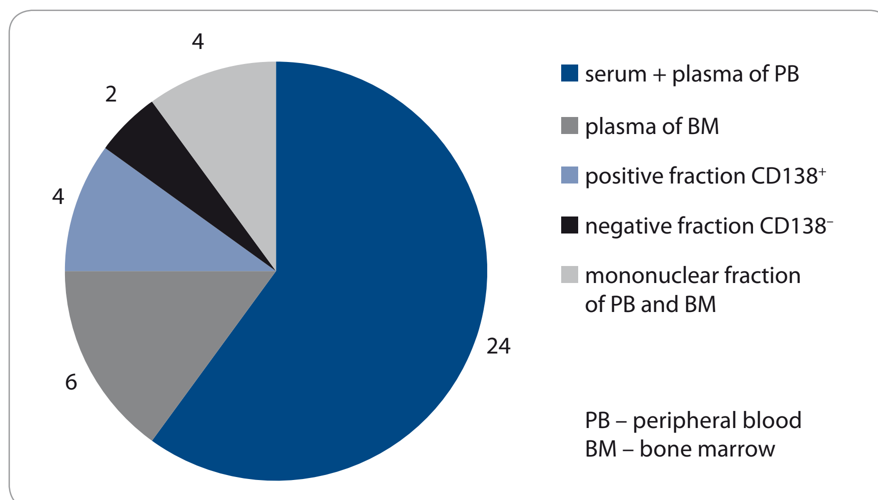
If calculation of theoretical yield is $\geq 0.5 \times 10^6$ separated CD138$^+$ cells, we use MACS separation, if it is lower, then BMMC are frozen at –80 °C in 1 mL of fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (DMSO) in a freezing container. For long-term storage, these samples are transferred into liquid nitrogen containers and stored at –196 °C. These cells may be later used for sorting by FACS as previously described [14].

MACS separation is based on labeling the cells with monoclonal antibody coupled to magnetic particles. Target cells bind to the antibody and are captured in a separation column which is placed in a magnetic field in the autoMACS-Pro Separator (Miltenyi Biotec). Thus, the positive fraction contains PC while negative fraction (cells that did not bind the antibody) contains the rest of mononuclear cells.

Based on flowcytometry determination of infiltration of more than 10% of PC, the program Possels is used. For lower PC infiltration, Posseld2 program for double selection is used. In the next step, the column is removed from the magnetic field and enriched positive fraction (PC) is eluted. Then, yield and purity of the fractions

are determined. The number of BMMC before separation and the number of cells in the positive and negative fraction are calculated in the Burker chamber after staining with trypan blue and counted on inversion microscope. Purity of the positive fraction is measured by flowcytometry. At least in our hands, the median purity of PC separated fraction (using program Possels) is 91.3% (range 22.6–99.6%) as calculated on samples obtained between January 2013 and March 2017 (n = 197). Median purity of PC separated fraction (using program Posseld2) is 92% (range 2.2–99.7%) calculated for samples obtained in the same time interval as mentioned above (n = 151). In total, median purity of separated PC of samples archived in this time period is 92.3% (range 2.2–99.9%) (total number of samples n = 348).

Then, aliquots of separated BM cells (positive fraction as a dry pellet and negative fractions in DMSO) and aliquots of plasma of BM are stored.



Graph 1. Number of archived aliquots of biological material for research purposes (in thousands).

Peripheral blood

Our attention is currently focused on liquid biopsies, meaning samples of PB. Both research teams in Ostrava and Brno have contributed to this Supplementum by several articles [15–17].

There are several pitfalls that can impact quality of samples and accuracy of further analyses. One of them is hemolysis of the specimen that may lead to release of cell material into serum or plasma. Therefore, it is important to note potential hemolysis for each sample so that these samples are not used for experiments that may be influenced by this fact.

Serum is the liquid portion of the blood without cells and clotting factors. The cells and clotting factors must be removed from the PB sample. Adequate time (30–60 min at room temperature) is required for the clot to form. Serum samples that are allowed to rest for less than 30 min are likely to retain cellular elements and other contaminants. On the other hand, samples that are allowed to rest for more than 60 min, are endangered by elevated lysis of cells in the clot [18].

On the other hand, plasma includes cellular material as well as clotting factors. Thus, plasma collection tubes contain different anticoagulants such as ethylenediaminetetraacetic acid (EDTA), sodium citrate and heparin. Each of these additives can impact the protein makeup of the sample in different ways. Based on our experience, heparin interferes with many kinds of analyses; therefore, we are currently using only EDTA collection tubes.

Recently, we have started separating circulating PC from PB. This cell fraction is found in very low quantities in PB and thus its analysis is a challenge. We use a two-step separation by MACS. In the first step, negative selection of non-PC is performed. Non-PC are indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies and anti-Biotin MicroBeads. Non-PC are retained in the magnetic field of a separation column. In the second step, $CD138^+$ PC are directly labeled with CD138 MicroBeads and isolated by positive selection from the previously enriched PC fraction. After the column is removed from the magnetic field, $CD138^+$ PC are eluted into the positive cell fraction. This depletion approach is faster and more dependable than FACS.

Proper biobanking – aliquots and storage temperature

Biobanking is a fast developing field using standard operating procedures (SOP). Quality criteria are based on the conditions needed for research purposes, therefore there is not a universal standard for sample processing, and the needs of the research team may change in time. Thus, biobanking need to flexibly adapt to these changes. At the same time, there are known conditions that directly influence quality of archived material.

All samples should be immediately transported for processing. Long and improper handling of sampled BM and PB have detrimental effect on quality of cells as well as isolated nucleic acids. SOP for cell isolation should be used to ensure best quality samples for further analyses. All SOP need to be regularly evaluated and quality controls need to be performed.

Cryogenic preservation of cells is a major downfall for further analysis if improper procedures are used – incorrect cryogenic media, not standardized ways of freezing and thawing of samples all influence viability of the cells as well as quality of isolated nucleic acids [19].

Optimal long-term storage temperature is at least −80 °C. Samples of serum and plasma are of better quality if smaller volume aliquots are prepared so that each aliquot is thawed only once as freezing and thawing cycles have a dramatic negative impact on sample quality. Smaller volume aliquots simplify sharing samples and usage for various analyses. PB samples (serum, plasma, whole blood) are usually stored at −80 °C, in 0.5–1 mL aliquots.

On average, we archive 5–10 mL of serum, 3 mL of plasma from PB using

EDTA, 3 mL of plasma of BM. Separated PC are divided into several aliquots based on the total number of separated cells, so for most samples, more than 1 aliquot is archived.

Separation of cells

For some biological analyses, separation of certain subpopulations are necessary (in the case of MG, these are malignant PC) that are found in the BM or PB only in a small portion. Thus, it is necessary to use a separation technique based on the amount and purity of the cell population, its percentages and presence of a specific marker necessary for the separation.

Every method has varying needs for amount of cells and their purity. For i-FISH (interphase fluorescence *in situ* hybridization), minimal purity is 70% and amount of separated $CD138^+$ cells fraction is 7×10^4. For other methods, optimal purity is above 90%.

For analysis using RNA, native cells are preferred as they allow for purification of high integrity RNA. Frozen samples may prove to be challenging for integrity and purity of isolated RNA and some changes in isolation procedures may be necessary. For successful isolation of RNA, the minimal amount of $CD138^+$ cells is usually 0.35×10^6 cells; on the other hand, for other analyses, dry pellets of $0.3–0.5 \times 10^6$ $CD138^+$ cells are used, frozen at liquid nitrogen at –196 °C. Generally, long-term storage of RNA is not recommended as RNA disintegrates even at lower temperature; thus, storage in RNA-stabilizing solutions or storage of cDNA is more appropriate.

While DNA is more stable, integrity and quality of DNA directly influences whole genome amplification [20] and may influence even detection of single nucleotide polymorphisms [21,22]. Repeated freeze-thaw samples are not recommended even for DNA.

Informed consents and ethical issues of biobanking

Ethical issues surround usage of human biological material, most often it is the protection of personal information of patients and anonymized usage of samples. For research purposes, coding of samples and clinical data without identifying information are necessary. Each patient is given a unique code so that direct identification of the patient is impossible and only a limited number of medical staff may identify the patient. This is a pseudo-anonymous form of protection of the patient and his/her data as no other person is capable of identification of the patient.

All research projects need to be approved by an ethics committee of the hospital and all patients participating in any research project need to sign an informed consent form. All volunteering patients have to be informed about research purposes of the project and may be enrolled into the project only after the form is signed. Otherwise, the samples may not be used for any research purposes whatsoever.

Conclusion

A key requirement for clinical research is sufficient amount of clinical material obtained from successful and high-quality initial processing of biological material. A general problem in solving many research questions is the availability of a critical amount of specimens for research projects to enable robust statistical analysis. That is why sample archiving and multicentric collaboration are key elements for many research analyses.

Presented biobanks in Brno and Ostrava provide sufficient amount of clinical material for research projects in multiple myeloma. At the same time, amount of archived material of rare diagnoses such as amyloidosis and Waldenstrom macroglobulinemia have been increasing thus creating a basis for further research in these rare and rarely studied diseases.

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Liquid Biopsies – the Clinics and the Molecules

Tekuté biopsie – klinika a molekuly

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Summary
Unlike bone marrow biopsies, liquid biopsies represent a gentler, more accessible, less painful, repeatable and more comprehensive approach to get biologically relevant information about the entire tumor but also about treatment response and level of minimal residual disease. This is all possible since peripheral blood contains not only circulating tumor cells but also many circulating molecules of nucleic acids (microRNA, cell-free DNA, long non-coding RNA etc.). Multiple myeloma is a genetically heterogeneous disease characterized by multifocal tumor deposits in the bone marrow but also focal lesions elsewhere. Single-site biopsy of the bone marrow creates a sampling bias that provides a limited molecular profile as the biopsy cannot capture all subclones. Moreover, during disease progression and treatment, molecular profile is changed and subclones of multiple myeloma cells resistant to treatment are formed. Likewise, various clones found in extramedullary sites that are not present in the bone marrow respond differently to treatment directly influencing survival of patients. Thus, liquid biopsies seem to be a relevant and necessary next step for diseases such as multiple myeloma.

Key words
multiple myeloma – minimal residual disease – prognosis – liquid biopsies – cell-free DNA – non-coding RNA

Souhrn
Na rozdíl od klasických biopsií představují tekuté biopsie jemnější, více dostupný, méně bolestivý a komplexnější přístup, který je možné opakovat častěji, a umožňují tak získání biologicky relevantních informací o celém nádoru, ale i monitorování léčebné odpovědi a detekci minimální reziduální choroby. To je možné díky tomu, že periferní krev obsahuje nejen cirkulující nádorové buňky, ale také různé cirkulující molekuly nukleových kyselin (mikroRNA, mimobuněčné DNA, dlouhé nekódující RNA atd.). Mnohočetný myelom je geneticky heterogenní onemocnění charakterizované multifokálními nádorovými ložisky v kostní dřeni, ale i fokálními ložisky mimo kostní dřeň. Biopsie kostní dřeně z jednoho místa ovlivňuje molekulární profil, který je limitovaný místem odběru, protože taková biopsie neposkytne informaci ze všech klonů. Navíc během progrese nemoci a léčby se molekulární profil mění a subklony buněk mnohočetného myelomu se mohou stát rezistentními k léčbě. Navíc, různé klony, které se objevují v extramedulárních oblastech, které se navíc nenachází v kostní dřeni, reagují na léčbu jinak a přímo ovlivňují přežití pacientů. Pro nemoci jako je mnohočetný myelom se vyšetření pomocí tekuté biopsie jeví jako relevantní a nutný další krok.

Klíčová slova
mnohočetný myelom – minimální reziduální choroba – prognóza – tekuté biopsie – mimobuněčné DNA – nekódující RNA

This work was supported by grant AZV 15-29508A.

Tato práce byla podpořena grantem AZV 15-29508A.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 15. 6. 2017
Accepted/Přijato: 22. 6. 2017

doi: 10.14735/amko20172S13

Introduction

Increasing knowledge of disease pathogenesis brings more attention to biomarkers that would improve diagnosis, prognosis and prediction. In monoclonal gammopathies, painful biopsies of the bone marrow (BM) are still the standard way of obtaining diagnostic samples. While these diagnostic biopsies are necessary, their discomfort to patients is indisputable. Liquid biopsies from peripheral blood (PB) are the latest, gentler approach to obtain information about the tumor, as PB contains not only blood cells, but also circulating tumor cells, as well as various DNA or RNA molecules that carry information from the tumor [1].

Multiple myeloma (MM) is a genetically heterogeneous disease characterized by multifocal tumor deposits throughout the BM and focal lesions elsewhere. Single-site biopsy of BM creates a sampling bias providing only a limited molecular profile as it cannot capture all subclones. Moreover, during disease progression and treatment, molecular profile is changed and subclones of MM cells resistant to treatment are formed. Likewise, various clones found in extramedullary sites that are not present in the BM respond differently to treatment and directly influence survival of patients [1].

This paper will summarize current information about liquid biopsies in MM with special focus on circulating non--coding RNA (ncRNA) molecules and cell-free DNA (cfDNA) molecules.

Liquid biopsies – the clinical point of view

Liquid biopsies are gaining more attention in research as well as in clinical setting. The 1996 FDA approval of cfDNA testing for non-small cell lung cancer patients has changed the field from purely research-oriented into clinical practice (www.fda.gov).

Liquid biopsies are most often characterized as biopsies of PB, where circulating cancer cells as well as circulating molecules, such as microRNA (miRNA), long ncRNA (lncRNA), cfDNA, can provide information that is easily accessible and complete (Fig. 1). As tumors are highly heterogenous and molecular profiles may depend on the site of biopsy, comprehensive information about the entire tumor genome is necessary [2].

Detection of circulating molecules in PB may be used for diagnostic assessment of the tumor, distinguishing cancer patients from healthy people at early stage with high sensitivity and specificity. Further, liquid biopsy can predict prognosis of cancer patients, even the risk of tumor metastasis and tumor recurrence after surgical operation, or provide an assessment index to evaluate whether operation is successful or not [3]. Apart from that, detection and capture of tumor cells in PB can be used for testing of sensitivity and resistance towards drugs, serving as a tool for personalized medicine. The main advantage of liquid biopsy is its repeatability – it can be performed as often as required; there is no need for the patient to travel to specialized clinics as it can be done in any medical center or hospital. The above mentioned circulating molecules were proven to be stable, so the samples can be collected and transported for evaluation in multicentric studies [2].

In MM, most work on liquid biopsies was carried out on circulating miRNA and cfDNA. Only very few papers have been published regarding lncRNA.

NcRNA molecules

The central dogma of molecular biology shows the flow of encoded genetic information from DNA into RNA and proteins. While it was suggested that most genetic information is translated into proteins, sequencing of the human genome showed that the number of genes coding for proteins is between 20 000 and 25 000 – only about 1.5% of the entire

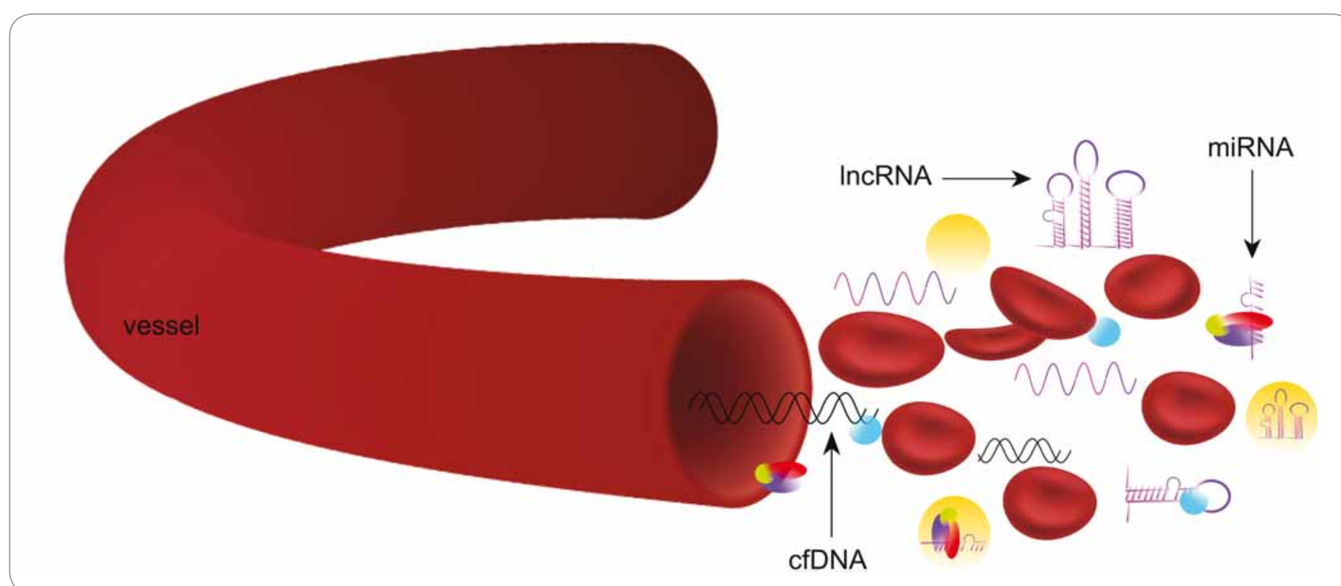

Fig. 1. Circulating molecules in bloodstream.

There are many circulating molecules found in the bloodstream – long non-coding (lncRNA), microRNA (miRNA), cell-free DNA (cfDNA) and many others.

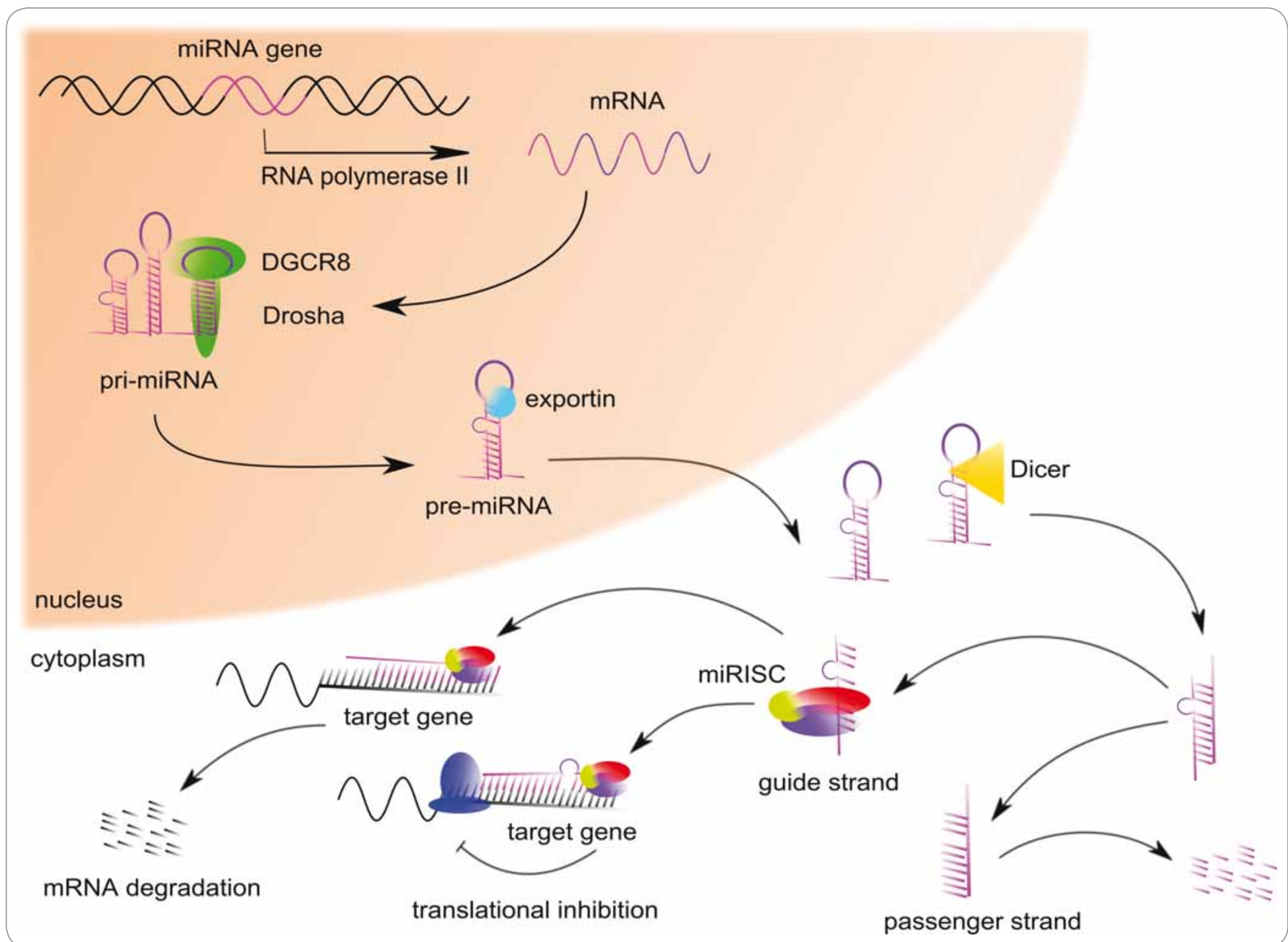

Fig. 2. Biogenesis of microRNA (miRNA).
MiRNA genes are transcribed in the nucleus, into long primary precursors called pri-miRNA. Then, they are cleaved into short secondary stem-loop precursors known as pre-miRNA by nuclear complex Drosha and Pasha. Pre-miRNA are further actively transported to cytoplasm by Exportin 5. In the cytoplasm, they are cleaved near the terminal loop by endonuclease Dicer generating mature miRNA duplex composed of functional "guide" strand and the "passenger" strand, which is degraded.

genome. While sequences coding for proteins are only such a small part of the genome, more than 90% is actually transcriptionally active [4]. Transcription of these sequences (the so-called junk DNA) creates a vast amount of ncRNA. These molecules function in their RNA form and have been described in various physiological as well as pathological processes. The most commonly known ncRNA are ribosomal RNA (rRNA) and transfer RNA (tRNA) that are both involved in translation of mediator RNA (mRNA) [5].

Interestingly, it was shown that the amount of DNA that is not translated into proteins is increasing with biological complexity of an organism meaning that the amount of ncRNA molecules is increasing [6].

NcRNA are divided into two groups based on their length – short ncRNA (sncRNA) and lncRNA. The arbitrary length was set at 200 nt; thus, sncRNA are shorter than 200 nt and lncRNA are longer than 200 nt. So far, several groups of molecules have been described in sncRNA – miRNA, short interfering RNA (siRNA), RNA interfering with PIWI proteins (piRNA) and small nucleolar RNA (snoRNA) [7–9].

MiRNA and circulating miRNA

MiRNA are short (20–23 nt), endogenous, single-stranded, ncRNA molecules that regulate gene expression. MiRNA genes are transcribed by RNA polymerase II or III into primary miRNA transcripts (pri-miRNA), which are characterized by a hairpin stem-loop structure. Then, the pri-miRNA are cleaved by the nuclear complex consisting of RNase III enzyme Drosha and Pasha protein (Partner of Drosha). The complex cleaves the pri-miRNA to pre-miRNA. Then, pre-miRNA are exported from the nucleus to the cytoplasm by Exportin 5. In the cytoplasm, pre-miRNA are processed in the RISC complex (RNA-induced silencing complex) composed of RNase III Dicer and core component Argonaute-2 (AGO2) protein. Dicer cleaves off the loop and generates 20–23 nt miRNA duplex with 2 nt overhangs at each 3′ end. The double-stranded duplex is then separa-

ted into the functional "guide" strand, which is complementary to the target sequence, and the "passenger" strand, which is degraded. Depending on the complementarity of miRNA in RISC, the target mRNA can be either translationally repressed at partial complementarity or degraded in the case of full complementarity (Fig. 2) [9,10].

MiRNA regulate a broad spectrum of physiological and pathological processes and can affect oncogenesis via possessing oncogenic and tumor-suppressor functions [11]. Several possible mechanisms how miRNA can affect oncogenesis have been described so far. For example, overexpression, amplification and epigenetic silencing, loss of miRNA gene that regulates expression of tumor suppressor gene were all observed [12]. On the contrary, deletion and epigenetic silencing of miRNA gene repressing oncogene expression can occur during tumorigenesis as well [3]. Moreover, mutations in target mRNA play an important role in oncogenesis since they are not repressed by their targeting miRNA as a consequence [13].

There is also a class of these molecules, so-called circulating miRNA, that have been discovered in a variety of body fluids (such as PB, urine, saliva, breast milk etc.) [14]. The main advantage of circulating miRNA is their stability since they can form complexes with proteins (such as AGO2) or lipoproteins (such as high-density lipoprotein) or may be packed into exosomes. Exosomes are small vesicles found in PB that contain promising markers of disease progression in cancer, including miRNA. In addition, various modifications of these molecules have been described that make them resistant to RNase activity [11]. Circulating miRNA have been proposed to play a role in cell-to-cell communication, proliferation, differentiation and metastasis, making them perfect putative markers for diagnosis and disease relapse. However, their origin and precise function is still unclear [15,16].

Circulating microRNA in MM

The very first study of circulating miRNA in MM was published in 2012 by Jones et al. evaluating miRNA expression profile in the serum of PB of MM patients, patients with monoclonal gammopathy of undetermined significance (MGUS) and healthy donors (HD). It revealed miR-720, miR-1246 and miR-1308 as potential MM biomarkers. Moreover, the combination of miR-720 and miR-1308 expression levels was able to distinguish MM and MGUS patients from HD, and the combination of miR-1246 and miR-1308 distinguished MGUS from MM patients [17].

Since then, many studies focused on circulating miRNA in MM. However, the studies were not uniform in sample types (serum vs. plasma), experiment design (patients vs. HD), used methods and platforms resulting in variable outcomes. Plasma samples were analyzed in the study of Yoshizawa et al. where significantly lower levels of miR-92a of newly diagnosed MM patients compared to HD were observed. Moreover, level of plasma miR-92a changed among patients according to the stage of the disease and response to treatment, suggesting its possible use as a biomarker for therapeutic response [18].

In another study, five plasma miRNA (miR-148a, miR-181a, miR-20a, miR-221 and miR-88b) were significantly up-regulated in MM patients compared with HD and correlated with patients' clinical data and survival. Interestingly, miR-20a and miR-148a expression levels were associated with shorter relapse-free survival of MM patients. Thus, the authors proposed that plasma miR-20a could serve as a biomarker of poor prognosis of MM [19].

On the other hand, in the study of Qu et al., most of the 12 differently expressed plasma miRNA were down-regulated in MM samples compared to HD. Plasma miR-483-5p and miR-20a expression levels were described to have a diagnostic and prognostic power in MM [20].

In MM, more studies of circulating miRNA used serum samples. Our research group was no exception. Our own observations revealed significantly increased levels of miR-29a, miR-660 and miR-142-5p in MM patients compared to HD. It was further proven that serum miR-29a expression level might serve as a useful biomarker for differentiating MM patients from HD [21].

In a related study, we analyzed groups of MM, MGUS patients and HD and revealed a dysregulation of five serum miRNA (miR-744, miR-130a, let-7d, let-7e and miR-34a). In a multivariate analysis, improved stratification power for combination of miR-34a and let-7e was shown. The serum level of let-7e correlated with Durie-Salmon stage, and levels of miR-744, miR-130a, let-7d and miR-34a associated with Durie-Salmon substage. However, there was no significant correlation between any studied serum miRNA and BM plasma cell (PC) infiltration. In the study, dynamics of miRNA levels during disease progression was analyzed showing increase of serum miR-34a and decrease of serum let-7d levels in relapsed samples compared to diagnostic samples. Serum miR-744 and let-7e were assessed as possible indicators of overall survival (OS), when their low expression levels were linked to shorter survival [22].

Rocci et al. evaluated serum miRNA levels in uniformly treated MM patients and correlated miRNA levels with clinical outcome in order to test their prognostic impact. They observed longer OS of MM patients with higher levels of serum miR-25, miR-16 and miR-30a when compared to patients with lower expression of these miRNA. However, only increased serum miR-25 expression levels were associated with better progression-free survival (PFS) [23].

Another study revealed 95 dysregulated serum miRNA in newly diagnosed MM patients. Further analysis showed that combination of miR-19a and miR-4254 was able to distinguish MM from HD with high sensitivity and specificity and that decreased expression of serum miR-19a was positively correlated with ISS stage, del(13q14) and 1q21 amplification. Moreover, down-regulation of serum miR-19a resulted in a significantly shortened PFS and OS. Interestingly, although miR-19a was connected with poor prognosis, patients with down-regulated serum miR-19a had a better response to bortezomib-based therapy [24].

Navarro et al. examined serum miRNA expression in MM patients at the time of diagnosis and complete remission (CR) after autologous stem cell transplantation (ASCT), and in MGUS patients and HD. They identified and validated five serum miRNA (miR-16, miR-17, miR-19b, miR-20a and miR-660) which were decreased in diagnostic samples compared to CR samples. MiRNA with lower expression in diagnostic serum samples partially recovered normal levels during CR. Expression levels of these miRNA in MGUS patients were similar to levels of MM patients in CR but lower than in HD. Patients with higher level of miR-19b and miR-331 had longer PFS after ASCT. Moreover, lower levels of these miRNA in combination showed shortened PFS. Level of miR-19b was significantly lower in samples at relapse than at CR [25].

MM often manifests via destructive lytic bone lesions. Hao et al. found miRNA potentially involved in myeloma-related bone disease in serum samples of MM patients. MiR-214 and miR-135b increased in serum of MM patients suffering from bone lesions; their levels correlated with the severity of bone lesions thus serving as a potential diagnostic tool for bone disease identification. Moreover, patients with higher expression levels of serum miR-214 had an abysmal survival and shortened PFS and OS [26].

In the study of Manier et al., miRNA were found to be predominant small RNA in exosomes isolated from serum samples of MM patients and HD by small RNA sequencing. Moreover, they observed that let-7b and miR-18a significantly correlated with both PFS and OS in univariate analysis and with ISS stage and adverse cytogenetic aberrations in multivariate analysis improving prediction of uniformly treated newly diagnosed MM patients with poor outcomes [27]. In addition, exosomal miRNA, miR-16-5p, miR-15a-5p, miR-20a-5p and miR-17-5p significantly decreased in MM patients resistant to bortezomib treatment suggesting correlation of exosomal miRNA with un/responsiveness to the novel agent treatment [28].

Circulating miRNA were the object of interest not only for MM in particular, but also for the extramedullary relapse (EM) of MM, which is defined as presence of extraskeletal PC infiltrates, either connected to the bone or infiltrating soft tissues [29]. Our group was first to identify circulating serum miR-130a as a marker distinguishing patients with EM from MM patients and from HD. This miRNA decreased in EM patients and distinguished EM from newly diagnosed and relapsed/progressed MM patients without EM, suggesting that miR-130a is a new putative minimally invasive marker of EM. Moreover, we observed cellular miR-130a expression level increased in PC from tumor site of EM patients when compared to PC from BM of these patients. Our data suggest a role of miR-130a in migration and invasiveness of PC of EM patients [30].

Our findings about circulating miRNA in MM and EM prompted us to test them also as a marker for rare immunoglobulin M (IgM) – monoclonal gammopathies – Waldenström macroglobulinemia (WM), IgM-MGUS and IgM-MM. We showed that combination of miR-320a and miR-320b is able to discriminate WM from IgM-MGUS as well as IgM-MM with high sensitivity and specificity. These results suggest that such miRNA-based biomarker might be a novel effective tool for WM diagnostics. Nevertheless, further validation is needed [31].

Some preliminary work has been done by the Ostrava team on circulating miRNA in AL amyloidosis where four miRNA (miR-134, miR-133a, miR-342, let-7b) were found to be overexpressed in these samples in comparison to MGUS and MM samples (presented at EHA 2016).

As mentioned above, many studies analyzed circulating miRNA in PB of MM patients but no study focused on miRNA in urine of MM patients as a truly non-invasive diagnostic biomarker of the disease. Our group performed profiling of miRNA expression levels in urine samples of MM patients and HD and determined 20 deregulated miRNA. Unfortunately, further validation did not confirm statistical significance of previously observed miRNA dysregulation suggesting that circulating urinary miRNA are not MM-specific and cannot serve as a non-invasive marker of the disease [32].

LncRNA molecules

LncRNA are present in all vertebrate species, and their sequences cover a larger fraction of the human genome than protein-coding genes. It has been estimated that nearly 15,000 lncRNA are present in the human genome [33,34]. LncRNA are mRNA-like transcripts longer than 200 nt located within the nucleus or cytoplasm of cells. They are transcribed by RNA polymerase II, less often by RNA polymerase III, but do not have open reading frames and generally are located in intronic and intergenic regions. Their expression levels seem to be lower than protein-coding genes, and they appear to be tissue-specific. Human lncRNA have been associated with a spectrum of biological processes, including alternative splicing or nuclear import, transcription, translation, differentiation, gene expression, cell cycle regulation and many others, both positively and negatively [35]. Moreover, they can serve as structural components, precursors to small RNA and even as regulators of mRNA decay. New reports of dysregulated lncRNA (HOTAIR, MALAT1, HULC, etc.) across numerous cancers suggest that aberrant lncRNA expression may be an important contributor to tumorigenesis. Additionally, lncRNA promoters are bound and regulated by transcriptional factors and epigenetically marked with specific histone modifications [35]. However, one of their primary tasks appears to be regulation of protein-coding gene expression. Recently, Wang et al. described four different mechanisms of lncRNA action. He proposes that these molecules can function as signals, decoys, guides or scaffolds [36]. In addition, dysregulation of lncRNA resulting in changed expression of tumor suppressors or oncogenes may be one of the "hits" that leads to oncogenesis. That is why lncRNA might be suitable as potential biomarkers and targets for novel therapeutic approaches in the future [4].

LncRNA are involved in hematopoietic development, including proliferation, differentiation and apoptosis of hematopoietic stem cells, as well as progenitors and precursors of multilineage mature blood cells, which include erythrocytes, megakaryocytes, myelocytes (monocy-

tes, macrophages and neutrophils), and B- and T-lymphocytes. It seems that dysregulation of lncRNA leads to abnormal modulation of multiple cellular pathways and results in various hematological malignancies, including lymphoma, MM and leukemia. Dysregulation of lncRNA can lead to anemia, which is the most common hematologic disorder. These studies suggest the potential clinical relevance of lncRNA in the diagnosis, prognosis, and therapy of these diseases [37].

In MM, only one paper about circulating lncRNA has been published so far. Five lncRNA (TUG1, MALAT1, HOTAIR, GAS5, LincRNA-p21) were selected and their levels were checked in plasma of PB of MM patients using quantitative real-time PCR (qPCR). TUG1, MALAT1, HOTAIR, GAS5 were found to be dysregulated in MM patients. In comparison to HD, only TUG1 was found to be significantly dysregulated [38].

Circulating lncRNA are a newer class of ncRNA molecules that show a lot of promise. However, very few studies have been published so far so their full potential has not been clarified.

Circulating cfDNA

Molecules of cfDNA are extracellular fragments of short double-stranded DNA found in PB and other body fluids, such as urine, saliva, breast milk and synovial fluid [39,40]. The first mention of these molecules dates back to 1948, when Mandel and Métais described their presence in human PB [41]. Thirty years later, these molecules were studied in malignant diseases. In 1977, Leon et al. described significantly elevated levels of cfDNA in PB of patients with various malignant diseases (lung, kidney, prostate and ovarian cancers, etc.) in comparison to HD [42].

In PB of HD, cfDNA is found in very low concentrations (10–100 ng/ml, with an average of 30 ng/ml). Elevated levels of cfDNA are present in various pathological conditions, such as inflammation, trauma, surgery, heart attack, stroke or autoimmune diseases [43,44]. The most significantly elevated cfDNA levels have been described in malignant diseases, especially in advanced stages and metastases, where levels of cfDNA reach up to 1,000 ng/ml [45,46].

CfDNA may be released from cells by several mechanisms. The first possibility is apoptosis – in this case, fragments are about 180 bp long and can be bound to the surface of other cells, for example to erythrocytes in PB [47]. In pathological conditions, including tumors, cfDNA is released from cells by necrosis. The fragments are longer than 180 bp (up to 1,000 bp) and can form complexes with proteins resembling nucleosome in PB [48]. The last possibility is active release from cells, when DNA fragments can be involved in certain cellular functions, such as transcription or intercellular communication [47,49].

Physiologically, dead cells are removed by phagocytosis by macrophages or by some other scavenger cells [50]. Such released DNA fragments have a short half-life and are degraded in about 10 to 15 min by liver and kidneys. In pathological conditions, elevated levels of cfDNA are caused by massive cell death or by insufficient removal of DNA fragments by liver [51]. In malignant diseases, cfDNA is released not only from tumor cells but also from surrounding cells [39].

Genetic and epigenetic aberrations can be detected in cfDNA, for example mutations of various genes, chromosomal aberrations, loss of heterozygosity (LOH), microsatellite instability or DNA methylation [52]. These aberrations are the same as in primary tumor cells, and they are tumor-specific [2]. Detection of these aberrations discriminates specific tumor cfDNA from cfDNA released from other cells [53]. The total amount of cfDNA contains only a small proportion (< 1%) of tumor cfDNA and depends on the tumor size, location, type and stage [47]. For example, Diehl et al. found that 3.3% of tumor DNA were released into PB during one day in patients with 100 g tumor (approximately 3×10^{10} tumor cells) [54].

So far, published studies have shown that cfDNA derived from tumor cells is potentially useful in diagnosis, prognosis, monitoring of tumor burden and response to treatment in patients with a broad range of malignant diseases [45,55,56].

CfDNA in MM

In contrast to single-site BM biopsy, cfDNA contains DNA released from multiple independent tumor sites and can reflect complex heterogeneity and provide a better description of MM [57].

While various abstracts have been presented at conferences, only a few papers on use of cfDNA in MM have been published so far. In 2015, Sata et al. examined tumor burden using various ways of detection and several sources of clinical material, such as mRNA from BM and PB mononuclear cells and $CD20^{+}CD38^{-}$ B-cell population in BM, as well as serum cfDNA at diagnosis and at follow-up, 6 and 12 months after the start of treatment. The allele-specific oligonucleotide (ASO) primers for *VDJ* sequence of immunoglobulin heavy chain (IgH) rearrangement specific for each patient were designed in 20/30 MM patients at diagnosis, and this tumor specific rearrangement was quantified by qPCR using ASO primers [58].

Levels of tumor rearrangement of BM mononuclear cells statistically significant and correlated with PB mononuclear cells at diagnosis. On the other hand, no correlation was found with percentage of PC in BM or serum monoclonal Ig (M-Ig) levels. Levels of tumor rearrangement of PB mononuclear cells rapidly decreased after treatment. These data indicated that PB mononuclear cells could be suitable biological material for monitoring of minimal residual disease (MRD). The changes of levels of tumor rearrangement were more expressive than M-Ig. Thus, tumor rearrangement levels in the BM could be used for monitoring of tumor burden in non-secretory MM [52].

As for cfDNA, identical DNA sequences from BM cells were found in cfDNA in 18/20 MM patients at diagnosis, 17/20 follow-up samples at 6 months and 16/20 at 12 months. Interestingly, the amount of cfDNA remained the same and sometimes increased during treatment and did not correlate with tumor rearrangement levels of mononuclear cells from PB and BM. Based on these pilot data, the authors suggested that detection of tumor *VDJ* sequence in cfDNA could reflect persistence of

MM cells in the patients. A longer follow-up and larger cohort of MM patients are needed to clarify clinical significance of cfDNA and its relationship with prognosis [58].

The main disadvantages of qPCR with ASO primers for *VDJ* sequence of IgH are 30% failure to design of primers and the dependency of efficiency of amplification on primer sequences. Next-generation sequencing (NGS) could resolve these problems and be more suitable for MRD monitoring.

In a recent study, Oberle et al. evaluated clinical utility of leukocytes and cfDNA from plasma of 27 patients for MM monitoring by NGS. Patient-specific clonal *V(D)J* rearrangement was identified in BM of 23 MM patients and then before and after treatment in follow-up plasma samples. Overall, in leukocytes, *V(D)J* rearrangement was detectable in 71% and plasma cfDNA-*V(D)J* in 100% of cases at baseline screening. At the follow-up time points after treatment initiation, leukocytes-*V(D)J* was detectable in 40% and cfDNA-*V(D)J* in 34% of samplings. In 47% of follow-up cases, the leukocytes-*V(D)J* and cfDNA-*V(D)J* were simultaneously present [59].

Clonal *V(D)J* rearrangement was detected in plasma cfDNA and genomic DNA (gDNA) from leukocytes in 91% of non-responders/progressors and 41% of responders to treatment. Clonal *V(D)J* rearrangement was not detected in approximately half of the patients with a partial response, unlike the persistent M-Ig levels. It suggests that these PB markers are more dependent on cell turnover and therefore provide faster information about the patient's response to treatment. There was a significant correlation ($p = 0.0042$) between simultaneous presence of leukocytes-*V(D)J* and cfDNA-*V(D)J* rearrangements, but in 30% of cases, no clonal rearrangement was found. Based on these findings, the authors indicated that tumor cfDNA may be released not only by circulating MM cells but may reflect tumor progression from multiple extravascular tumor sites. However, real predictive value of the disappearance of VDJ rearrangement in cfDNA or gDNA from leukocytes or their persistence cannot be assessed because of the limitations of the study (small number of patients and heterogeneous treatment) [59].

In a recent study, proof-of-concept evaluation of presence of activating mutations in *KRAS, NRAS, BRAF* and *TP53* genes in paired DNA samples from CD138+ PCBM and plasma-derived cfDNA of 33 relapsed/refractory (RR) and 15 newly diagnosed (ND) MM patients were analyzed by NGS. Mithraprabhu et al. detected 128 mutations (31 in cfDNA, 59 in BM DNA and 38 in both). It was interesting that 10 patients had mutations in cfDNA which were not present in BM [1].

The results from screening phase were validated by droplet digital PCR. Higher frequency of mutations in cfDNA was detected in RR than in ND patients reflecting an increase in number of mutations in advanced stages of the disease. The presence and number of mutations did not correlate with presence of high-risk cytogenetics. Activating mutations of the *RAS-MAPK* pathway, especially *RAS* mutation, were highly prevalent, as they were detected in 69% of patients. In contrast, previous reports showed only 40–50% of cases to have this mutation [60]. *KRAS* mutations had the highest incidence in RR patients and *NRAS* in ND patients. Interestingly, *TP53* mutations were present only in RR patients.

This study also evaluated the amounts of cfDNA which were higher in MM patients (range 5–195 ng/ml) when compared to HD (6–32 ng/ml) ($p = 0.0085$). Levels of cfDNA correlated with disease stage but not with clinical parameters (infiltration of BM by malignant PC, serum M-Ig levels). In this study, specific mutant clones were longitudinally tracked in sequential plasma cfDNA samples of seven patients by droplet digital PCR. Presence of changes in representation of clones was observed reflecting disease progression. In three MM patients, fractional abundance of cfDNA correlated with changes of serological biomarkers and reflected disease status. For example, two mutations *KRAS* G12S and *KRAS* G12V were detected in cfDNA of one patient. *KRAS* G12S mutation changed minimally over time, while fractional abundance of *KRAS* G12V changed together with light chain levels. This patient did not respond to treatment. In four other patients, cfDNA seemed to be a better biomarker of disease status with emergence of new tumor clones during therapy. For example, in one patient, two identified tumor clones were reduced by therapy. Then, this patient relapsed and rapid increase of light chain levels correlated with emergence of two new tumor clones, which were not detected previously. This study concludes that cfDNA analysis, as an adjunct to BM biopsy, represents a minimally invasive strategy for improved mutational characterization and therapeutic monitoring of MM [1].

Conclusion

MM is a clonal heterogeneous disease. Commonly used BM biopsies sample a single site and thus cannot contain all clonal information. Liquid biopsies show a real promise for such a disease as circulating molecules mirror the entire heterogeneity of the tumor and may be used as diagnostic markers. In the near future, it is possible that circulating molecules will be used more often than standard biopsies.

While there are only a few papers about the possibilities of circulating lncRNA as markers of the disease, circulating miRNA have been extensively studied as diagnostic as well as prognostic markers. Unification of analysis and platforms for these molecules is necessary for the field to advance into clinics.

Despite little published data, the promise of cfDNA carrying a specific marker derived from tumor cells is huge. CfDNA have a great potential as a minimally invasive marker especially in analysis of tumor burden monitoring and MRD detection. But more studies are needed for clarification of the importance of these molecules.

It is predicted that circulating molecules (cfDNA, miRNA, lncRNA and others) could bring major benefits in clinical use and improve patient lives in the future.

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Minimal Residual Disease Assessment in Multiple Myeloma by Multiparametric Flow Cytometry

Analýza minimální reziduální nemoci u mnohočetného myelomu pomocí multiparametrické průtokové cytometrie

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Summary
Background: Progress in treatment of multiple myeloma extensively increased patient remission rates, so minimal residual disease (MRD) detection becomes essential to assess the effectivity of treatment and depth of complete response. Nowadays, multiparametric flow cytometry (MFC) is the most used method for monitoring of MRD presence in the bone marrow of multiple myeloma patients; however, detection on molecular level can be used as well. It is evident that choice of protocol used for MFC-MRD assessment can significantly affect required results; nevertheless, standardized and highly sensitive approach of "next generation flow" is already available. Although benefit of MRD assessment as an independent predictor of progression-free survival and overall survival is known, very recent research showed that MRD-negative status surpasses the prognostic value of complete response achievement for progression-free survival and overall survival. ***Aim:*** This review is focused on use MFC in MRD assessment in multiple myeloma. The technical aspects and clinical benefits of this approach are mentioned as well. ***Conclusion:*** The information about MRD level detected by highly sensitive and reproducible MFC can be potentially used as a biomarker to evaluate the efficacy of different treatment strategies, help on treatment decisions and act as a surrogate for overall survival in multiple myeloma patients.

Key words
multiple myeloma – minimal residual disease – flow cytometry – plasma cells

Supported by Ministry of Health of the Czech Republic, grant No. 17-30089A.

Podpořeno z programového projektu Ministerstva zdravotnictví ČR s reg. č. 17-30089A.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 25. 6. 2017
Accepted/Přijato: 29. 6. 2017

doi: 10.14735/amko20172S21

Souhrn

Východiska: Díky pokrokům v léčbě mnohočetného myelomu se značně zvýšil počet pacientů dosahujících remise onemocnění, a tedy detekce minimální reziduální choroby (minimal residual disease – MRD) se stala nepostradatelnou ke zhodnocení účinnosti léčby a k posouzení hloubky kompletní léčebné odpovědi. Multiparametrická průtoková cytometrie (multiparametric flow cytometry – MFC) je v současnosti nejpoužívanější metodou pro stanovení a monitorování přítomnosti MRD v kostní dřeni pacientů s mnohočetným myelomem, nicméně mohou být využity také metody na molekulární úrovni. Je zřejmé, že protokol použitý pro stanovení MFC-MRD může významně ovlivnit získané výsledky, přičemž standardizovaný a vysoce senzitivní přístup „next generation flow" je již dostupný. Přínos stanovení MRD pro predikci přežití bez progrese onemocnění a celkového přežití je znám, nicméně nedávné výzkumy ukazují, že MRD negativita dokonce překonává prognostickou hodnotu dosažení kompletní léčebné odpovědi pro přežití bez progrese a celkové přežití. ***Cíl:*** Souhrnný článek je zaměřen na využití MFC v analýze MRD u mnohočetného myelomu. Zmíněny jsou také technické aspekty a zhodnocení klinického přínosu tohoto stanovení. ***Závěr:*** Informace o hladině MRD stanovené pomocí vysoce senzitivní a reprodukovatelné MFC může být využita jako biomarker k hodnocení účinnosti rozdílných léčebných přístupů, k rozhodování o vhodnosti léčby, ale také může sloužit jako parametr nahrazující celkové přežití u pacientů s mnohočetným myelomem.

Klíčová slova

mnohočetný myelom – minimální reziduální nemoc – průtoková cytometrie – plazmatické buňky

Introduction

Multiple myeloma (MM) is a hematologic malignancy characterized by presence of clonal bone marrow (BM) plasma cells (PCs). Development of new therapies led to the significantly prolonged overall survival (OS) in newly diagnosed patients [1]. Effective treatment comes along with the need for more sensitive approaches to compare the efficacy of different treatment strategies and implementation of individualized therapy monitoring strategies to prevent both under- and overtreatment [2]. New therapies are being developed but the transition to mainstream availability is much slower as randomized phase III clinical trials take years to show a benefit when using progression-free survival (PFS) and OS as study endpoints [3]. Extensive data indicate that minimal residual disease (MRD) information can potentially be used as a biomarker to evaluate the efficacy of different treatment strategies, help make treatment decisions, and act as a surrogate for OS [2–4]. Confirmation of the elimination of myeloma residual cell clones resistant to the therapy should be the way to cure MM.

Available techniques on cellular (multiparametric flow cytometry – MFC; next generation flow – NGF) and/or molecular (quantitative polymerase chain reaction – qPCR; next generation sequencing – NGS) level including imaging methods showed that persistent MRD means worse survival in MM [2,5–7]. MFC seems to be the most effective of existing approaches. Development of protocols for MFC-MRD followed technical progress of cytometry itself, availability of new antigens and fluorochromes together with standardization requirements [2,8]. A novel validated NGF assay for highly sensitive, fast and standardized quantification of MRD in MM that overcomes previous limitations of conventional MFC-MRD approaches and improves prediction of patient outcome, is ready-to-use and well suited for implementation in clinical trials to establish the diagnostic role of MRD in MM [7].

Needs for MRD detection

The indicator of treatment effectivity is the number of residual clonal cells. Assessment of MRD is becoming a standard diagnostic care for potentially curable neoplasms, such as acute lymphoblastic leukemia. In MM, the majority of patients will inevitably relapse despite achievement of progressively higher complete response (CR) rates, but new treatment approaches might further increase remission rates and potentially cure rates [2]. Interestingly, few of the patients that reach suboptimal response (near CR and/or very good partial response) are relapse-free at 10 years [9]. In 2008, Paiva et al. already demonstrated the clinical importance of MRD evaluation by MFC and illustrated the need for further refinement of MM response criteria [10]. Analysis of contribution of the serum free light chain ratio (sFLCr) or BM clonality to the prognosis of MM revealed that the sFLCr does not identify patients in CR at distinct risk; by contrast, flow cytometry revealed patients with a significantly inferior outcome. Thus, achieving CR does not mean achieving cure, and new definition of CR is needed even as stringent CR (sCR) is insufficiently informative in terms of expected PFS and/or OS [6]. However, the definition of clinical response criteria and clinical end points largely remained the same over the past 15 years. It was proven that MRD detection is a sensitive and fast approach and an independent predictor of PFS and OS [11,12]. Even more, as was recently demonstrated, MRD-negative status surpasses the prognostic value of CR achievement for PFS and OS across the disease spectrum, regardless of the type of treatment or patient risk group. Thus, MRD negativity should be considered as one of the most relevant end points for transplant-eligible and elderly fit patients with MM [13].

Analysis of PCs and their phenotype profile by MFC

MFC has been used in MM analyses for a long time. Mostly DNA analysis with cytoplasmic immunoglobulin detection was done [14–15]. Discovery of new monoclonal antibodies (MoAb) against PC antigens helped in the development of immunophenotyping of monoclonal gammopathies (MGs) [16–17]. Combination of surface CD138, CD38 and CD45 allowed identification of whole PC population in BM. The most useful antigens for basic orientation in context of PC normality are CD19 and CD56 which relatively easily discriminate immuno-

Tab. 1. List of the most useful antigens allowing normal and abnormal PC discrimination [21,22].

Antigen	Normal expression	Abnormal expression	Patient's expression	Diagnostics/ /monitoring
CD19	positive (> 70%)	negative	95%	essential
CD56	negative (< 15%)	strongly positive	75%	essential
CD20	negative (0%)	positive	15%*	recommended
CD28	negative* (< 15%)	strongly positive	15–45%	recommended
CD27	strongly positive (100%)	weak/negative	30–45%	recommended
CD81	positive (100%)	weak/negative	20–50%*	recommended
CD117	negative (0%)	positive	30%	recommended
CD200	negative (0%)	positive	75%*	useful

*own results

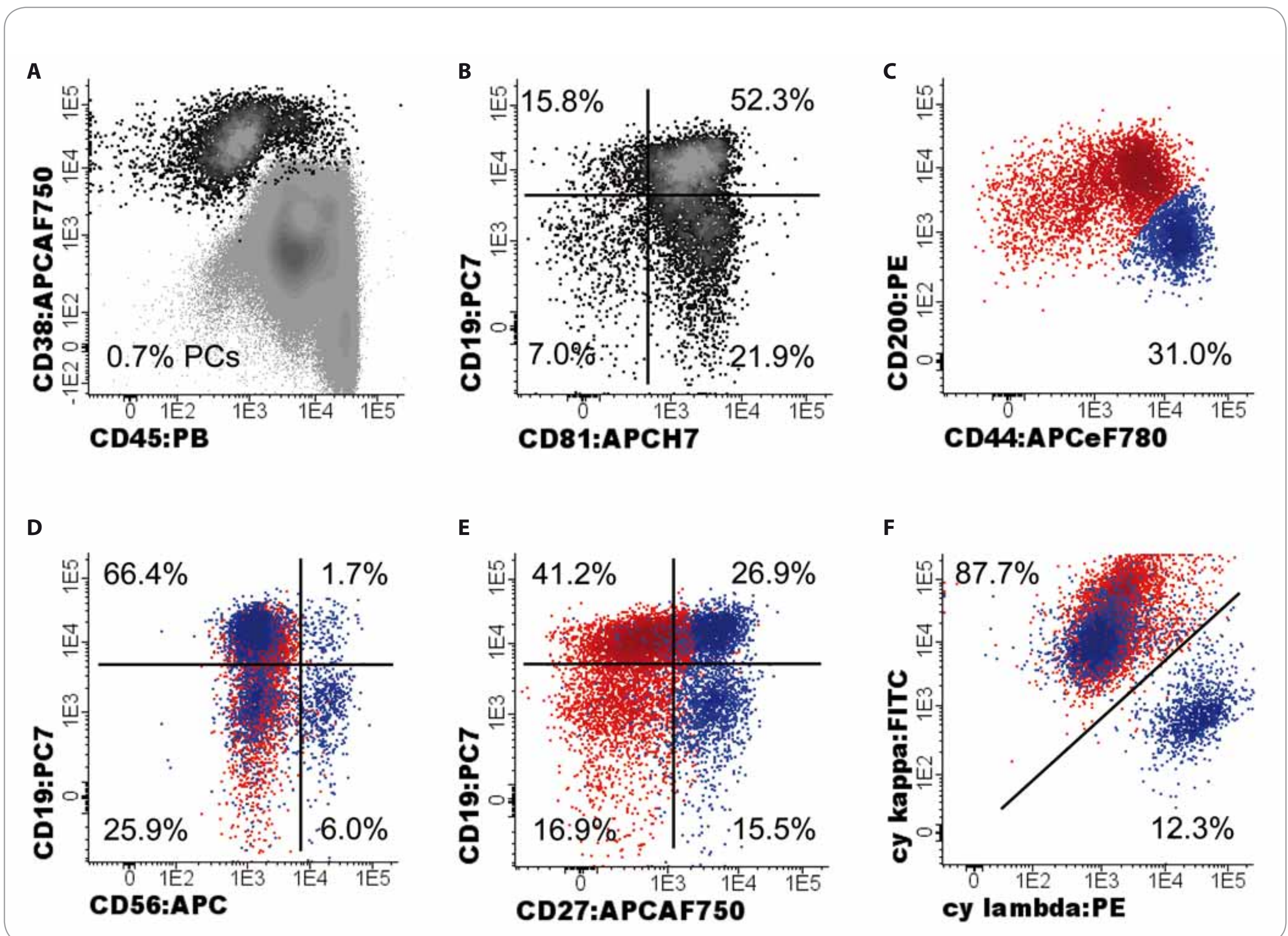

Fig. 1. Polychromatic analysis of PCs in newly diagnosed MM.

Clonal CD45⁻ PCs (A) with atypical phenotype profile CD19⁺CD81^dim+ (B) are visualised. Using other surface markers and clonality assessment revealed CD56⁻CD27⁻CD200⁺cykappa⁺ clonal PCs (red dots; C–F) with slightly lower expression of CD44 (C). Data acquired on BD FACSCanto II with Diva SW (BD Biosciences) and reanalysed by Infinicyt SW (Cytognos).

phenotypically normal (N-PC; $CD19^{+}CD56^{-}$) from aberrant/pathological (A-PC; $CD19^{-}CD56^{+/-}$) PCs [18–19]. Combination with other surface Ag further identifies phenotypic profile of pathological PCs (Tab. 1) [20–21]. Detection of immunoglobulin light chains kappa and lambda cytoplasmic expression is used for confirmation of PC clonality.

Thus, polychromatic MFC (minimum of six markers, but usually eight markers) is required for sufficient PC analysis and combination of surface and intracellular antigens is necessary for identification and clonality assessment of pathological myelomatous PCs (Fig. 1) [22–24]. Peripheral blood circulating clonal PCs are not detectable by routine analysis in all MM in time of diagnosis or are present in very low number, so their further monitoring during treatment needs a highly sensitive approach [25].

Flow cytometric assessment of MRD

Although flow cytometry is not a diagnostic tool in MG analyses (morphology is widely available and plays an irreplaceable role; on the other hand, the underestimation of PC number by flow cytometry is known), it provides important information about the presence and number of especially clonal PCs. MFC is generally applicable to majority of MM patients with sensitivity ranging from 10^{-4} to 10^{-6}. Progress in MFC technology and wide availability of used antibodies allows MFC to be an integral part of laboratory analyses and management of plasma-cell disorders (PCD). It can play an important part in diagnosis, prognostic stratification and monitoring of response to therapy via MRD detection, understanding of biology of disease progression, study of the role of the tumor microenvironment in PCD and identification of potential therapeutic targets on malignant PCs [2,26–28]. Valid and/or even better standardized MRD detection will ensure superior uniform assessment of response and clinical prognostication.

MFC in MRD definition

The use of MFC in the detection of MRD in BM has been demonstrated by several studies since 2002. The sensitivity of the flow cytometry assay was highlighted by the presence of detectable PCs in nearly a third of the patients with negative immunofixation (IFx$^-$) results and patients who were MRD-positive (MRD$^+$) had a worse outcome [29–30]. Flow cytometry was mentioned in sCR definition for the first time, where absence of BM clonal cells by immunohistochemistry or immunofluorescence was required [31]. Presence/absence of clonal cells was based on the κ/λ ratio which required a minimum of 100 PCs for analysis, an abnormal ratio reflecting presence of an abnormal clone was κ/λ of > 4 : 1 or < 1 : 2 [31]. Then, the term MFC remission was used and patients were considered to be in MFC remission when MM-PCs were undetectable in the BM sample at the MFC sensitivity limit of 10^{-4} (i.e., 1 MM-PC in 10^4 N-PCs). Only 4-colour MFC was used and a minimum of 3×10^5 BM cells was acquired [10]. Later, the updated International Myeloma Working Group (IMWG) response criteria in 2011 incorporated some new designations to traditional CR definitions and immunophenotypic CR (iCR, sCR$^+$) was defined as mentioned in previous sentence [10,32]. Recently published IMWG MRD criteria defines flow MRD-negative (MRD$^-$) sample as an absence of phenotypically aberrant clonal PCs by NGF on BM aspirates using the EuroFlow standard operation procedure for MRD detection in MM (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher [28].

Highly sensitive MFC and standardization

MFC seems to be the most perspective approach for detection of MRD in MM in terms of speed, price and availability of method. Applicability of MFC is over 90% of MM patients regardless of the knowledge of the phenotype at the time of diagnosis. The initial lower sensitivity of the method (when 4-colour flow cytometry was used) was increased by simultaneous detection of eight markers/fluorochromes and acquisition of a sufficient number of leukocytes in one run up to 10^{-7}, thus MFC is comparable with molecular techniques based on molecular level [7]. The technique has been modified to include an initial bulk lysis step to measure consistently more than 5×10^6 leukocytes per tube. Transition to quantitative assessment of residual clonal PCs will improve predictive potential of analyses as higher logarithms of clonal PC depletion significantly improved length of OS [12]. However, harmonization and/or standardization of MFC in MM are still relatively open as majority of clinical labs use their own protocols, which are comparable and sufficiently sensitive [4].

Project EuroFlow offers a standardized process of sample preparation and data acquisition when defined validated panels of selected and verified markers are used for every type of hematological malignancy. In addition, innovative software Infinicyt for data analysis is used [33]. Original EuroFlow panel designed for PCD was not sufficient for MRD detection, so 2nd generation of panel was developed (Tab. 2). This panel can identify clonal PCs on a background of normal regenerating BM (Fig. 2) [8]. The novel NGF-MRD approach takes advantage of innovative tools and procedures recently developed by the EuroFlow Consortium for sample preparation, antibody panel construction (including choice of type of antibody and fluorochrome), and automatic identification of PCs against reference databases of normal and patient BM. An optimized 2-tube 8-color antibody panel was constructed in five cycles of design-evaluation-redesign. In addition, a bulk-lysis procedure was established for acquisition of $\geq 10^7$ cells/sample. Prospective validation of the whole procedure at two distinct centers confirmed its robustness and significantly greater sensitivity vs. conventional 8-color MFC-MRD approaches, comparable to current NGS methods, with an improved prediction of patient outcome [7]. On the other hand, semi-standardized approach is available from Beckman Coulter Company as premixed dry tubes combining eight surface markers (CD38/CD45/CD81/CD27/ /CD19/CD200/CD138/CD56) for effective detection of clonal PCs without clonality assessment.

Preanalytical rules

It is very important to obtain BM sample not diluted by peripheral blood, where marrow elements must be present, to receive high quality results. There is a preference of EDTA anticoagulants as heparin decreases CD138 intensity and PCs should be less recognizable in context of

Tab. 2. Development of EuroFlow PCD panel [7,34].

1st PCD generation	PB	PO	FITC	PE	PerCP-Cy5.5	PC7	APC	APC-H7
PCD1	CD45	CD138	CD38	CD28	CD27	CD19	CD117	CD81
PCD2	CD45	CD138	CD38	CD56	β²m	CD19	cy Igκ	cy Igλ
2nd PCD generation	**BV421**	**BV510**	**FITC**	**PE**	**PerCP-Cy5.5**	**PC7**	**APC**	**APC-C750**
PCD1	CD138	CD27	CD38	CD56	CD45	CD19	CD117	CD81
PCD2	CD138	CD27	CD38	CD56	CD45	CD19	cy Igκ	cy Igλ

PB – Pacific Blue, PO – Pacific Orange, FITC – Fluoresceinisothiocyanate, PE – Phycoerythrin, PerCP-Cy5.5 – Perridin Chlorophyll--Cyanine 5.5, PC7 – Phycoerythrin-Cyanine 7, APC – Allophycocyanin, APC-H7 – Allophycocyanin-H7, BV421 – Brilliant Violet 421, BV510 – Brilliant Violet 510, APC-C750 – Allophycocyanin-C750

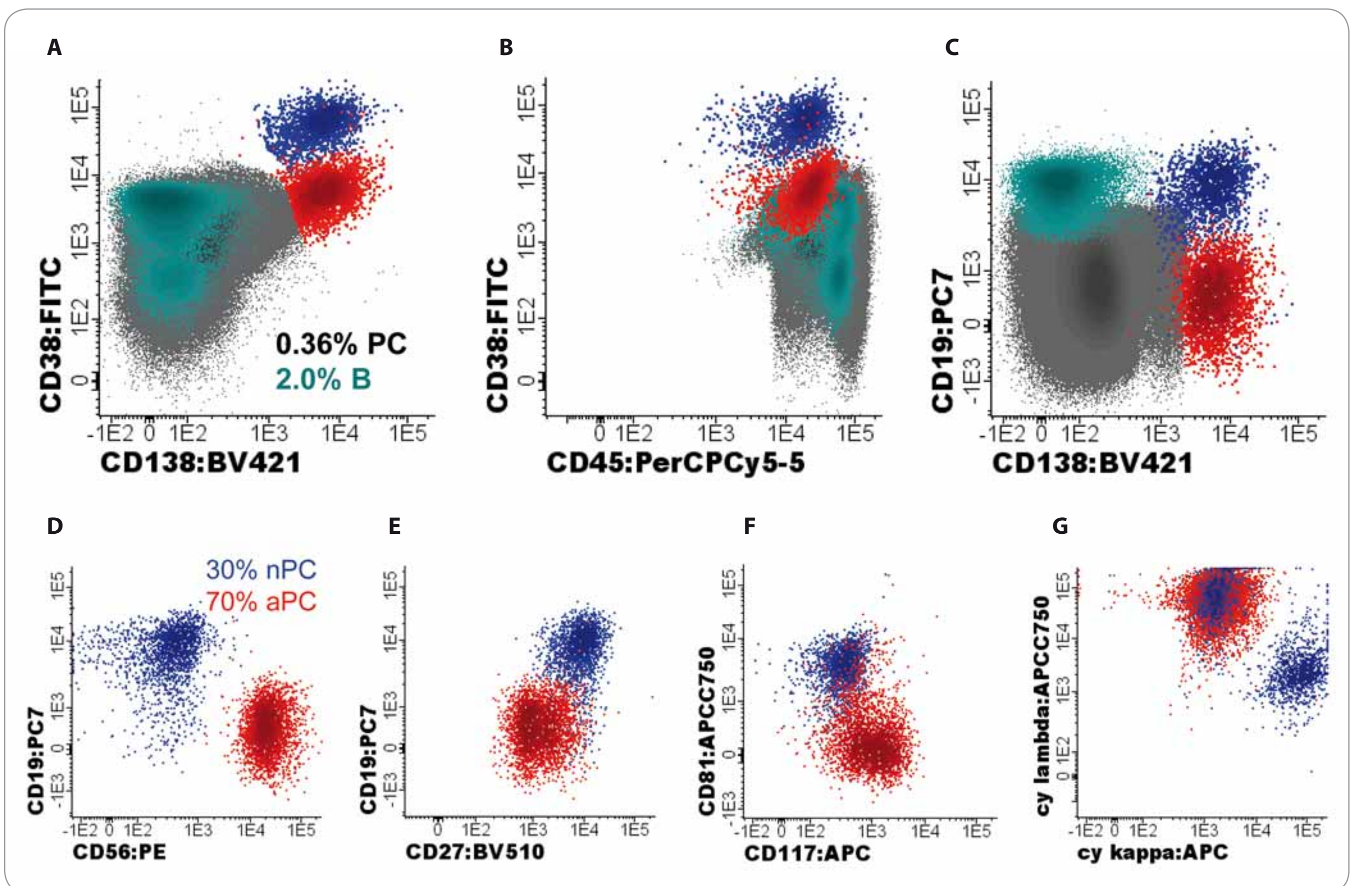

Fig. 2. Standardized analysis by 2nd generation of EuroFlow PCD protocol.

Polyclonal (nPCs, blue dots) and clonal (aPCs, red dots) PCs together with total B-cells (turquoise dots) are visualised in context of whole BM leukocytes (A–C). Typical phenotype of aPCs CD19⁻CD56⁺CD27⁻CD81⁻CD117het+ with cytoplasmic lambda expression was detected (D–G). Data acquired on BD FACSCanto II with Diva SW (BD Biosciences) and reanalysed by Infinicyt SW (Cytognos).

whole leukocytes. When transportation of sample is needed, only room temperature must be used. Analysis must be done within 36 hours from sample acquisition [4,34].

Trouble shooting

As a result of reduced number of PC after treatment, the procedure for cell concentration must be used (bulk lysis), to allow MRD assessment without loss of cell subpopulation, and to acquire at least limit of detection (LOD) and better limit of quantification (LOQ) of method [35]. Using targeted therapies as anti-CD38 (daratumumab) may complicate analy-

sis by homemade protocols, but replacement of CD38 by CD229 and/or using multiepitope CD38 resolve that problem [36]. Therapy-induced clonal selection could be already present at the MRD stage, where chemoresistant PCs show a singular phenotypic signature that may result from the persistence of clones with different genetic and gene expression profiles [37]. Although characteristic phenotypic profile of clonal PCs is already known, their detection should be impossible in a small subgroup of patients with atypical and/or changed profile (diagnostic vs. MRD antigenic profile), where different spectrum of antibodies should be used. Analysis of listmodes without SW assistance requires a well-educated operator and it is relatively time consuming.

Results report

Reporting of results to the clinicians should be descriptive and clear including percentage of PCs from leukocytes, percentage of clonal PCs from whole PC population, phenotype of clonal cells and sensitivity of analysis. Unsuitable and/or not representative samples should be reported.

Clinical relevancy of MFC-MRD assessment

Previous approaches to measurement of MRD levels were based on morphological assessment of BM, analysis of the paraprotein levels, or polymerase chain reaction (PCR) analysis of the immunoglobulin heavy chain variable-diversity-joining (VDJ) region. Historically, first results demonstrating the clinical importance of MFC in MRD detection were published in 2002, when Rawstron at al. showed that analysis of normal and neoplastic PC levels is more sensitive than IFx [29]. Patients with detected neoplastic PCs had a significantly shorter PFS than those with no detectable disease (median 20 vs. > 35 months; p = 0.003). Neoplastic PCs were detectable in 27% (9 of 33) of IFx$^-$ CR patients. These patients had a significantly shorter PFS than IFx$^-$ patients with no detectable neoplastic PCs (p = 0.04) [29]. Similarly, San Miguel at el. found that treatment-induced changes in the PC compartment correlated with disease outcome and patients in whom at least 30% of gated PCs had a normal phenotype after treatment had a significantly longer PFS (60 ± 6 vs. 34 ± 12 months; p = 0.02) [30]. Detailed analysis of newly diagnosed MM patients treated in Spanish GEM2000 protocol showed that PFS (median 71 vs. 37 months; p < 0.001) and OS (median not reached vs. 89 months; p = 0.002) were longer in patients who were MRD$^-$ vs. MRD$^+$ at day 100 after autologous stem cell transplantation (ASCT). Similar prognostic differentiation was seen in patients who achieved IFx$^-$ complete response after ASCT. Moreover, MRD$^-$IFx$^-$ patients and MRD$^-$IFx$^+$ patients had significantly longer PFS than MRD$^+$IFx$^-$ patients. Multivariate analysis identified MRD status by MFC at day 100 after ASCT as the most important independent prognostic factor for PFS (hazard ratio (HR) 3.64; p = 0.002) and OS (HR 2.02; p = 0.02) [10]. Results from Medical Research Council (MRC) Myeloma IX trial showed that absence of MRD at day 100 after ASCT was highly predictive of a favorable outcome (PFS, p < 0.001; OS, p = 0.0183). This outcome advantage was demonstrable in patients with favorable and adverse cytogenetics (PFS, p = 0.014 and p < 0.001, resp.) and in patients achieving IFx$^-$ CR (PFS, p < 0.0068). The effect of maintenance thalidomide was assessed, with the shortest PFS demonstrable in those MRD$^+$ patients who did not receive maintenance and longest in those who were MRD$^-$ and did receive thalidomide (p < 0.001). Further analysis demonstrated that 28% of MRD$^+$ patients who received maintenance thalidomide became MRD$^-$. MRD assessment after induction therapy in the non-intensive-pathway patients did not seem to be predictive of outcome (PFS, p = 0.1) [38]. In addition, it was demonstrated that prognostic impact of MRD following ASCT is independent of the induction therapy received [39,40]. Surprisingly, MFC-MRD monitoring has a prognostic value also in relapsed MM and is one of the most relevant prognostic factors in elderly MM patients, irrespectively of age or cytogenetic risk [11,40]. Using NGF in multicenter evaluation of 110 follow-up BM from MM patients in very good partial response (VGPR) or CR showed a higher sensitivity for NGF-MRD vs. conventional 8-color MFC-MRD with MRD$^+$ rate of 47 vs. 34% (p = 0.003). Thus, 25% of patients classified as MRD$^-$ by conventional 8-color MFC were MRD$^+$ by NGF, translating into a significantly longer PFS for MRD$^-$ vs. MRD$^+$ CR patients by NGF (75% PFS not reached vs. 7 months; p = 0.02) [7]. Very recent publication showed that achievement of CR in the absence of MRD negativity was not associated with prolonged PFS and OS compared with near-CR or partial response (median PFS 27, 27, and 29 months, resp.; median OS, 59, 64, and 65 months, resp.). MRD$^-$ status was strongly associated with prolonged PFS (median 63 months; p < 0.001) and OS (median not reached; p < 0.001) and in subgroups defined by prior transplantation, disease stage, and cytogenetics, with prognostic superiority of MRD negativity vs. CR particularly evident in patients with high-risk cytogenetics [13].

Implementation of MFC-MRD assessment in the Czech Republic – a single center experience and cooperation

Our laboratory provides flow cytometry analyses of MGs for almost 15 years. MRD monitoring began at our department in 2006 when 4-colour flow cytometry (CD38/CD56/CD45/CD19) was used for detection of pathological PCs in MM patients after ASCT. Switch to 5-colours in 2007 (CD38/CD138/CD45/CD56/CD19) helped to better discriminate subpopulations of PCs. Although surface expression of CD19 and CD56 on CD38$^+$CD138$^+$ PC is able to discriminate N-PCs from A-PCs, in some cases more detailed analysis using other markers was necessary. Therefore, other markers, which are aberrantly expressed on PCs (CD28, CD117 and CD20), were analyzed, but these were present in only a limited cohort of MM patients. CD27 was used as well, as its higher expression is specific for N-PCs, while lower intensity and/or lack of its expression are typical for A-PCs. Unfortunately, there was still no clear evidence which subpopulation is really polyclonal and/or clonal in selected cases. Thanks to new labora-

tory equipment, we replaced "minimalistic" 4-color flow cytometry analysis with 8-color MFC including also cytoplasmic kappa and lambda immunoglobulin light chains expression (CD38/CD45/ /cyκ/cyλ/CD138/CD19/CD56/CD27) in March 2011. Together with a progressive increase in number of acquired events, we are now able to reach sensitivity up to 10^{-6}.

As Czech central laboratory for EMN02 HOVON study, we have repeatedly analyzed 29 patients achieving CR by EuroFlow 1st generation protocol and MRD negativity was confirmed in 44.8% (13/29) of them. Unfortunately, only about 2×10^6 leukocytes are acquired for MRD assessment in that study, so sensitivity of analyses is only 10^{-5}. Interestingly, when we compared our MFC-MRD results to other European laboratories in this study (the same BM sample was sent, prepared and analyzed by others), there was an absolute concordance [41].

Nowadays, we are testing EuroFlow 2nd generation protocol for research purposes and clinical studies as well [42].

We meet other university laboratories in the Czech Republic involved in clinical studies during annual meetings of the Czech myeloma Group (CMG) and Myeloma Workshops and discuss innovations and problems in MFC-MRD analyses. They usually use homemade methods, EuroFlow-like and/or standardized EuroFlow approach (1st generation) and commonly acquire about 2×10^6 leukocytes. When tested similarity of acquired data from one patient BM in two different laboratories using the same EuroFlow protocol of 1st generation but different flow cytometers (BD FACS Canto vs. Navios, both set up according to EuroFlow rules), these looked similar. So rather harmonized than standardized MFC-MRD is going to be provided by different centers in Czech Republic.

Conclusion

As treatment strategies for MM become more effective and PFS becomes longer, assessing treatment efficacy according to MRD levels becomes increasingly important. Different approaches for MFC-MRD monitoring are available. Implementation of highly sensitive automated MFC-MRD assessment by NGF should confirm a new biomarker for treatment effectivity assessment and replace obsolete indicators defining clinical response and prediction of OS in MM. The prerequisite for that is standardization of sample processing, sample and data analysis and verification of this approach in clinical studies.

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Circulating Plasma Cells in Monoclonal Gammopathies

Cirkulující plazmocyty u monoklonálních gamapatií

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Summary

Background: Monoclonal gammopathies are characterized by presence of clonal plasma cells in the bone marrow, although peripheral blood circulating plasma cells can be found in a significant proportion of patients. The number of circulating plasma cells is an independent prognostic marker associated with shorter survival, but it can also help to predict early relapse. The reason and mechanism of plasma cell expansion from the bone marrow to enter peripheral blood is still not entirely clear, but possible changes in the expression of adhesion molecules are probably involved. Multiparametric flow cytometry allows simple and exact enumeration of circulating plasma cells in different types of cell suspensions, even in their low quantity. The phenotype profile and confirmation of clonality regarding to their bone marrow clonal counterparts should be verified as well. There is no uniform method used in clinical laboratories for circulating plasma cells analyses at this moment. ***Aim:*** Review is focused on use of multiparametric flow cytometry for circulating plasma cells analysis in peripheral blood. It is comparing possibilities of their detection by different methods and on clinical relevance of that assessment. The standardization of analyses is the main goal. ***Conclusion:*** Multiparametric flow cytometry is a very sensitive method for detection of circulating plasma cells, so using a standardized approach can lead to determination and implementation of the flow cytometry diagnostic threshold in plasma cell leukemia suspicious cases as well as in prognostication of monoclonal gammopathies patients. Moreover, analysis of plasma cells phenotypic profile could probably clarify their future behaviour.

Key words

monoclonal gammopathies – circulating plasma cells – plasma cell leukemia – flow cytometry

Souhrn

Východiska: Monoklonální gamapatie jsou charakteristické přítomností klonálních plazmocytů v kostní dřeni, nicméně cirkulující plazmatické buňky lze u významné části pacientů nalézt i v periferní krvi. Počet cirkulujících plazmatických buněk je nezávislým prognostickým faktorem asociovaným s kratším přežíváním, ale také může napomoci předvídat časný relaps. Příčina a mechanizmus vycestování klonálních plazmocytů z kostní dřeně stále není objasněna, nicméně může zahrnovat např. změny v expresi adhezivních molekul. Multiparametrická průtoková cytometrie umožňuje jednoduché a přesné stanovení zastoupení cirkulujících plazmocytů v jakékoli buněčné suspenzi, a to i při velmi nízkých počtech, vč. stanovení jejich fenotypu a potvrzení příslušnosti ke klonálním plazmocytům kostní dřeně. V současnosti však v klinických laboratořích není používán jednotný postup k analýze cirkulujících plazmocytů. ***Cíl:*** Souhrnná práce popisuje využití průtokové cytometrie v analýze cirkulujících plazmocytů v periferní krvi. Zaměřuje se na možnosti detekce pomocí různých přístupů a také na klinický význam stanovení těchto buněk s cílem standardizace analýz. ***Závěr:*** Multiparametrická průtoková cytometrie je vhodnou a dostatečně citlivou metodou pro detekci cirkulujících myelomových klonálních plazmocytů. Využití standardizovaného přístupu může vést ke stanovení a zavedení nového „průtokově cytometrického" diagnostického kritéria u suspektních případů plazmocelulární leukemie, a může být využito také v rámci prognostikace pacientů s monoklonální gamapatií. Mimoto, stanovení fenotypového profilu klonálních plazmocytů by mohlo napomoci objasnit jejich budoucí chování.

Klíčová slova

monoklonální gamapatie – cirkulující plazmatické buňky – plazmocelulární leukemie – průtoková cytometrie

This work was supported by grant of the Ministry of Health, Czech Republic – Conceptual development of research organization (FNBr, 65269705).

Práce byla podpořena grantem MZ ČR – RVO (FNBr, 65269705).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 23. 6. 2017
Accepted/Přijato: 30. 6. 2017

doi: 10.14735/amko20172S29

Introduction

Presence of circulating "myelomatous" plasma cells (PCs) has an independent prognostic value in multiple myeloma (MM) [1,2]. Plasma cell leukemia (PCL) represents a unique subset of MM; it is generally a rare and aggressive PC proliferative disorder [3–5]. Existing methods of circulating plasma cell (cPC) detection provide correlating results in some cases but they are not comparable in manner of sensitivity and number of identified cPCs [6]. Although phenotypic profile of cPCs is at least partially known, information explaining behaviour of cPCs is needed. New sensitive multiparametric flow cytometry (MFC) based techniques are able to detect even low number of cPCs together with clonality assessment, so using a standardized approach may lead to improvement in diagnostics and clinical research [7].

Development and localization of PCs under normal and pathological conditions

PCs are terminal stage of B-lymphocytes differentiation with ability to produce antibodies. Their development takes place in secondary lymphoid organs, where B-cells are activated by the antigen and differentiate into plasmablasts. These progenitors of PCs migrate to the bone marrow (BM) where they interact with the BM microenvironment and differentiate into long-lived PCs [8–12]. Some studies described the presence of circulating antibody-secreting cells and especially their increasing number after immunization. Although part of the $CD19^{+}CD20^{-}CD38^{++}$ cells in blood might express CD138, a marker of PCs, they are probably not fully mature PCs but rather a transitional stage between plasmablasts and BM PCs [9–11]. These cells have homogenous $CD45^{bright}$ expression in all cases [12]. Typical transient expansions of polyclonal plasmablasts/early PCs can be observed in peripheral blood (PB) in case of reactive plasmacytosis in both neoplastic and non-neoplastic conditions [12,13]. Mature long-lived PCs in healthy donors persist only in the BM compartment, and they are not detectable in PB [14].

On the other hand, in monoclonal gammopathies (MG), characterized by presence of clonal PCs in BM, pathological PCs can expand into PB as cPCs [15,16]. Presence of cPCs was documented in various cases, not only in symptomatic MM, but also in asymptomatic smoldering MM (SMM), benign monoclonal gammopathy of undetermined significance (MGUS) [2,17,18] and also in patients with primary amyloidosis [19]. The cause and mechanism of PC expansion from the BM into circulation remains largely unknown, but the critical feature of cPCs is their independence from the BM microenvironment. Thus, some possible reasons of their migration may include changes in angiogenesis and microvessel density with subsequent increase in proliferative rate, higher incidence of cytogenetic abnormalities and changes in expression profile of adhesion molecules [14,20–23]. The question is if presence of cPCs is connected with natural development of the disease to the late phase of MM or forms a completely distinct biological group [21–23]. It was shown that cPCs are mostly quiescent, but surprisingly they have a higher clonogenic potential than their paired BM counterparts. This fact could explain their ability to disseminate into various locations in the BM. This suggests that cPCs represent a unique subpopulation coming from BM clonal PCs [22].

Detection of cPCs

Conventional morphology is able to detect cPCs in around 15% of all newly diagnosed MM patients [21,24]. Immunofluorescence microscopy (IM) detects cPCs in approximately 19–28.5% of MGUS patients, 25–38% SMM and more than 70% of newly diagnosed MM patients [1,15,25,26]. On the other hand, MFC can revealed presence of cPCs in 20–25% of MGUS patients, 40–69% of SMM patients and in 70–84% of newly diagnosed MM patients [2,14,25,27–29] and more than 90% of patients in relapse [14]. Using routine diagnostic immunophenotyping with sensitivity 10^{-4} was able to detect cPCs in 69% (78/113) newly diagnosed MM (median 0.2% and range 0.05–36.05) (own unpublished results). Using the more sensitive next generation flow (NGF) method, based on standardized Euroflow settings, presence of cPCs was documented in 60% of MGUS, 75% SMM and 96% of MM; and very recently cPCs were detected even in 100% SMM and MM new diagnosed patients [30,31]. Above that, using NGF and gene expression profiling (GEP) demonstrated that genetic features of cPCs are in concordance with BM clonal PCs [30]. It was recently published that CD138-based microfluidic PC capture is a potentially useful tool in MM as it permits quantitation of rare cPCs in blood and subsequent fluorescence-based assays [32]. Underestimation of PC number by MFC when compared to morphology evaluation is known, but results obtained by both methods correlate, and the percentage of PCs provided by MFC is also an independent prognostic factor affecting the overall survival (OS) of patients [6]. Hence, new "flow cytometric" criterion for PCL diagnostics should be established.

Characterization of cPCs by MFC

PCs are commonly identified using markers CD45, CD38 and specific CD138. Despite downregulation of CD138 on abnormal cPCs [22], they are clearly distinguishable by specific SSC/FSC position, usually bright expression of CD38 and low or absent expression of CD45 [14,33]. Discrimination of cPC from normal plasmablasts is important, especially in clonal $CD19^{+}$ MG cases (Fig. 1).

Aberrantly expressed or missing markers on clonal PCs have been described, as well as their relationship to prognosis of MG patients [34,35]. In comparison to BM PCs, cPCs show downregulation of some surface markers, such as CD11a, CD11c, CD29, CD49d, CD49e, CD33, CD56, CD117, CD138, CD28, CD38, CD81 and upregulation of CD44 and CD97, but their basic phenotype profile is mostly the same as in BM [14,22,36].

Adhesive molecule CD56, neural cell adhesion molecule (NCAM), seems to have a specific significance in abnormal PCs. CD56 is mainly involved during normal embryogenesis and has an important role in migration, mediates cell-to-cell interactions and cell-matrix interaction [37–39]. CD56 is commonly

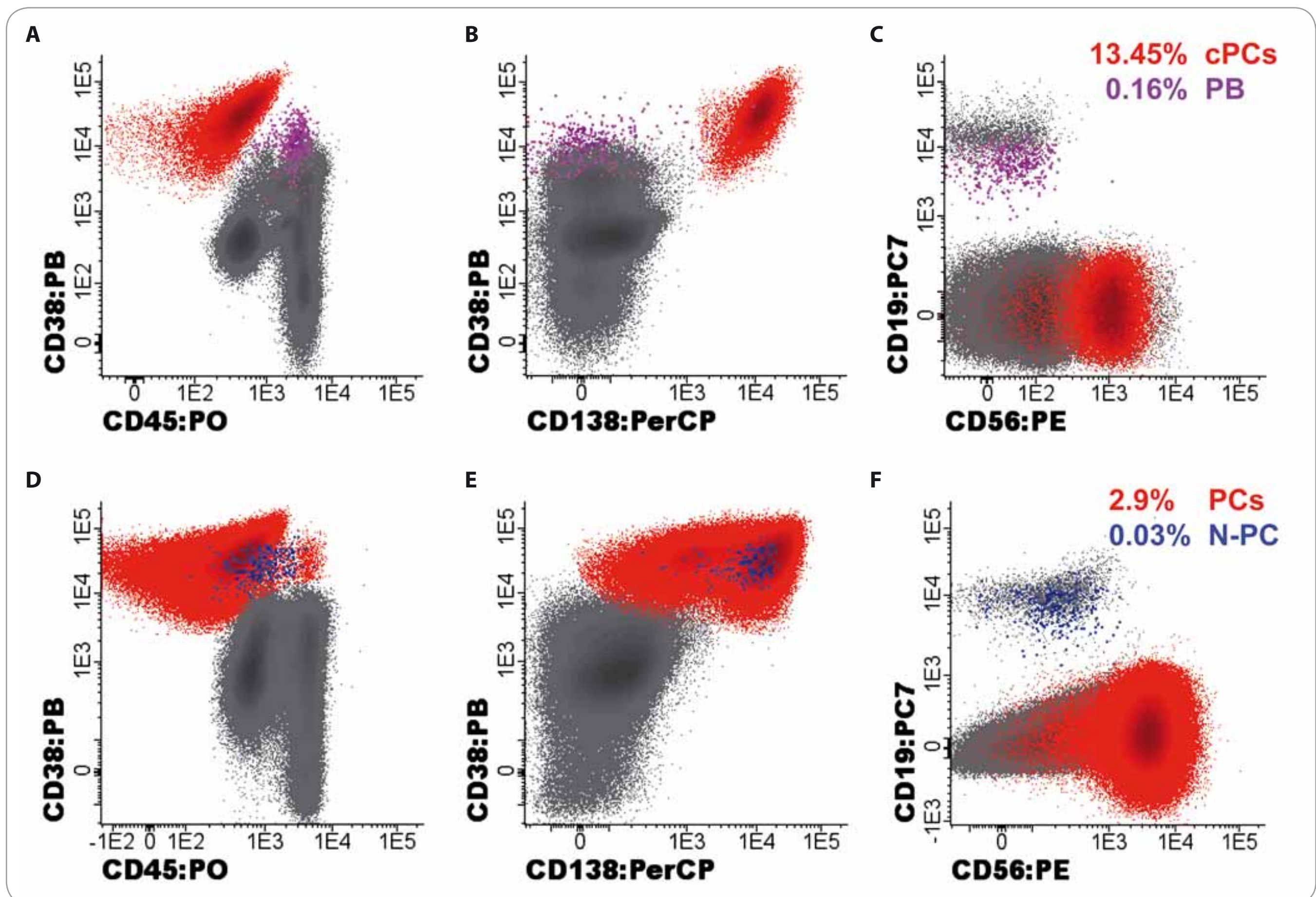

Fig. 1. Comparison of cPCs and PCs in a single patient.

The same phenotypic profile as CD19⁻CD56⁺CD45⁻ was found when cPCs and PCs (red dots) were analyzed in whole PB (A–C) and BM (D–F), resp. Presence of plasmablasts is visible in PB (purple dots) and rezidual normal PCs (N-PCs) are present in BM (blue dots). Data acquired on BD FACS Canto II (BD Biosciences), reanalysis done in SW Infinicyt (Cytognos).

present on NK cells, but it is usually expressed on abnormal PCs in majority of MM [23,37,40]. There is no significant alteration of CD56 expression over the course of the disease, so it is possible that weak expression and/or absence of this molecule on PCs at the time of diagnosis may be connected to PCL development and can help predict dissemination of PCs into PB [23]. This is consistent with downregulation of CD56 in patients with extramedullary involvement [39,40]. Interestingly, CD56 negativity is related to the absence of lytic bone lesions [23,41] and is also associated with more aggressive disease and shorter OS in patients treated by conventional therapy [37,40]. However, lack of CD56 is not a prognostic marker in patients treated with high-dose chemotherapy, but is associated with t(11;14) [42]. Although decreasing and/or missing expression of CD56 was thought to be responsible for extramedullary spread, there are MG cases with clearly CD56⁺ cPCs [39,40,43,44]. Also our results showed that CD56 was expressed in majority of cPC⁺ MM cases although median fluorescence intensity of CD56 in PB was lower than in BM. In our hands, the phenotypic profile of cPCs in a single patient is comparable to their clonal BM PCs (unpublished data).

Clinical relevance of cPCs

Presence of cPCs is associated with remarkably increased risk of malignant transformation of asymptomatic MGUS [1] and SMM into symptomatic MM [45]. Thus, MFC could be a convenient method to identify SMM patients with high-risk of progression before they develop end-organ damage [18]. It has also been shown that presence of cPCs in MGUS patients is related to shorter progression-free survival (PFS) and OS [1]. SMM patients with high levels of cPCs have significantly shorter OS compared to patients without cPCs or low number of cPCs [18,45]. It was demonstrated that MM patients with standard risk cytogenetics have better prognosis when associated with a lower number of cPCs, whereas those with high-risk cytogenetics had poor prognosis independently of number of cPCs [27]. Presence of cPCs positively correlates with higher level of beta2microglobulin, advanced ISS and Durie-Salmon stage and lower hemoglobin [21]. Moreover, their presence is also associated with shortened survival, so they could predict more aggressive disease and early relapse as well [20,46,47]. Thus, cPCs are a marker of

disease activity [48]. Number of cPC correlates with disease stage, and patients responding to therapy show reduction of cPCs [14]. Further, negative prognostic impact of cPCs in relapsed or refractory MM was observed [49]. Median time to progression (TTP) was the shortest in patients with no reduction of cPCs compared to patients with cPCs reduction or no detectable cPCs after one cycle of therapy (51 vs. 258 vs. 581 days); median OS was the shortest in the group of patients without reduction of cPCs in comparison to other groups (308 vs. 856 vs. 1,006 days) [49]. Therefore, monitoring of cPCs reduction after first cycle of therapy can be a useful tool for identification of patients resistant to treatment [49]. Negative prognostic impact of cPCs presence prior to transplant on post-transplant response has been observed. Achievement of stringent complete response (sCR) in the group with cPCs presence was 15 vs. 38% in patients with no detectable cPCs before autologous stem cell transplantation (ASCT) [50]. Patients without cPCs were associated with better PFS and OS in comparison to patients with detectable cPCs before ASCT (median PFS in the group with cPCs/without cPCs was 15.1 vs. 29.6 months and OS was 41.0 months vs. not reached). Thus, monitoring of cPCs presence before transplant by FC could predict survival in newly diagnosed MM [50]. Additionally, there were no PCs detected in patients who reached complete remission (CR) [14,51]. Although some studies found statistically significant positive correlation between higher presence of cPCs and the number of PCs in the BM [21,25,52], it was not confirmed by other research groups [2,36,45,53]. So, presence of cPC probably cannot be predicted on the basis of high BM infiltration when different methods of analyses are used (morphology vs. flow cytometry etc.).

Surprisingly, Paiva et al. demonstrated that numbers of clonal cPCs fluctuate in circadian rhythms [22]. In addition to that, patients with low or no cPCs were more likely to have bone lesions than those with high cPC numbers [25]. Monitoring of cPCs kinetics during treatment can help predict early relapse or refractory myeloma and reveal resistance to therapy [49].

Detection, analysis and separation of cPCs is a much less invasive way for their further characterization and useful PB "liquid biopsy" should be a part of routine examination of MG patients which can supply BM aspiration.

Plasma cell leukemia

PCL is a very rare and aggressive form of plasma cell dyscrasias characterized by presence of cPCs. The diagnostic criteria are based on a relative number of cPCs exceeding 20% of the total leukocytes and/or on the absolute number of cPCs exceeding 2×10^9/L in PB [3–5].

PCL can be divided in two different groups. Primary PCL (pPCL) originates *de novo* with no previous evidence of MM and represents approximately 60% of all PCL cases with median age at diagnosis 55 years [54,55]. On the other hand, secondary PCL (sPCL) is a leukemic transformation of pre-existing MM with median age at diagnosis 65 years and significantly shorter OS than pPCL (1.3 vs. 11.1 months) [55]. The clinical course of both PCLs is very aggressive with bad prognosis, significantly shorter OS than MM and adverse prognostic factors in comparison to MM [56,57]. Treatment of PCL is similar to MM, but despite aggressive new treatment strategies which have improved OS of pPCL patients, it is still worse than MM patients [56–58]. Interestingly, patients with pPCL achieve CR after transplantation more likely, but their OS remains worse than MM patients [59]. On the contrary, sPCL is usually not responsive to any treatment modality [60].

Analogous to MM, PCs of both PCL types express CD38, CD138 and typically have no CD19 [23,56,61]. Despite low incidence of PCLs, some studies demonstrated differences in their PC phenotypic profile. In addition to markedly reduced levels of CD27, which is associated with an aggressive clinical course of the disease [62], lack of or weak expression of CD56 seems to be a characteristic feature of pPCL in both compartments (BM and PB) [23,62]. Increased levels of soluble CD95 and beta2microglobulin have been observed in PCL [63]. Primary PCL shows higher expression of CD20 and lower CD9, CD56, HLA-DR and CD117 compared to BM PCs in MM patients [56,64] and lower expression of CD40 in comparison to MGUS [63]. Interestingly, expression of CD23, which is specific for abnormalities of chromosome 11, is associated with pPCL [65]. As well as in pPCL, typical absence of CD19 and negativity or low expression of CD56 in PB or BM was found in sPCL. CD56$^{-/weak}$ phenotype is stable from time of diagnosis to disease progression [23]. As mentioned above, majority of MM patients express CD56 [23,62], so the group of patients with CD56$^{-/weak}$ expression can possibly delineate a special subset of MM [23,42]. However, even in the era of new drugs, there is no clear connection between dissemination of PCs and loss of CD56, but CD56^{-} group might preferentially be the source of sPCL [23]. In addition to that, PCL shows high expressions of CD54, CD49d, CD29, CD126; in contrast to MM; PCL has uniform expression of CD44. Moreover, overexpression of nestin – a protein marker for neural stem cells – was found in both PCLs when compared to MM [66]. Surprisingly, expression of molecules CD18, CD11a, CD11b was lower on BM PCs and higher on cPCs, so these differences may explain hematogenic dissemination characterizing PCLs [61]. In comparison to untreated MM, PCs from progressing MM patients and BM PCs or cPCs from sPCL patients expressed lower levels or were negative for CD106 and the activated form of CD29, resp., with a weaker or zero ability to adhere to human fibronectin [67]. Phenotypic profile of pPCL and sPCL is not very different, except for a decrease of CD117 in pPCL and loss of CD19 and CD20 expression in sPCL [66]. CD28 seems to be able to distinguish pPCL from sPCL. It was found that expression of CD28 can be found in PB, but not in all cases in BM, and these PCs are more proliferative. Furthermore, in contrast with other markers, CD28 can be upregulated during disease evolution and correlates with tumor progression [23,64].

Using conventional morphology, a group of patients with the presence of

5–20% cPCs was observed to have worse OS independently of other prognostic factors, such as age, creatinine and the grade of D-S and ISS staging system [24]. Above that, this group of patients had lower platelets number together with higher BM infiltration by PCs. Consistently, another study found that OS of MM patients with more than 2% of cPCs was comparable to pPCL [21,24]. Based on this observation, it seems that the time has come to reconsider the diagnostic criteria of PCL. Also, due to the well-known phenomenon of underestimation of PC number by MFC when compared to morphology evaluation [6], and together with important role of MFC in PCL diagnostics [68], there is a need to determine the new flow cytometry criterion for PCL diagnosis which reduces previously established morphologic cut--off to (at least) 10%.

Conclusion

Presence and clinical significance of cPCs is still a partially unsolved phenomenon in majority of MGs. MFC is a very sensitive method not only for detection of cPCs, but also for determination of their phenotypic profile. Technical development of MFC together with development of novel standardized NGF analysis allows standardization of this approach. Further analysis of cPC using other methods on cellular and molecular level may further explore their characteristics regarding their possible future behaviour. Moreover, definition of the "flow cytometry criterion" for PCL diagnostics as well as the prognostication of patients according to cPCs presence and phenotype should be validated in the future.

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Epidemiology of Multiple Myeloma in the Czech Republic

Epidemiologie mnohočetného myelomu v České republice

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Summary

Background: Multiple myeloma (MM) is a cancer of plasma cells with an incidence of 4.8 cases per 100,000 population in the Czech Republic in 2014; the burden of MM in the Czech Republic is moderate when compared to other European countries. This work brings the latest information on MM epidemiology in the Czech population. ***Material and Methods:*** The Czech National Cancer Registry is the basic source of data for the population-based evaluation of MM epidemiology. This database also makes it possible to assess patient survival and to predict probable short-term as well as long-term trends in the treatment burden of the entire population. ***Results:*** According to the latest Czech National Cancer Registry data, there were 504 new cases of MM and 376 deaths from MM in 2014. Since 2004, there has been a 26.9% increase in MM incidence and an 8.3% increase in MM mortality. In 2014, there were 1,982 persons living with MM or a history of MM, corresponding to a 74.4% increase when compared to MM prevalence in 2004. The 5-year survival of patients treated in the period 2010–2014 was nearly 40%. ***Conclusion:*** The available data make it possible to analyse long-term trends in MM epidemiology and to predict the future treatment burden as well as treatment results.

Key words

multiple myeloma – epidemiology – Czech National Cancer Registry – Registry of Monoclonal Gammopathies – Czech Republic

Souhrn

Východiska: Mnohočetný myelom (MM) je maligní nádorové onemocnění plazmatických buněk s incidencí 4,8 případů na 100 000 obyvatel v České republice v roce 2014; Česká republika patří mezi země se středním výskytem tohoto onemocnění v Evropě. Práce přináší aktuální informace v oblasti epidemiologie MM v české populaci. ***Materiál a metody:*** Základním zdrojem údajů pro populační hodnocení epidemiologie MM je Národní onkologický registr ČR. Tato databáze rovněž umožňuje hodnotit přežití dosahované u pacientů a predikovat pravděpodobné krátkodobé a dlouhodobé trendy v léčebné zátěži celé populace. ***Výsledky:*** Dle posledních dostupných dat Národního onkologického registru ČR bylo v roce 2014 nově diagnostikováno 504 nádorů MM a 376 pacientů na toto onemocnění zemřelo. Incidence stoupla od roku 2004 o +26,9 %, u mortality došlo ve stejném období k vzrůstu o +8,3 %. Prevalence dosáhla v roce 2014 hodnoty 1 982 osob a ve srovnání s rokem 2004 tak vzrostl počet žijících osob s tímto onemocněním o 74,4 %. Pětileté relativní přežití u léčených pacientů z období 2010–2014 bylo necelých 40 %. ***Závěr:*** Dostupná data umožňují analyzovat dlouhodobé trendy v epidemiologii MM, predikovat budoucí léčebnou zátěž i výsledky péče o pacienty.

Klíčová slova

mnohočetný myelom – epidemiologie – Národní onkologický registr – Registr monoklonálních gamapatií – Česká republika

This work was supported by the project Development of the NHIS Technology Platform – CZ.03.4.74/0.0/0.0/15_019/0002748.

Práce byla podpořena projektem ESF – Rozvoj technologické platformy NZIS (CZ.03.4.74/0.0/ /0.0/15_019/0002748).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 9. 6. 2017
Accepted/Přijato: 17. 6. 2017

doi: 10.14735/amko20172S35

Introduction

The importance of cancer epidemiology has been growing steadily, partly as a result of a high cancer incidence worldwide. The Czech Republic is no exception in this regard; on the contrary, it is one of the countries with the highest burden in several cancer types. Tens of thousands of cancer patients are newly diagnosed in the Czech Republic each year, and hundreds of thousands of them are followed-up due to a history of cancer in the past. This work aims to describe epidemiological trends of multiple myeloma (MM) in the Czech population, to assess patient survival and to predict probable short-term as well as long-term trends in the treatment burden of the entire population.

Material and methods

High-quality data play a key role in the evaluation of cancer epidemiology; in particular, the following data sources [1] are used for this purpose in the Czech Republic:

- Data on demographic structure of the Czech Republic population are processed by the Czech Statistical Office (CZSO) as part of its monitoring of the Czech population. These data cover demographic characteristics of the population, such as the total number of population, age structure, life expectancy etc. [2,3]. Population-based cancer mortality data are stored in a database of causes of death which is also processed by CZSO, in accordance with international methodology and based on data from death certificates (ICD-10 classification).
- The Czech National Cancer Registry (CNCR) is the main source of data on cancer epidemiology. CNCR is nowadays an integral part of comprehensive cancer care, covering 100% of the Czech population and containing more than 2.2 million records from the period 1977–2014. MM accounts for 0.6% of these records. The registration of malignant tumours is enshrined in the Czech legislation and is obligatory. CNCR is also the source of data for the assessment of basic performance indicators of cancer care, particularly for the assessment of patient survival. This work uses the estimate of 5-year relative survival [4] for this purpose. The Czech Society for Oncology has used CNCR data to develop an information system that predicts both the population burden of cancer as well as treatment burden for future periods. These predictions resulted from modelling of the development of demographic structure and cancer incidence in the population; survival models leading to predictions of prevalence of cancer patients to be treated in near future are another important component. The methodology and applied prediction scenarios are described in a publication by Dušek et al. [5].

The Registry of Monoclonal Gammopathies (RMG), established in 2007 by the Czech Myeloma Group, is another source of data. RMG represents an international database designed for the collection of data on patients with MM and other types of monoclonal gammopathies. Nineteen Czech centres and four Slovak centres currently contribute to the registry. The registry already contains data on more than 5,000 patients with MM (visit https://rmg.healthregistry.org to find more information). RMG not only contains data on MM incidence, but also makes it possible to monitor the use of individual treatment modalities, the occurrence of adverse events and drug toxicity [6]. RMG is therefore one of the largest and most comprehensive registries with regular monitoring in Europe. Treatment with all available therapies is recorded into the registry, thus making RMG a valuable source of data from real clinical practice. RMG represents a data basis that makes it possible to monitor the disease course on the population level. Data from RMG are utilised in the preparation of national guidelines for treatment of monoclonal gammopathies, serve as a basis for negotiations with the State Institute for Drug Control, and are also used for subsequent negotiations on reimbursements of new drugs with healthcare payers.

The database GLOBOCAN 2012, which is available at http://globocan.iarc.fr/, is the international source of data on cancer epidemiology. In view of the fact that these data are always available with a certain delay, this work presents estimated values for the year 2012, based on the most recent data available at the time of processing [7,8].

Results

International comparison of MM epidemiology

The estimated incidence of MM in 2012 was 114,251 new cases globally (23rd most common cancer, accounting for 0.8% of all cancers excluding non-melanoma skin cancer) and 38,956 new cases in Europe (21st most common cancer, accounting for 1.1% of all cancers excluding non-melanoma skin cancer). The cumulative risk of developing MM before the age of 74 is 0.17% globally (0.20% in men, 0.15% in women) and 0.31% in Europe (0.38% in men, 0.26% in women). The estimated mortality of MM in 2012 was 80,019 deaths globally (20th most common cause of cancer deaths, accounting for 1.0% of all cancer deaths excluding non-melanoma skin cancer) and 24,300 deaths in Europe (16th most common cause of cancer deaths, accounting for 1.4% of all cancer deaths excluding non-melanoma skin cancer). The estimated 5-year prevalence of MM in 2012 (i.e. the number of persons diagnosed within 5 previous years and still alive in 2012) was 229,468 globally (4.4 per 100,000 population) and 89,187 in Europe (14.3 per 100,000 population). Table 1 provides the basic overview of epidemiological characteristics of MM in Europe. Recent statistics have also clearly shown that the burden of MM in the Czech Republic – in terms of both incidence and mortality – is moderate when compared to other European countries [7,8].

Epidemiology of MM in the Czech Republic

For the purpose of analysis of data from CNCR, neoplasms with diagnosis C90.0 (according to the International Statistical Classification of Diseases and Related Health Problems – 10th Revision, ICD-10) and neoplasms with morphology code 9732/3 (according to the International Classification of Diseases for Oncology – 3rd edition, ICD-O-3) were included in the group of MM.

Trends in MM incidence and mortality in the Czech Republic over more

Tab. 1. Epidemiology of MM and immunoproliferative diseases (C90 + C88) in Europe (source: GLOBOCAN 2012).

	Men	Women	Both sexes
Incidence			
number of new cases	20,513	18,443	38,956
number of new cases per 100,000 population	5.7	4.8	5.3
ASR(W)	3.2	2.1	2.6
proportion of all newly diagnosed cancers (apart from non-melanoma skin cancer)	1.1%	1.1%	1.1%
Mortality			
number of deaths	12,220	12,080	24,300
number of deaths per 100,000 population	3.4	3.1	3.3
ASR(W)	1.7	1.2	1.4
proportion of all cancer deaths (apart from non-melanoma skin cancer)	1.3%	1.5%	1.4%
Prevalence rates (patients alive 5 years after diagnosis)			
absolute number of patients	47,507	41,680	89,187
rate per 100,000 population	15.9	12.7	14.3
Cumulative risk			
from birth until the age of 74	0.38%	0.26%	0.31%

ASR(W) – age-standardised rate (world standard population)

than three decades are shown in Fig. 1A. There has been a significant and consistent increase not only in incidence rates, but also in mortality rates (however, European age-standardised mortality rate is stabilised). Between 1980 and 2014, MM incidence increased from 2.3 to 4.8 new cases per 100,000 population and MM mortality increased from 1.4 to 3.6 deaths per 100,000 population. In absolute numbers in 2014, MM incidence was 504 new cases, MM mortality was 376 deaths and MM prevalence (i.e. all cases diagnosed in the past and still alive in 2014) was 1,982 persons (corresponding to 18.8 per 100,000 population). Although the incidence of MM in the Czech Republic is low when compared to other cancer types, its prevalence has been growing over the years (Fig. 1B) and the absolute number of persons with a history of MM, who need to be followed-up, is certainly not negligible. The overall epidemiological situation of MM in the Czech Republic in the period 2010–2014 is summarised in Tab. 2.

MM is mostly diagnosed in older people – the median age is 68 years in men and 70 years in women. Incidence rates are highest between the age of 61 and 75 years in men and between the age of 62 and 78 years in women (Fig. 2A). At the time of diagnosis, less than 1% of all MM patients are under the age of 40, and 18.6% of all MM patients are under the age of 60. Age-specific incidence rates, shown in Fig. 2B, demonstrate the growing risk of developing MM in older age. Age-specific incidence rates rise sharply from around age 45.

MM as a subsequent primary cancer in the same patient

The occurrence of subsequent primary cancers in the same patient significantly contributes to the overall epidemiological burden of the Czech population. A detailed analysis of historical data from CNCR has revealed a significant increase in the number of cases of MM as subsequent primary cancers over time (Tab. 3). Until 1994, these cases accounted for less than 5% of the overall number of new cases of MM. This proportion grew to almost 9% in the period 1995–2004, and available data from 2014 have shown that as much as 17% of all newly diagnosed cases of MM were subsequent primary cancers. Nowadays, MM is expected to be diagnosed as a subsequent primary cancer in about 80 persons each year.

MM incidence according to data from CNCR vs. RMG

Comparison of the number of patients according to the CNCR with the number of patients according to the RMG shows that RMG covers approximately 80% of data contained in CNCR for the period 2007–2014.

Predicted numbers of MM patients to be treated

Table 4 shows the estimates of incidence, prevalence and number of MM patients who will probably be treated in 2017. The estimate of overall prevalence

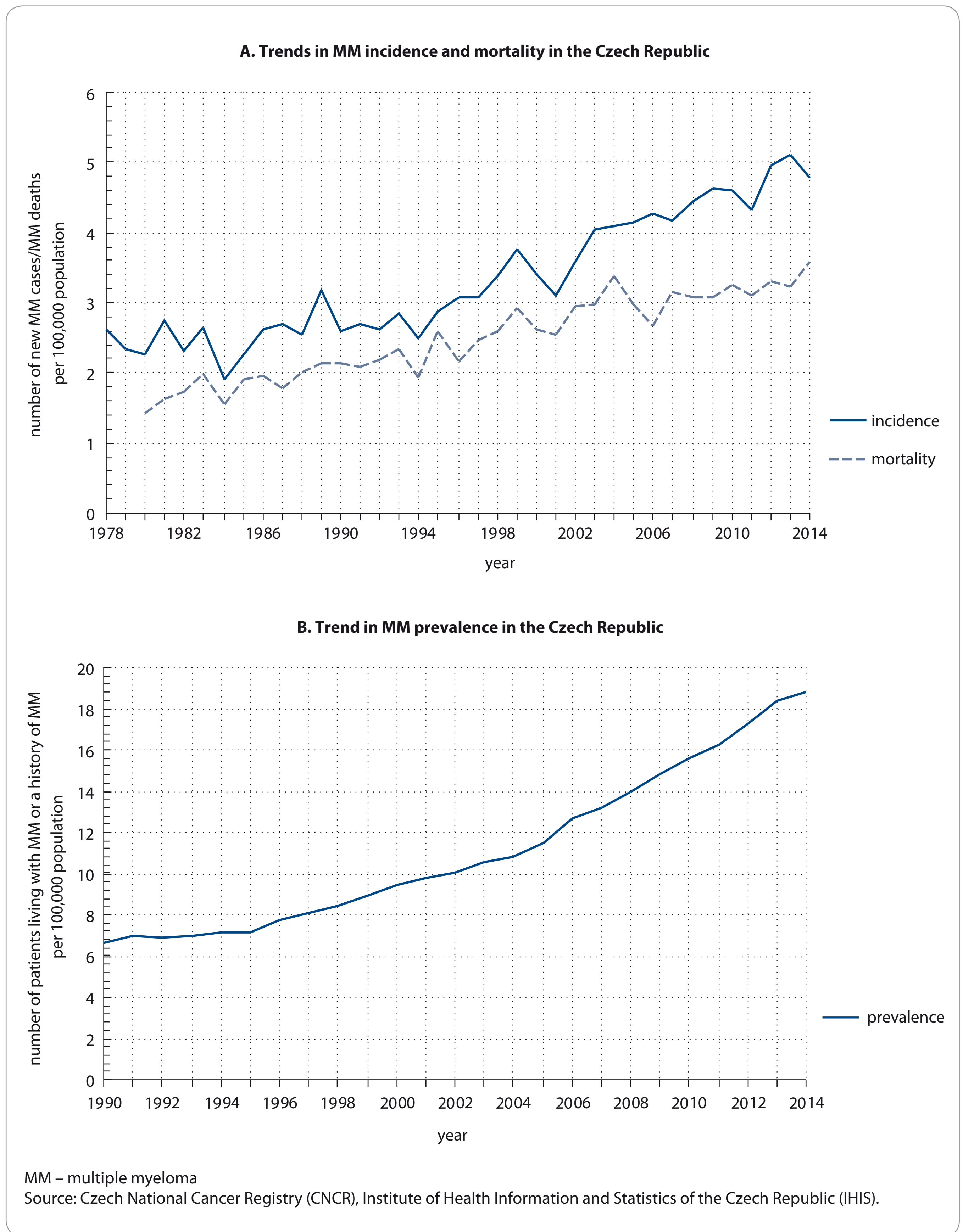

Fig. 1. Epidemiological trends of MM in the Czech Republic.

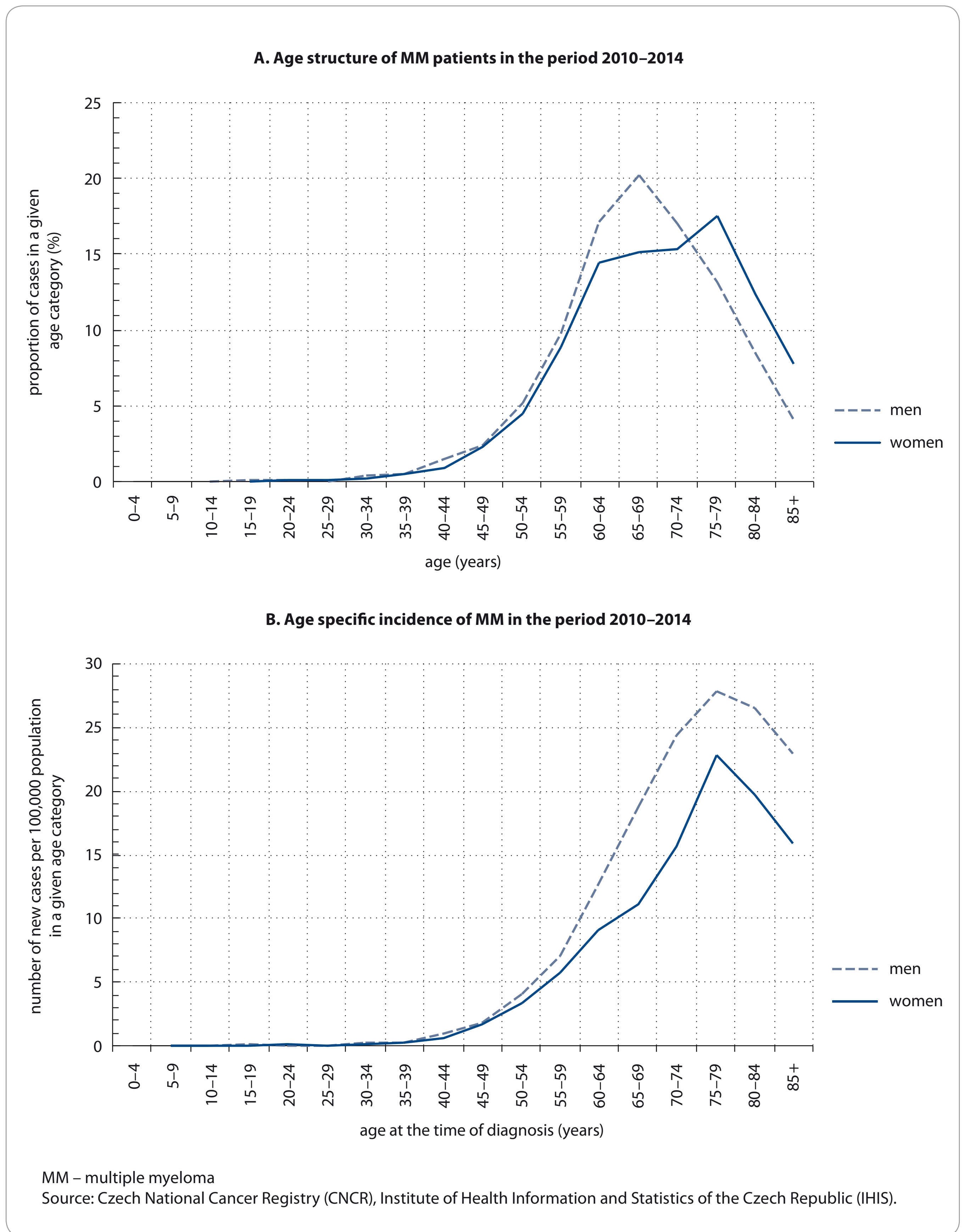

Fig. 2. Age of MM patients.

Tab. 2. Basic epidemiological characteristics of MM in the Czech Republic.

	Men	Women	Both sexes
Incidence			
absolute number of new cases (period 2010–2014, annual average)	257	242	500
rate per 100,000 population (period 2010–2014)	5.0	4.5	4.8
proportion of all newly diagnosed cancers apart from non-melanoma skin cancer (period 2010–2014)	0.84%	0.91%	0.87%
proportion of all newly diagnosed hemato-oncological malignancies (period 2010–2014)	11.4%	12.0%	11.7%
trend in the period 2004–2014	+33.0%	+20.8%	+26.9%
typical age of patients – median (25th to 75th percentile) (period 2010–2014)	68 (61–75) years	70 (62–78) years	69 (62–77) years
occurrence men : women (period 2010–2014)			1.1 : 1
Mortality			
absolute number of deaths (period 2010–2014, annual average)	175	171	346
deaths per 100,000 population (period 2010–2014)	3.4	3.2	3.3
proportion of the overall mortality (period 2010–2014)	0.32%	0.32%	0.32%
trend in the period 2004–2014	+13.9%	+3.3%	+8.3%
Prevalence			
absolute number of living patients (year 2014)	979	1,003	1,982
living patients per 100,000 population (year 2014)	18.9	18.7	18.8
trend in the period 2004–2014	+80.4%	+69.0%	+74.4%

Tab. 3. MM as a subsequent primary cancer in the same patient according to CNCR data.

Assessed category	**Period 1985–1994** n = 2,745	**Period 1995–2004** n = 3,528	**Period 2005–2014** n = 4,741
MM is the first cancer in a given patient	2,621 (95.5%)	3,214 (91.1%)	4,010 (84.6%)
MM is a subsequent primary cancer in a given patient	124 (4.5%)	314 (8.9%)	731 (15.4%)

MM – multiple myeloma

involves both the number of newly diagnosed patients in 2017 and the estimated number of living patients who were diagnosed and treated in previous years (calculated by population survival models). The overall sum for 2017 expects at least 2,079 newly treated patients (with a 90% CI ranging between 1,876 and 2,303); in case of optimally full reimbursements, the predicted number of newly treated patients is 2,680 (2,442; 2,936).

The predicted numbers of patients are updated each year and provided to healthcare payers as a background for their planning of treatment costs. Determination of the number of patients to be treated, which is based on the epidemiological situation, provides population-based reference data for a subsequent monitoring of clinical practice. In this way, the Czech Society for Oncology and healthcare payers are informed about the availability of therapy nationwide and in individual regions. It has been repeatedly shown that the numbers of actually treated patients do not substantially exceed the epidemiologically estimated burden.

Assessment of results of care provided to MM patients

The 5-year relative survival is the most common measure to describe the seriousness of a given cancer type on a population level, expressing the proportion of patients who have not died from that cancer type within 5 years of diagnosis. The relative survival is calculated as the ratio of the overall survi-

Tab. 4. MM – predicted epidemiological characteristics and numbers of patients to be treated in the Czech Republic in 2017.

MM	Predictions for the year 2017 (90% CI)
incidence	546[1] (508; 586)
prevalence	2,738 (2,653; 2,826)
clinically adjusted prediction of newly started treatments[2] with targeted therapies	2,079 (1,876; 2,303)

[1] The overall incidence of MM is provided, including new cases of MM diagnosed in cancer patients.

[2] Based on epidemiological models (incidence and prevalence of treated patients) corrected on the basis of clinical decision support models.

MM – multiple myeloma

Tab. 5. Reference values of 5-year relative survival of MM patients in the Czech Republic. The rates are age-standardised.

Type of record	5-year relative survival in different periods (95% CI)				
	Comparison of two periods further in the past[1]		Comparison of three recent periods with available data[2]		
	1990–1994	1995–1999	2000–2004	2005–2009	2010–2014
all CNCR records with non-zero survival values[3]	24.7 (21.9–27.7)	27.0 (24.4–29.7)	27.7 (25.2–30.3)	34.5 (29.8–39.2)	37.3 (35.0–39.5)
only patients treated with anticancer therapy	25.4 (22.0–28.8)	27.1 (24.0–30.3)	28.0 (25.0–31.1)	38.0 (32.3–43.7)	39.3 (36.6–42.0)

[1] Cohort analysis of patients diagnosed in a given time period.

[2] Period analysis – information on the survival of patients diagnosed in a recent period is involved in the calculation.

[3] Records on diagnosis based on autopsy or DCO (death certificate only) were left out from the overall population database. All other records were involved in the analysis.

CNRC – Czech National Cancer Registry

val (representing the overall observed mortality in a cohort of patients with a given cancer type) and the so-called expected survival (representing the mortality in a general population which corresponds in age and sex to the monitored group of patients). In other words, the relative survival describes the overall survival adjusted to mortality related to other diseases which the patient might die of. Furthermore, relative survival rates are age-standardised due to different age structures of patients in individual time periods; this means that the resulting survival is obtained as a weighted mean of relative survival rates in defined age categories [9,10].

In this regard, population-based data from CNCR provide the possibility of a very valuable assessment of the overall survival of cancer patients. Table 5 shows the assessment of relative survival of MM patients in the Czech Republic. Comparison of different time periods shows that there has been a significant increase in the 5-year survival of MM patients over time: the 5-year relative survival of MM patients who had been diagnosed before 2000 was 27.0%; by contrast, the 5-year relative survival of MM patients who were treated with anticancer therapy was almost 40% in the period 2010–2014.

Conclusion

The aim of this work was to describe epidemiological trends of MM in the Czech population, to assess patient survival and to predict probable short-term trends in the treatment burden of the entire population. This work proves that adequate data sources are available for the above-mentioned analyses. Each year in the Czech Republic, about 500 people are newly diagnosed with MM and about 350 people die of it. MM prevalence – i.e. the number of persons living with MM or a history of MM – was 1,982 in 2014, corresponding to a 74.4% increase when compared to MM prevalence in 2004. The probability of 5-year survival of MM patients has significantly increased over the last 20 years.

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Czech Registry of Monoclonal Gammopathies – Technical Solution, Data Collection and Visualisation

Český Registr monoklonálních gamapatií – technické řešení, sběr dat a jejich vizualizace

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Summary

Background: The Registry of Monoclonal Gammopathies (RMG) was established by the Czech Myeloma Group in 2007. RMG is a registry designed for the collection of clinical data concerning diagnosis, treatment, treatment results and survival of patients with monoclonal gammopathies. Data on patients with monoclonal gammopathy of undetermined significance (MGUS), Waldenström macroglobulinaemia (WM), multiple myeloma (MM) or primary AL ("amyloid light-chain") amyloidosis are collected in the registry. ***Data:*** Nineteen Czech centres and four Slovak centres currently contribute to the registry. The registry currently contains records on more than 5,000 patients with MM, almost 3,000 patients with MGUS, 170 patients with WM and 26 patients with primary AL amyloidosis, i.e. more than 8,000 records on patients with monoclonal gammopathies altogether. ***Results:*** This paper describes technology employed for the collection, storage and subsequent online visualisation of data. The CLADE-IS platform is introduced as a new system for the collection and storage of data from the registry. The form structure and functions of the new system are described for all diagnoses in general; these functions facilitate data entry to the registry and minimise the error rate in data. Publicly available online visualisations of data on patients with MGUS, WM, MM or primary AL amyloidosis from all Czech or Slovak centres are introduced, together with authenticated visualisations of data on patients with MM from selected centres. ***Conclusion:*** The RMG represents a data basis that makes it possible to monitor the disease course in patients with monoclonal gammopathies on the population level.

Key words

Registry of Monoclonal Gammopathies – RMG – registries – monoclonal gammopathies – CLADE-IS – data visualisation – database

This study was supported by the Ministry of Health of the Czech Republic (project identifier: DRO-FNOs/2017).

Práce byla podpořena projektem Ministerstva zdravotnictví České republiky (DRO-FNOs/2017).

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 13. 6. 2017
Accepted/Přijato: 19. 6. 2017

doi: 10.14735/amko20172S43

Souhrn

Východiska: V roce 2007 byl Českou myelomovou skupinou založen Registr monoklonálních gamapatií (RMG). RMG je registr určený pro sběr klinických dat týkajících se diagnózy, léčby, jejích výsledků a přežití u pacientů s monoklonálními gamapatiemi. V registru jsou sbírána data pacientů s monoklonální gamapatií nejasného významu (monoclonal gammopathy of undetermined significance – MGUS), Waldenströmovou makroglobulinemií (WM), mnohočetným myelomem (MM) nebo primární AL („amyloid light-chain“) amyloidózou. ***Data:*** V současné době do registru přispívá 19 českých a 4 slovenská centra. V registru je v současnosti evidováno více než 5 000 pacientů s MM, téměř 3 000 pacientů s MGUS, 170 pacientů s WM a 26 pacientů s primární AL amyloidózou; registr tak disponuje více než 8 000 pacienty s monoklonálními gamapatiemi. ***Výsledky:*** Článek je věnován popisu technologií využitých pro sběr, uložení dat a jejich následné online vizualizace. Představena je platforma CLADE-IS jako nový systém pro sběr a uchování dat z registru. Obecně pro všechny diagnózy je popsána struktura formulářů a funkcionality nového systému, které usnadňují zadávání nových dat do registru a minimalizují chybovost v datech. Představena je veřejně dostupná online vizualizace dat pacientů s MGUS, WM, MM nebo primární AL amyloidózou pro všechna česká nebo slovenská centra a autentizovaná vizualizace dat pacientů s MM z vybraných center. ***Závěr:*** RMG představuje datovou základnu, díky které lze monitorovat průběh onemocnění u pacientů s monoklonálními gamapatiemi na populační úrovni.

Klíčová slova

Registr monoklonálních gamapatií – RMG – registry – monoklonální gamapatie – CLADE-IS – vizualizace dat – databáze

Introduction

The Registry of Monoclonal Gammopathies (RMG) is one of the main projects of the Czech Myeloma Group (http://www.myeloma.cz). The registry is run by the Institute of Biostatistics and Analyses of the Masaryk University (http://www.iba.muni.cz) in cooperation with the Institute of Biostatistics and Analyses Ltd, a spin-off company of the Masaryk University (http://www.biostatistika.cz/), hereinafter referred to as IBA. IBA is also responsible for the technological solution of the registry. RMG is one of many clinical registries run by IBA (visit http://www.registry.cz to find out more). IBA activities include scientific research, solving research project and providing related services, particularly in the areas of analysis of biological and clinical data, organisation and management of clinical trials, development of software and ICT (information and communication technology) applications. Four IBA departments contribute to a smooth running of each registry – data management, project management, department for data analysis and IT department.

The main objectives of this paper are:

1. to introduce the RMG project,
2. to introduce the new database system as a new data basis,
3. to describe data collection, functions of the new system and structure of the registry,
4. to demonstrate and to describe interactive browsers for online visualisation of data from the registry.

Registry of Monoclonal Gammopathies

The RMG was established by the Czech Myeloma Group in 2007 [1]. The registry was established in order to collect data on patients with monoclonal gammopathies in order to monitor the disease incidence, how individual treatment modalities are used, what are their results including adverse effects, and to monitor the patients' survival in the long term. Monoclonal gammopathies are a collective name for a heterogeneous group of diseases that are characterised by the proliferation of one or more clones of differentiated B-cells producing a monoclonal immunoglobulin, which is sometimes referred to as the monoclonal protein (M-protein) or paraprotein [2]. The registry was originally focused on the collection of data on two different gammopathies – monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM), i.e. a malignancy of plasma cells characterised by very diverse clinical symptoms, such as headaches caused by osteolysis, disorders of kidney function, reduced blood cell formation, frequent infections etc. The registry was extended in 2014 – forms for the collection of data on patients with Waldenström macroglobulinaemia (WM) and primary or MM-associated AL ("amyloid light-chain") amyloidosis were added.

RMG is a database suitable for parametric monitoring of patients. Cooperation of important treatment centres is key to success, because an adequate amount of representative data needs to be collected. A total of 23 treatment centres in the Czech Republic and in Slovakia currently contribute to the registry (Fig. 1). The main Czech centres involve University Hospital Brno, University Hospital Hradec Kralove, University Hospital Olomouc, University Hospital Ostrava, University Hospital Plzen, University Hospital Kralovske Vinohrady and General University Hospital in Prague. Each centre has access to its data at any time; summary analyses are based on data from those centres which have agreed to provide their data for analysis. The project is open for cooperation to all treatment centres in the Czech Republic, Slovakia and other European countries. The project has been designed as a study with both retrospective and prospective patient recruitment. All patients are asked to sign an informed consent approving the registration of their clinical data into the registry. The registry contains data on more than 8,000 patients; specifically, data on more than 5,000 patients with MM, almost 3,000 patients with MGUS, 170 patients with WM and 26 patients with primary AL amyloidosis have been recorded into the registry so far. RMG is therefore one of the largest and most comprehensive registries collecting data on patients with monoclonal gammopathies. RMG has been designed as a purely observational, research-focused and epidemiological study. Treatment with all available therapies is recorded into the registry, thus making RMG a valuable

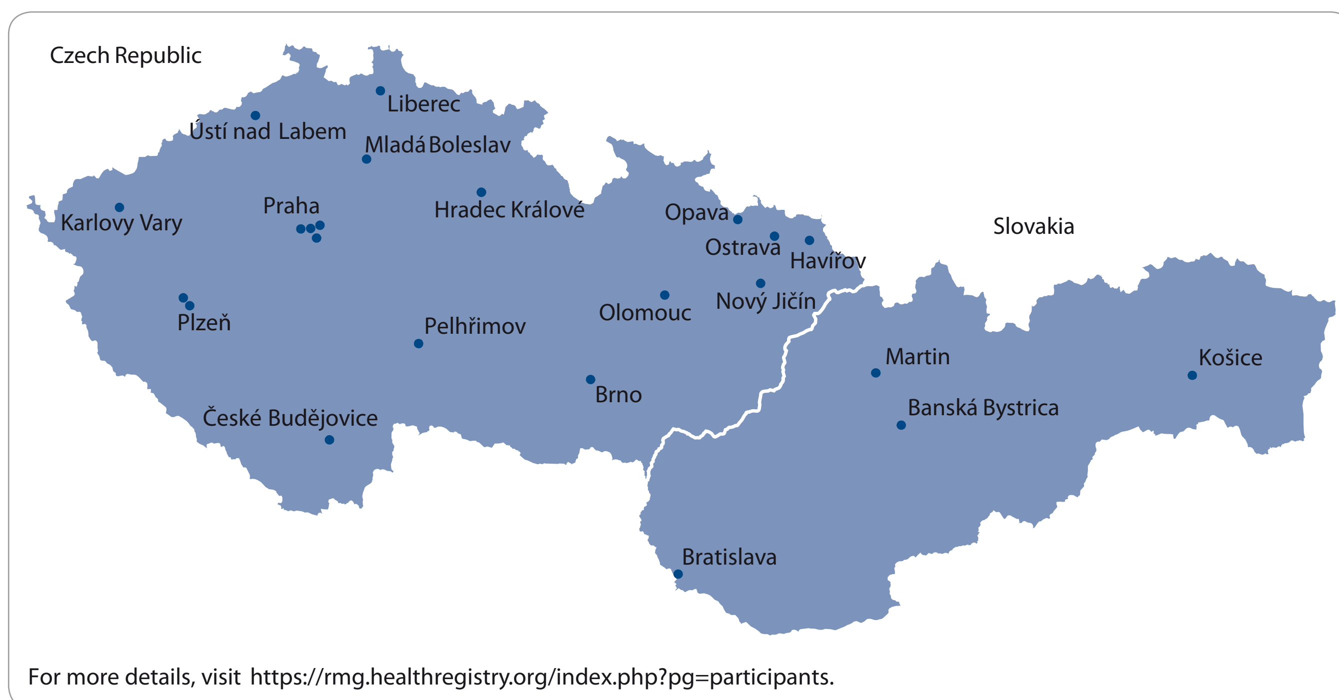

Fig. 1. Participating centres from the Czech Republic and Slovakia.

source of data from real clinical practice. Visit https://rmg.healthregistry.org to find more information on the registry itself, the list of centres with respective numbers of diagnosed patients, registry entry for authorised users, online visualisations of data, a form requesting data analysis, list of publications based on data from the registry etc.

CLADE-IS – a new online platform for data registration

The technological background of the registry has been built on the CLADE-IS platform (Clinical Data Warehousing Information System), which is software specifically designed for a repeated generation of electronic data capture (EDC) systems used in research management in clinical practice and in healthcare in general. Apart from classic randomised control trials (RCTs), CLADE-IS also supports the design and running of observational non-interventional studies, recently also referred to as "RWD/RWE" (real-world data/real-world evidence) studies. In terms of processes, the CLADE-IS platform covers all necessary steps to be made when organising a clinical trial, in accordance with the so-called good clinical practice, among others. All modules within this platform (Adminer, Designer, Reporter, Datavisor) – controlling various agendas such as the management of users and centres, design of forms with skip logic and validation rules, operating reports, data exports or graphic visualisation of data – are available online via a web browser, and therefore do not require any desktop application to be installed by the user (i.e. investigator).

In the long-term perspective, software development within the CLADE-IS platform is oriented towards open-source technologies and products (database layer – PostgreSQL, application layer – PHP/Symfony, front-end – ZURB Foundation, operation system – Linux Ubuntu LTS, deployment and virtualisation – KVM). Institute of Biostatistics and Analyses Ltd, provider of the CLADE-IS platform, prefers to work with open-source software and chooses technologies with an already existing long-term support by developer communities or even by large companies releasing their source codes as an attmpt to enhance the development dynamics. The CLADE-IS platform has replaced the formerly used system based on a proprietary technology (Microsoft, Oracle) which, although it had good results, did not meet the growing requirements anymore, namely the implementation of new functions and orientation to open-source solutions.

The CLADE-IS platform has dealt with the requirements and needs of the registry thanks to three important characteristics – 1. robustness, 2. usability, 3. openness. The robustness aspect describes the ability to define the structure of a wide range of study types – from randomised control trials (RCTs) to non-interventional observational trials or registries of qualitative data describing RWD/RWE trends. Data for RWE in clinical research cannot currently be referred to as big data because they fail to meet the definition of big data (volume, velocity, variety); nevertheless, they have many properties in common. Data variety is one of them; CLADE-IS deals with this aspect by using an original hybrid data model combining the properties of a data warehouse and a NoSQL database for unstructured data. Usability of the CLADE-IS platform is based on the application of front-end technologies that meet the requirements on responsive web design (i.e. an optimal display in a wide range of devices, from smartphones to widescreen desktop mon-

itors) and on the adherence to principles of designing the user interface as "mobile first". In this way, CLADE-IS brings breakthrough elements from the "Philosophy of UX" (User Experience) to the healthcare segment, which is often based on outdated IT solutions. This feature is important for the automation of selected agendas, such as online calculations or adverse event reporting. Openness of the system lies in its ability to communicate with other parties' systems and in the possibility of further extensions. The communication is carried out via REST API (Representational State Transfer Application Interface) and involves the possibility to obtain and to process various data from wearable technology (various biosensors or devices used in fitness and well-being) or to exchange data with international registries.

Further development of CLADE-IS is directed at improving functions in the domains of visual analytics (see below) and virtual patients, supported by unstructured data mining and by machine learning.

Structure and principles of data collection in the RMG registry

Data collection

The frequency of patients' visits to their physicians depends on their diagnosis and disease severity. At the beginning, MGUS patients have appointments every 2 or 3 months. If the patient's condition is stable, follow-up intervals increase to once every 6 to 12 months. Patients with MM, WM or AL amyloidosis visit their physicians depending on their individual condition and treatment. Patients in remission (i.e. without treatment) are usually invited to follow-up visits every 3 months. As regards patients enrolled in clinical trials, the procedure is individual, depending on the standard operating procedure (SOP) of a given trial.

Collection and updates of data in the registry are controlled by the registry coordinator. Data are entered into the registry by data managers from individual centres. In MGUS diagnosis, data in the registry are updated once a year; in WM, MM and AL amyloidosis, updates are required twice a year (each record should be updated before 30 June and before 31 December of a given year). A random check of entered data for selected patients is performed once a year by the registry coordinator, who compares these data from the registry with medical documentation in the participating centre. Apart from these tasks, the registry coordinator arranges training of new registry users as well as current users (aiming to inform them of new recommendations by CMG on data collection), helping users with entering data into the registry, having new user accounts created or existing user accounts deleted, etc.

Important functions of the system

Data originally stored in a former database system (based on a modified version of the TrialDB system) were converted to a new CLADE-IS system in 2017. Each individual user can use his/her username and password to enter the registry at https://rmg.data-warehouse.cz/login. Users have different rights according to their roles – for example, representatives of a given treatment centre can only see data from that centre. Data in the registry are de-identified, i.e. each patient in the registry is represented by an identification code that makes it impossible to reveal the patient's identity. Entered data can be exported from the registry to a table in Excel format. The IBA helpdesk sends regular monthly reports to registry users, summing up the numbers of patients with individual diagnoses recorded by the respective centre.

The new CLADE-IS offers important functions that not only make data entry into the registry easier and faster for users, but also minimise the error rate in data:

1. The system enables users to search for patients according to defined characteristics (for example, when the user needs to find patients with a specific type of treatment in order update their data).
2. Skip logic rules contribute to shortening of otherwise lengthy forms and require specific items to be filled in. For example, if the user indicates that a cytogenetic examination was carried out, the system displays individual items (presence of translocations, gains etc.) and requires them to be filled in. By contrast, if the user indicates that the cytogenetic examination was not carried out, the related items remain hidden. In this way, the form length changes dynamically.
3. Validation rules are set in some important parts of individual forms, making it impossible for the user to enter wrong data into the registry (for example, a patient's treatment cannot be terminated before it is started). Limits are set in selected parameters, requiring the entered value to fall between them (for example, percentages are limited to a minimum of 0 and a maximum of 100).
4. All items in the registry can be classified into two categories – mandatory and optional. If an item is marked as mandatory, the expert group (CMG) requires it to be filled in. Not filling such an item will prevent saving the form as "valid", which is an indication for the user that not all important items have been filled in. If the user considers the form to be completely filled in, he/she saves it as "completed". The system will subsequently evaluate whether all mandatory items are filled in; if so, the state of the form is changed to "valid"; if not, the system will prompt the user to fill the mandatory items (the text "this field is required" will be displayed in a red box under the item title). In situations when the user does not know the value of a mandatory item, the form must be saved as "pending". Some parameters can be marked as non-evaluable (for example, this can be used in a situation where a given treatment centre does not perform a specific test to find the value of a certain parameter); after marking the mandatory item as non-evaluable, the form can be saved as "completed".

Structure of forms

When working with the RMG database, users create and fill in forms for four groups of diagnoses – MGUS, WM, MM and primary AL amyloidosis. In general, three types of forms are filled in for each of these diagnoses:

1. **Diagnostics** – Characteristics of the patient's condition at the time of diagnosis. The evaluated parameters usually involve – date of diagnosis, characteristics describing the M-protein, performance status, disease stage, cytological and histological examination of bone marrow, evaluation of presence of bone lesions and extramedullary mass, results of biochemical, flow-cytometric and cytogenetic examinations.
2. **Follow-up/Treatment** – Description of a patient's follow-up over time. In MGUS patients, one form is repeatedly used to record paraprotein levels over time; date of evaluation, level of M protein and potential comorbidities are recorded. In WM, MM or primary AL amyloidosis patients, the form is created repeatedly for each treatment line. In general, each Treatment form describes – line of treatment, clinical symptoms of the disease, patient's characteristics at the time of treatment initiation (e.g. biochemistry), dates of treatment initiation and treatment termination, description of the treatment itself (drugs, dosage, whether a transplantation was carried out etc.), assessment of treatment effectiveness (treatment response), adverse effects/treatment toxicity and date of progression after treatment. One treatment switch can be recorded into the form; a switch should be mostly performed due to toxicity of the previous therapy. If the treatment is not successful, change of treatment regimen should always lead to the creation of another form for a new treatment line.
3. **Current status** – Description of a patient's condition at the last follow-up visit. In MGUS patients, progression to malignancy is evaluated; in WM patients, transformation to another disease and the presence of another malignancy is evaluated. The patient's condition as of the date of the last visit is recorded for all diagnoses; if the patient was alive, it is recorded whether treatment was administered; if the patient died, date and cause of death are recorded.

The content of forms is always adapted to the diagnosis of interest; for example, the diagnostic form for primary AL amyloidosis also includes the biopsy of amyloid. The diagnostic form for MM is also created for patients diagnosed with smouldering MM (SMM). The user then ticks off in the form whether the patient meets the criteria for SMM diagnosis. If MM and AL amyloidosis were diagnosed in a patient at the same time, the user should also fill in a diagnostic form for secondary MM-associated AL amyloidosis (apart from forms related to MM).

Online visualisation of data

With the growing amount of information available across the Internet, inevitably there is a growing need not only to process and to store data, but also to present data in a comprehensible manner. The latter issue is addressed by visual analytics, a dynamically developing field of data analysis and visualisation. Using advanced statistical methods, available data are searched for interesting and at first glance invisible features and relations, and these are subsequently presented to the target audience in the form of a summary report. The domain of data visualisation is characterised by a wide range of possibilities arising from modern technologies that are used in the development and subsequent implementation of web applications. Online visualisations provide a well-arranged view of required information, together with interactive elements and animated effects. A navigation panel for the specification of data selection and for setting the view itself (in the form of data tables, graphs or maps) usually forms an integral part of such visualisations. As part of the RMG project, an interactive browser has been implemented over data that had been collected via the CLADE-IS platform. With regard to the nature of published information, the visualisation is divided into a public part (Fig. 2 – available online at www.linkos.cz) and an authenticated part (Fig. 3A–C – available online at www.linkos.cz), revealing only relevant information to a given user, according to his/her level of access rights. Access to both types of visualisation is available at the RMG project website, namely in the section "Interactive browser" (https://rmg.healthregistry.org/index.php?pg=data). All outputs are automatically processed, solely based on data contained in the registry; validity of these data is controlled only by the skip logic and validation rules, both of which had been previously set in the system.

Technology

The RMG project website, including the interactive browser, has been developed using the following technologies:

- the LAMP platform for the implementation of web applications (Linux, Apache, MySQL, PHP),
- scripting languages JavaScript and R (support for the OpenCPU system) to process and to present data online,
- WKHTML component to convert the outputs into PDF format in the authenticated part of visualisations.

Our own library of visualisation components has been used to develop the individual graphical components. This library loads data in the JSON format (JavaScript Object Notation) and uses the d3.js component to display the graphical representation in the SVG format (Scalable Vector Graphics). Fonts, colours and other design attributes are defined via CSS 3 (Cascading Style Sheets). This technology has been used to compose pie charts and bar charts into the interactive browser. Furthermore, the authenticated version of data visualisation is supplemented with survival analysis, which is presented via Kaplan-Meier curves and their descriptive statistics. In order to calculate a survival curve, the application sends a query to the R package (described in the following section), which is made accessible by the OpenCPU system. The result, which is received in the form of JSON data, is subsequently processed and displayed online.

RMG package

In order to perform calculations related to survival analysis, the integrated development environment RStudio 1.0.136 was used to develop and to implement an R package which employs the non-parametric Kaplan-Meier

estimate to evaluate the probability of event-free survival in the following four time-to-event endpoints – overall survival (OS), time to progression (TTP), progression-free survival (PFS) and duration of response (DOR). Data processing for visualisation is performed by a script running on an OpenCPU server, which provides the HTTP API (Hypertext Transfer Protocol Application Programming Interface) to calculate survival analysis, receives input data via the POST method and returns results both in the form of source data for graph display and in the form of statistics. The package itself contains a single function entitled rmg(), which is called by a specific address at the OpenCPU server (implemented via a OpenCPU package in the R language) and which employs the POST method to receive an array of eight vectors (in the JSON format) representing the values of parameters needed to calculate the time-to-event endpoints in individual patients.

The rmg() function calculates the time-to-event endpoints in accordance with IMWG (International Myeloma Working Group) definitions [3]. In event-free patients, the interval length is calculated as of the date of the last follow-up and these patients are censored in the analysis. Patients with missing values of parameters needed for classification into groups (such as age or treatment line) or with negative values of time to event (as a result of incorrectly entered data) are excluded from the analysis.

The RMG package uses previously published packages survival, splines and RColorBrewer, which are publicly available via the CRAN (Comprehensive R Archive Network) repository. In order to calculate the Kaplan-Meier estimate, the following functions from the survival package are used: 1. the Surv() function, which creates an object of class "survival"; 2. the survfit() function, which considers this object as a dependent variable to define the survival model as a background for the calculation of survival curves. The graphs contain a single curve if survival is evaluated together for all patients in a given selection; or more curves if patients are classified into groups (such as two curves for two groups of patients, e.g. those with transplantation versus those without transplantation in a given line of treatment). If more curves are presented in a graph, the statistical significance of the difference between the curves is quantified via the p-value of log-rank test; this value is obtained from the survdiff() function. This procedure is repeated four times, separately for each time-to-event endpoint.

This process results in a graphical output for each time-to-event endpoint, in the form of one or more (differently coloured) curves drawn over a time interval from 0 to 96 months. Each graph is accompanied by a table, which describes the course of individual curves in more detail. Specifically, the table provides the overall number of patients, the overall number of events, median survival for a given time interval supplemented with 95% CI, and the probability of event-free survival in 12, 24, 36, 60 and 120 months, again supplemented with 95% CI.

Contents and work with browsers

From the user's point of view, work with the browser can be described as follows – 1. starting the application in a web browser; 2. in authenticated version, the user's identity is verified and predefined user rights are assigned; 3. data are selected using a set of filters; 4. visualisations are displayed. Updates of data to be presented are performed once a day, in nighttime hours of CET (Central European Time). Apart from several views of data on the webpage, the authenticated version also offers a full export of all available visualisations in the PDF format. Reports creation and their conversion to PDF format is done by a service on a dedicated server; due to high computational demands, requests on exports are lined up in a queue, which is subsequently processed by the service. Downloading the PDF export and putting it into the repository is the last step. The initial page shows the list of reports, with ten most recent ones being archived.

Figure 2 (online) demonstrates the web environment of a publicly available visualisation. In terms of contents, the publicly available browser describes only basic data on patients with monoclonal gammopathies (MGUS, WM separately for asymptomatic and symptomatic forms, MM, primary AL amyloidosis). Filters in publicly available visualisations involve the type of diagnosis and the selection of treatment centres (all centres/all Czech centres/all Slovak centres). Taking into account these filters, the browser displays:

1. Basic overview – numbers of newly diagnosed patients in individual years, by sex and in total; the total number of men and women over the entire monitored period. For the MM diagnosis, these results are supplemented with the total number of patients who had met the criteria for SMM at the time of diagnosis, and the total number of patients who developed MM-associated AL amyloidosis during the follow-up period. For the primary AL amyloidosis, the results are supplemented with the total number of patients with a given type of amyloidosis (either systemic or localised).
2. Follow-up – duration of follow-up from the date of selected diagnosis to either the last evaluation or the patient's death.
3. Age structure – age structure by sex and in total.
4. Paraproteins – type of heavy chain in the paraprotein.
5. Clinical stages – ISS (International Staging System) [4] and Durie Salmon staging system [5], only available for the MM diagnosis.
6. Risk score – in patients with MGUS or MM, the proportion of groups of patients with various numbers of risk factors according to the Mayo stratification system [6] is presented.

Unlike the publicly available visualisation, the authenticated version of the browser provides more detailed overviews focused on the MM diagnosis. Access to these visualisations has been granted to representatives of all treatment centres, each of whom is entitled to display data from his/her centre only, and to top representatives of CMG, who have extended rights and are entitled to display data from any individual

centre or overall data for either Czech or Slovak centres. Figures 3A–C (online) demonstrate the environment in this part of visualisations. After login, the user can set various filters for the analysis, i.e. selects criteria to be met by patients involved in the analysis. These criteria include:

1. Centre – a centre (or a group of centres) can be selected only in cases when the user is entitled to display data from more centres.
2. Year of treatment initiation – a scroll bar can be used to select the range of years; for example, all treatments initiated in the period 2007–2010. It is important to keep in mind that treatments initiated in 2006, for example, which carry on in the period 2007–2010, would not be involved in such analysis.
3. Age (treatment initiation) – those aged ≤ 70 years or those aged > 70 years.
4. Line of treatment – 1st, 2nd, 3rd, 4th, 5th and higher lines of treatment. Each patient is presented in a data set as many times as the number of treatment lines he/she initiated. The visualisation makes it possible to select more treatment lines.
5. Clinical trial – selection of treatments that were – or were not – administered as part of clinical trials.
6. Autologous stem cell transplantation (ASCT) – selection of induction regimens which were – or were not – followed by ASCT.
7. Treatment regimen – this option currently makes it possible to select treatments in which a new therapy has been used – bortezomib, thalidomide, lenalidomide, carfilzomib, pomalidomide, ixazomib or another therapy, which involves all therapies other than the above-mentioned ones. More therapies can be selected in the filter. If a treatment combination involved bortezomib and thalidomide, for example, this combination would be included in the analysis both in the situation where only bortezomib was selected and also in the situation where only thalidomide was selected. The selection of drugs is going to be expanded together with the development of new drugs.

By default, all parameters are set to "total", indicating that the user selects all data from the centre(s) of choice. The user can also tick off the "generate PDF report" option, if he/she wants the results to be exported to a PDF file; after clicking on the "display" button, the user is redirected on the main visualisation page. The upper part of this page shows the list of ten most recent reports – if the item "state" is marked as "finished", the report is ready for download. The blue box on the main page sums up all previously set filters for patient selection as well as the overall number of patients and treatment lines involved in the evaluation. An interactive list of items is displayed below the summary of predefined filters; results are displayed after clicking on a specific item.

The items are divided into five sections:

1. **Summary lists** – this section involves the total numbers of newly diagnosed patients in individual years, numbers of newly initiated treatments in years depending on the line of treatment, treatment regimen and in total; and the year of last update of records in the registry for patients who are alive.
2. **Basic overview** – patients – basic description of patients diagnosed with MM. Visualisations are available for sex, age, duration of follow-up, ISS and performance status. Although more than one treatment line for the same patient might meet inclusion criteria, any given patient is only involved once for the evaluation in this section.
3. **Basic overview** – treatment lines – basic description of patients at the time of treatment initiation with a given treatment line; this means that the patient is involved in the evaluation as many times as the number of his/her treatment lines which met the inclusion criteria. The same characteristics are evaluated as in Section 2; furthermore, the evaluation also involves the treatment line, treatment regimen, whether ASCT was performed and whether clinical trial was involved.
4. **Final treatment response and toxicity** – this section evaluates the success of treatment by the means of treatment response (defined by the current IMWG criteria [3]) and treatment toxicity (for example, anaemia or infections).
5. **Patient survival Kaplan-Meier curves** are used to present the results of treatment intervals OS, TTP, PFS and DOR. The first part displays the survival for all treatment lines that meet inclusion criteria. In other parts, treatment lines are classified to multiple curves, according to patient characteristics (for example, ISS stage I–III, cytogenetical examination positive/negative).

All outputs available in the web environment are also included in the PDF report, where each output is presented on a separate slide. By clicking on "log out", the user leaves the visualisation and returns to the RMG project website.

Conclusion

The RMG was established in 2007. Data originally stored in a former database system (based on a modified version of the TrialDB system) were converted to a new CLADE-IS system in 2017. Revision of form structure and contents was performed together with database conversion. New validation criteria were set in order to minimise the error rate in data. Data are recorded into the system by data managers, who are supervised by the registry coordinator. Data from the registry are evaluated after the approval by representatives of participating centres. A basic overview of data on individual diagnoses from all Czech or Slovak centres together is publicly available on the registry website. A detailed online visualisation of data on MM patients from selected centres, which can be also exported to a PDF file, is available for representatives of individual centres as well as for top representatives of CMG.

RMG represents an international database designed for the collection of data on patients with monoclonal gammopathies. Nineteen Czech centres and four Slovak centres currently contribute to the registry. The registry already contains data on more than 8,000 patients with monoclonal gammopathies. RMG is therefore one of the largest registries with

regular monitoring in Europe. Data from RMG are utilised in the preparation of national guidelines for treatment of monoclonal gammopathies, serve as a basis for negotiations with the State Institute for Drug Control (SUKL), and are also used for subsequent negotiations on reimbursements of new drugs with healthcare payers. The RMG represents a data basis that makes it possible to monitor the disease course in patients with monoclonal gammopathies on the population level.

Conflicts of interest

D. S., B. P. and P. N. declare an employment relationship with the Institute of Biostatistics and Analyses Ltd. – the owner and operator of the CLADE-IS system. R. H. has a consultant or advisory relationship and received honoraria from Amgen, Bristol-Myers Squibb, Takeda, Celgene and Janssen-Cilag; conducted a clinical research project(s) funded by Takeda, Novartis, Amgen and Janssen-Cilag. V. M. consulted for Amgen, Bristol-Myers Squibb, Celgene, Janssen-Cilag and Takeda; received grant support from The Binding Site, honoraria from Amgen, Bristol-Myers Squibb, Celgene and Janssen-Cilag, and has been involved in advisory boards for Amgen, Bristol-Myers Squibb, Celgene, Janssen-Cilag and Takeda. Other authors declare they have no conflicts of interest concerning drugs and other medicinal products used in the study.

Střet zájmů

D. S., B. P. a P. N. deklarují pracovněprávní vztah ke společnosti Institut biostatistiky a analýz s.r.o. – vlastníkovi a provozovateli systému CLADE-IS. R. H. je konzultantem nebo poradcem u společností Amgen, Bristol-Myers Squibb, Takeda, Celgene a Janssen-Cilag, od kterých obdržel honoráře; provádí klinické výzkumné projekty financované společnostmi Takeda, Novartis, Amgen a Janssen-Cilag. V. M. je konzultantem u společností Amgen, Bristol-Myers Squibb, Celgene, Janssen-Cilag a Takeda; přijal grantovou podporu od The Binding Site a honoráře od Amgen, Bristol-Myers Squibb, Celgene and Janssen-Cilag; je členem poradních výborů u společností Amgen, Bristol-Myers Squibb, Celgene, Janssen-Cilag a Takeda. Ostatní autoři deklarují, že v souvislosti s předmětem studie ve smyslu léků a jiných léčivých přípravků nemají střet zájmů.

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Asymptomatic and Treatment-requiring Multiple Myeloma – Data from the Czech Registry of Monoclonal Gammopathies

Asymptomatický a léčbu vyžadující mnohočetný myelom – data z českého Registru monoklonálních gamapatií

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Summary

Background: Monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM) are premalignant stages of multiple myeloma (MM). MM is a malignancy of plasma cells, which is associated with a median overall survival of 5 to 7 years. MM accounts for approximately 10% of hematological malignancies. ***Patients and Methods:*** Descriptive analysis of data from 19 Czech centres collected in the Registry of Monoclonal Gammopathies (RMG) was performed. ***Results:*** Over the last 10 years of prospective collection of data, together with retrospectively recorded data on patients diagnosed before the registry establishment, data on 7,467 patients with either asymptomatic or symptomatic form of MM have been gathered. Validation criteria for the analysis were met by 2,506 MGUS patients, 400 SMM patients and 4,738 MM patients. The median duration of follow-up was 4.3 years in MGUS patients and 2.4 years in SMM patients. The overall risk of progression from MGUS to malignancy was 1.7% per year. The risk of progression from SMM to MM was highest in the 1st years after diagnosis: overall, this risk was 16.6% per year. The median duration of follow-up was 2.8 years in MM patients. The median overall survival from the diagnosis was 5.7 years. The median OS from treatment initiation/progression-free survival decreased from 60.5/21.0 months in the 1st line therapy to 34.3/12.4 months in the 2nd line therapy, 22.6/8.9 months in the 3rd line therapy and 13.8/5.8 months in the 4th or higher line therapies. Thanks to the availability of novel drugs for MM treatment in the Czech Republic, treatment strategies have changed dramatically over the last decade. ***Conclusion:*** RMG is a registry designated for the collection of data on diagnosis, treatment, treatment results and survival of patients with monoclonal gammopathies in the long-term follow-up. RMG is a valuable source of data from real clinical practice.

Key words

registries – monoclonal gammopathy of undetermined significance – smouldering multiple myeloma – multiple myeloma – progression – treatment – survival

This study was supported by the Ministry of Health of the Czech Republic (project identifier: DRO-FNOs/2017).

Práce byla podpořena projektem Ministerstva zdravotnictví České republiky (DRO-FNOs/2017).

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 30. 5. 2017
Accepted/Přijato: 22. 6. 2017

doi: 10.14735/amko20172S51

Souhrn

Východiska: Monoklonální gamapatie nejasného významu (monoclonal gammopathy of undetermined significance – MGUS) a doutnající mnohočetný myelom (smouldering multiple myeloma – SMM) jsou prekancerózními stadii mnohočetného myelomu (MM). MM je malignita plazmatických buněk s mediánem přežití od 5 do 7 let. MM tvoří zhruba 10 % diagnóz v oblasti hematoonkologie. ***Pacienti a metody:*** Na datech z 19 českých center zadaných v Registru monoklonálních gamapatií (Registry of Monoclonal Gammopathies – RMG) byla provedena popisná analýza. ***Výsledky:*** Za posledních 10 let sběru dat, spolu s retrospektivně zadanými daty pacientů diagnostikovaných před založením registru, registr disponuje daty o 7 467 pacientech se asymptomatickou nebo symptomatickou formou MM. Validační kritéria pro analýzu splňovalo 2 506 pacientů s MGUS, 400 pacientů s SMM a 4 378 pacientů s MM. Medián délky sledování pacientů byl 4,3 roku u MGUS a 2,4 roku u SMM. Celkové roční riziko progrese z MGUS do maligního onemocnění bylo 1,7 %. Riziko progrese z SMM do MM bylo nejvyšší první roky po diagnóze; za celou dobu sledování bylo riziko progrese 16,6 % každý rok. Medián délky sledování od diagnózy MM byl 2,8 roku. Medián celkového přežití (overall survival – OS) od diagnózy byl 5,7 roku. Medián OS od zahájení léčby/doby bez progrese klesl z 60,5/21,0 měsíce u 1. linie léčby na 34,3/12,4 měsíce u 2. linie, 22,6/8,9 měsíce u 3. linie a 13,8/5,8 měsíce u 4. nebo vyšší linie léčby. Díky dostupnosti nových léků pro léčbu MM v České republice došlo v posledním desetiletí k dramatickým změnám v léčebných postupech. ***Závěr:*** RMG je registr určený pro sběr klinických dat týkajících se diagnózy, léčby, jejích výsledků a přežití pacientů s monoklonálními gamapatiemi. RMG je cenným zdrojem dat z reálné klinické praxe.

Klíčová slova

registry – monoklonální gamapatie nejasného významu – doutnající mnohočetný myelom – mnohočetný myelom – progrese – léčba – přežití

Introduction

Monoclonal gammopathy of undetermined significance (MGUS), smouldering multiple myeloma (SMM; previously called asymptomatic multiple myeloma) and multiple myeloma (MM) are considered to be plasma cell (PC) dyscrasias. Their diagnosis is determined according to the current IMWG (International Myeloma Working Group) criteria [1]. MGUS represents a benign condition characterised by the presence of M-protein (monoclonal protein) in a concentration lower than 30 g/L, by less than 10% of clonal PCs in the bone marrow, and by the absence of myeloma-defining events. The prevalence of MGUS is approximately 3% among the general population aged over 50 [2]. SMM is defined as the presence of M-protein in a concentration of 30 g/L or higher and/or 10–60% bone marrow PC infiltration with no evidence of end-organ damage. MGUS and SMM are considered to be precancerous conditions of MM. MGUS is associated with a 1% risk of progression to MM or related PC disorders per year; patients are at risk of progression even after 25 years of follow-up [3]. IgM (immunoglobulin – Ig) MGUS usually evolves into Waldenström macroglobulinaemia, whereas IgA or IgG variants progress to MM, primary amyloidosis or related PC disorders [4]. On the other hand, MGUS is assumed to precede MM in almost all cases [5]. According to one study, the risk of progression from SMM to MM was 10% per year in the first 5 years, 3% per year in the next 5 years and 1% per year in the last 10 years of a 20-year follow-up [6]. A population-based Scandinavian study revealed that SMM accounts for about 14% of patients with MM [7]. Regular monitoring is the standard of care in MGUS and in SMM. Prognostic models have been proposed to discriminate between patients with a low-risk and a high-risk of progression [8]. An early intervention in high-risk SMM patients has been shown to delay the progression to active MM and to increase the overall survival (OS) [9]. The current IMWG criteria thus reclassified the high-risk SMM patients as patients with MM, which led to the availability of early treatment for these patients [1].

MM is a malignancy of PCs accounting for 1% of all cancers and approximately 10% of all hematological malignancies [10]. MM diagnosis is associated with the presence of clinical symptoms usually described by the acronym CRAB (C – hypercalcaemia in serum, R – renal insufficiency, A – anaemia, B – lytic bone lesions). The median survival is approximately 5 to 7 years, but there are dramatic variations in the survival depending on the patient's characteristics (e.g. age, comorbidities), tumour burden (e.g. stage) and biological characteristics of the disease (e.g. cytogenetic abnormalities) [11]. In 2005, a simple International Staging System (ISS) based on two parameters describing the tumour burden (serum β2 microglubin and serum albumin) was developed [12]. In 2015, the ISS was revised and combined with parameters of disease biology (presence of chromosomal abnormalities and elevation of lactate dehydrogenase) [13]. From the chromosomal abnormalities, deletion on 17p and/or translocation t(4;14) and/or t(14;16) were considered as high-risk factors. Such a stratification systems helps physicians choose the optimal treatment strategy. With the novel drugs, the treatment of MM has advanced dramatically in the last decade [14]. The use of thalidomide, lenalidomide and bortezomib improved the patients' survival rates [15–17]. More recently, pomalidomide, carfilzomib, ixazomib and daratumumab have been used in the treatment of MM in the Czech Republic. The novel drugs are usually combined with chemotherapy and/or corticosteroids. The critical point in the choice of therapy is the patient's eligibility for autologous stem cell transplantation (ASCT); factors such as age, performance status and comorbidities influence the eligibility for ASCT. The upper age limit for ASCT eligibility was increased to 70 years from previous 65 years in the Czech Republic; this is in contrast to the United States, for example, where the age limit is 75 years [14]. After the 1st line therapy, relapse usually occurs and a next-line therapy of the disease is required. The periods between

Tab. 1. Basic characteristics of MGUS, SMM and MM patients.

Characteristics at diagnosis	MGUS (n = 2,506)	SMM (n = 400)	MM (n = 4,738)
sex	n = 2,506	n = 400	n = 4,738
women	1,363 (54.4)	219 (54.8)	2,281 (48.1)
men	1,143 (45.6)	181 (45.3)	2,457 (51.9)
age at diagnosis (years)	n = 2,506	n = 400	n = 4,738
≤ 50	430 (17.2)	49 (12.3)	446 (9.4)
51–60	606 (24.2)	100 (25.0)	1,103 (23.3)
61–70	767 (30.6)	128 (32.0)	1,642 (34.7)
71–80	574 (22.9)	104 (26.0)	1,248 (26.3)
> 80	129 (5.1)	19 (4.8)	299 (6.3)
median (min.–max.)	63 (22–93)	64 (28–88)	65 (18–92)
ECOG	n = 2,405	n = 374	n = 4,438
0	1,403 (58.3)	166 (44.4)	866 (19.5)
1	935 (38.9)	193 (51.6)	2,395 (54.0)
2	59 (2.5)	13 (3.5)	792 (17.8)
≥ 3	8 (0.3)	2 (0.5)	385 (8.7)
ISS	not available	n = 380	n = 4,302
stage I	–	297 (78.2)	1,563 (36.3)
stage II	–	70 (18.4)	1,383 (32.1)
stage III	–	13 (3.4)	1,356 (31.5)
M-protein type	n = 2,497	n = 400	n = 4,702
IgG	1,740 (69.7)	276 (69.0)	2,823 (60.0)
IgA	297 (11.9)	103 (25.8)	974 (20.7)
LC only	25 (1.0)	8 (2.0)	689 (14.7)
IgM	357 (14.3)	5 (1.3)	34 (0.7)
biclonal	74 (3.0)	5 (1.3)	57 (1.2)
non-secretory	2 (0.1)	1 (0.3)	90 (1.9)
other (IgD, triclonal)	2 (0.1)	2 (0.6)	35 (0.7)
Progression status	n = 2,506	n = 400	not available
no progression	2,275 (90.8)	172 (43.0)	–
MM	173 (6.9)	228 (57.0)	–
WM	20 (0.8)	–	–
lymphoma	18 (0.7)	–	–
other	20 (0.9)	–	–
Follow-up (years)	n = 2,506	n = 400	n = 4,738
median (min.–max.)	4.3 (0.0–34.8)	2.4 (0.3–20.6)	2.8 (0.0–32.1)
Death	n = 2,506	n = 400	n = 4,738
no	2,219 (88.5)	370 (92.5)	2,517 (53.1)
yes	287 (11.5)	30 (7.5)	2,221 (46.9)

Data are presented as n (%) for categorical variables and as median values (min.–max.) for continuous variables.
MGUS – monoclonal gammopathy of undetermined significance, SMM – smouldering multiple myeloma, MM – multiple myeloma, ECOG – Eastern Cooperative Oncology Group, ISS – international staging system, M-protein – monoclonal protein, Ig – immunoglobulin, WM – Waldenström macroglobulinaemia

relapses and remissions are usually increasingly shorter [6]. If the patient is eligible, ASCT can be repeated at the time of relapse. Treatment history is considered in the choice of therapy in higher-line therapies. Despite improvements in treatment strategies, MM remains to be an incurable disease; nevertheless, there is already a small number of patients who have been in a complete remission for more than 10 years [18,19].

Patients and methods

Data from Czech and Slovak centres have been collected in the Registry of Monoclonal Gammopathies (RMG) since 2007, the year of establishment of this registry. Only data from Czech centres were used in the analysis. Characteristics of patients with asymptomatic (MGUS, SMM) and symptomatic MM were analysed. Patients with asymptomatic MM have not received any therapy until the progression to a symptomatic disease. The diagnosis, treatment response and time to event endpoints were assessed according to the current IMWG (International Myeloma Working Group) criteria [20,21]. All patients signed the informed consent form, which had been approved by ethical committees of the respective hospitals.

Our analysis had four main objectives: 1. to describe patients' characteristics at the time of MGUS, SMM or MM diagnosis; 2. to evaluate the risk of progression from an asymptomatic MM to a symptomatic MM; 3. to describe the OS from the time of MM diagnosis and 4. to describe treatment of MM and its success in terms of OS and progression-free survival (PFS) in individual lines of therapy. All analyses were descriptive; no hypotheses were tested. Data were described by absolute and relative frequencies for categorical variables and by median values supplemented with range (min.–max.) for continuous variables. Treatment intervals were plotted using the Kaplan-Meier (K-M) methodology. The K-M estimates were completed by the Greenwood confidence interval (CI). Death was censored in the evaluation of time from an asymptomatic MM to disease progression. The annual risk of progression was evaluated as the ratio of the total number of patients with disease progression to the sum of person-years of follow-up in the cohort of MGUS or SMM patients. The analysis was performed using the SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) and the software R, version 3.3.0 (www.r-project.org).

Results

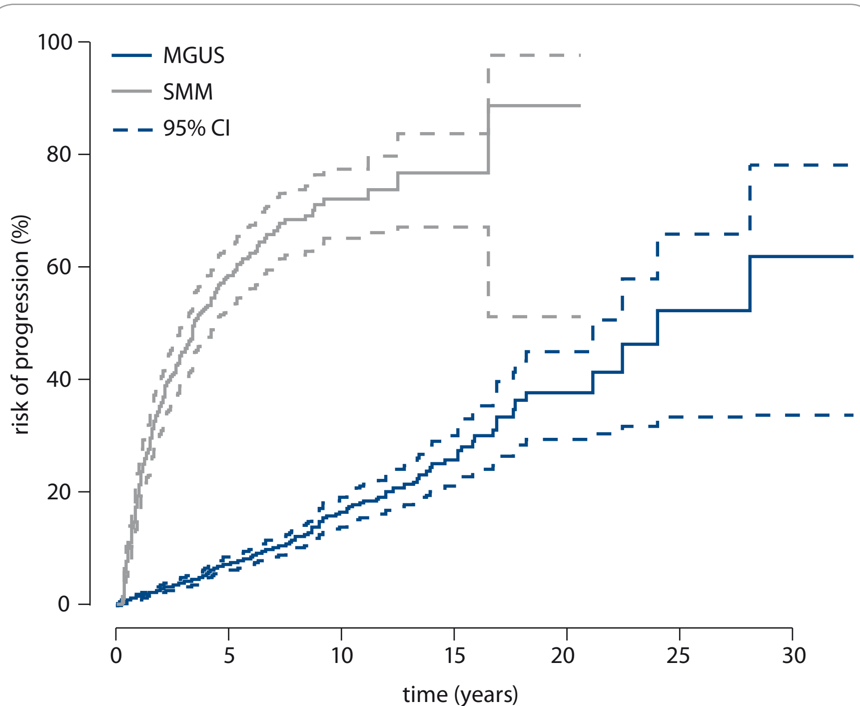
A total of 7,467 patients were diagnosed with monoclonal gammopathies in 19 Czech centres up to March 2017 (prospective collection of data was initiated in 2007). At that time, RMG contained data on 2,759 patients diagnosed with MGUS and 4,888 patients diagnosed with MM; MGUS diagnosis was followed by MM diagnosis in 180 (2.4%) patients. Because RMG does not include any diagnostic form for SMM, SMM patients were selected as a subset of MM patients who met the current IMWG criteria for SMM diagnosis [1]. Moreover, at least a 3-month follow-up without progression was required for SMM patients. From the total number of 4,888 patients diagnosed with MM, SMM criteria were met in 400 (8.2%) patients. Validation criteria for a more detailed analysis were considered and patients who did not meet those criteria were excluded. Validation criteria were defined as the availability of records on the patient's age, follow-up since diagnosis and the progression status (in case of MGUS or SMM). Finally, records on 2,506 MGUS (90.8% from a total of 2,759), 400 SMM (100% from a total of 400) and 4,738 MM (96.9% from a total of 4,888) patients were evaluated in the analysis.

Table 1 shows the basic characteristics of patients diagnosed with MGUS, SMM and MM. Data on patients diagnosed before 2007 (the year of registry establishment) were collected retrospectively. From the total number of patients with a given diagnosis, 600 (23.9%) MGUS, 91 (22.8%) SMM and 777 (16.4%) MM patients were diagnosed before 2007. On average, there were 190, 31 and 393 newly diagnosed patients per year (evaluated since 2007 to 2016) with MGUS, SMM and MM diagnoses, resp. A predominance of women in the group of patients with asymptomatic MM was observed (54.4% in MGUS, 54.8% in SMM) in contrast to MM, which seemed to be slightly more common in men (51.9%). The median age at diagnosis was 63, 64 and 65 years for MGUS, SMM and MM, resp. The diagnosis was established before the age of 50 in 430 (17.2%) MGUS patients and in 46 (12.3%) SMM patients. Although MM is generally considered as a disease associated with old age, 2.1% (99 patients) were diagnosed before the age of 40. Approximately one third of patients were older than 70 years at the time of diagnosis. The majority of MGUS and SMM patients had the ECOG grade (i.e. performance status developed by Eastern Cooperative Oncology Group) ranging between 0 and 1, which corresponds to the range from fully active patients to patients restricted in physically strenuous activities only [22]. ECOG of grade 3 or higher was recorded in 385 (8.7% from 4,438 patients with ECOG available) MM patients; these grades refer to patients with a limited self-care. There were only limited numbers of patients in grade 3 or higher in MGUS and SMM groups. ISS stages were rather uniformly distributed in MM patients (36%, 32% and 32% in stages I, II and III, resp.). The majority of SMM patients (78.2%) had the ISS stage I. Patients with MGUS are usually stratified by the Mayo risk stratification system, which is based on M-protein quantity (high-risk: ≥ 15 g/L), M-protein type (high-risk: non-IgG) and abnormal free light chain ratio (high-risk: < 0.26 or > 1.65) [8]. All three risk factors were recorded in 2,104 (84.0%) patients. MGUS patients were classified into four risk groups with none, one, two and all three risk factors according to the Mayo model; these groups involved 804 (38.2%), 862 (41.0%), 411 (19.5%) and 27 (1.3%) patients, resp. (data not shown). The type of M-protein was available in almost all patients: 2,497 (99.6%) in MGUS, 400 (100%) in SMM and 4,702 (99.2%) in MM. IgG was the most abundant type of M-protein, with a frequency of 70% in MGUS and SMM and 60% in MM. IgM was the second most abundant M-protein in MGUS (n = 357; 14.3%); by contrast, IgM was observed in

less than 2% of patients in SMM and MM groups. IgA M-protein was observed in 11.9%, 25.8% and 20.7% in MGUS, SMM and MM, resp. Light-chain only (LC only) MM or non-secretory MM were more frequently present at the time of MM diagnosis (LC only in 14.7%, non-secretory in 1.9% patients) when compared to MGUS and SMM groups (LC only in ≤ 2%, non--secretory in ≤ 0.3% patients).

The serum level of M-protein was measured repeatedly in the vast majority of MGUS patients (n = 2,438; 97.3%). After 1 year of MGUS diagnosis, the median number of patients' visits to their hematologist was two visits per year. Overall, the median number of check-ups in patients' follow-up was 7, ranging from 1 to 51 (data not shown). Data on progression status and patients' follow-up in MGUS and SMM diagnoses are presented in Tab. 1. The median follow-up was 4.3 years in the cohort of MGUS patients. Progression in MGUS occurred in 231 (9.2%) patients. From the overall number of 231 patients, MGUS developed to MM (n = 173; 74.9%), Waldenström macroglobulinaemia (n = 20; 8.7%), lymphoma (n = 18; 7.8%) or another disorder (n = 20; 8.7%). The overall risk of progression per year was 1.7%. The probability of progression (number of patients at risk) was 7.5% (n = 1,100) at 5 years, 16.7% (n = 329) at 10 years and 25.8% (n = 97) at 15 years (Fig. 1). Only 25 patients were followed for more than 20 years. Death occurred before progression in 287 (11.5%) patients. In SMM patients, only data at the time of diagnosis were collected in RMG. The median duration of follow-up from the SMM diagnosis to the progression to MM or the last date of evaluation was 2.4 years. SMM developed to MM in 228 (57.0%) patients. The overall risk of progression per year was 16.6%. The probability of progression (number of patients at risk) was 20.8% (n = 299) at 1 year, 35.9% (n = 223) at 2 years, 58.3% (n = 96) at 5 years and 71.7% (n = 23) at 10 years (Fig. 1). Only four patients were alive and without progression 15 years after SMM diagnosis. From the cohort of SMM patients, 30 (7.5%) patients died before progression.

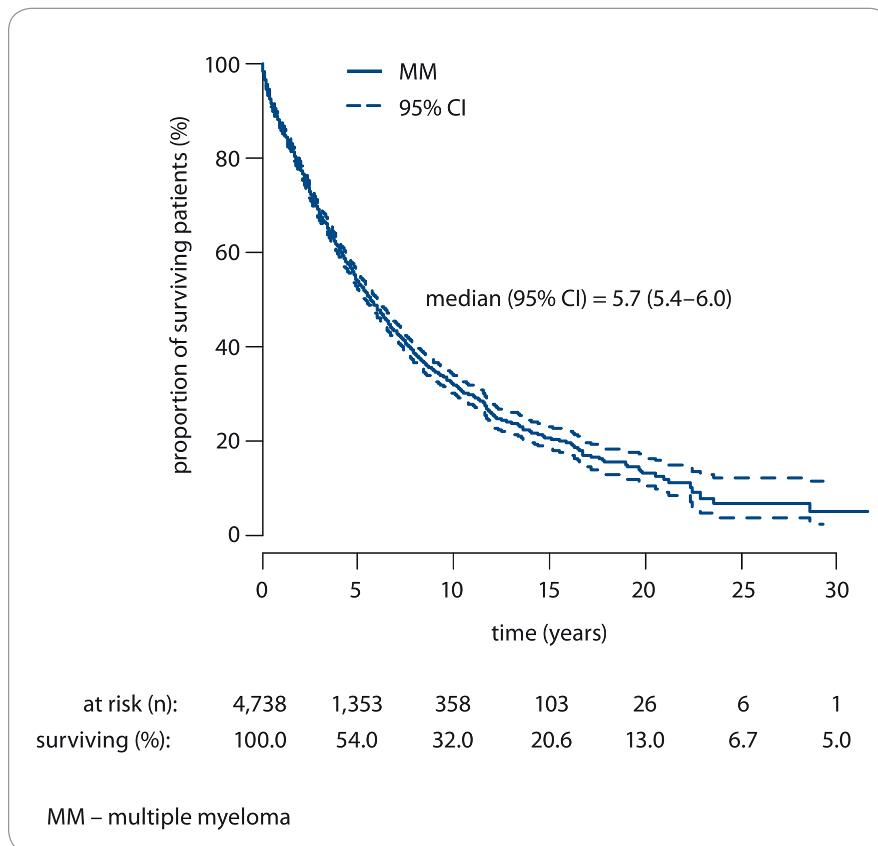
The median follow-up from MM diagnosis to a patient's death or the last date



	MGUS	SMM
total n	2,506	400
progression (n)	231	228
median (95% CI)	24.0 (18.5–29.5)	3.4 (2.7–4.1)
at risk (years) (n)		
1	2,137	299
2	1,840	223
5	1,100	96
10	329	23
15	97	4
20	25	1
25	7	0
risk of progression (years)	**% (95% CI)**	**% (95% CI)**
1	1.6 (1.1–2.1)	20.8 (17.1–25.2)
2	3.0 (2.4–3.8)	35.9 (31.3–41.0)
5	7.5 (6.3–8.8)	58.3 (52.9–63.8)
10	16.7 (14.3–19.5)	71.7 (65.5–77.7)
15	25.8 (21.6–30.6)	76.5 (68.1–84.1)
20	37.9 (30.7–46.2)	88.3 (66.7–98.5)
25	52.4 (37.6–68.8)	–

*Event for MGUS patients defined as progression to MM or related disorders; event for SMM patients defined as progression to MM.
MGUS – monoclonal gammopathy of undetermined signifikance,
SMM – smouldering multiple myeloma

Fig. 1. Time to progression* from MGUS and SMM diagnosis.



Graph 1. Overall survival from MM diagnosis.

of evaluation was 2.8 years. Almost half of the cohort of MM patients have already died (n = 2,221; 46.9%). The probability of survival (number of patients at risk) was 54.0% (n = 1,353) at 5 years, 32.0% (n = 358) at 10 years and 20.6% (n = 103) at 15 years (Graph 1). The median OS from the time of diagnosis was 5.7 years (95% CI 5.4–6.0 years).

From the total number of MM patients who met the validation criteria (n = 4,738), 4,375 (92.3%) patients had at least one line of therapy recorded. Only patients with initiated treatment were evaluated in this section. In sum, the total number of lines of therapy for the treated patients was 10,255. In terms of patient numbers, 4,375 (100.0%), 2,452 (56.0%), 1,503 (34.4%) and 872 (19.9%) patients initiated the 1st line, 2nd line, 3rd line and 4th or higher-line therapy, resp. The median of total number of lines of therapy per patient was 2, ranging from 1 to 15. The period between two lines of therapy was generally getting shorter with the increasing number of lines of therapy. The median time to the next line of therapy was 19, 13, 10 and 8 months in the 1st line, 2nd line, 3rd line and 4th line therapy, resp. From the 10,255 lines of therapy, 1,094 (10.7%) therapies were conducted within clinical trials. ASCT was performed in 1,640 (37.5%) patients. If the patient was eligible, ASCT was most frequently performed in the 1st line therapy (n = 1,488; 34.0%). Nevertheless, ASCT was performed in 332 (13.5%), 142 (9.4%) and 145 (16.6%) patients in the 2nd line, 3rd line and 4th or higher-line therapy, resp. ASCT was performed more than once in a patient's follow-up in 394 (9.0%) patients.

The OS from treatment initiation and PFS decreases with higher lines of therapy (Fig. 2). The median OS was 60.5 months (95% CI 57.3–63.7 months) in the 1st line therapy, 34.3 months (95% CI 31.9–36.7 months) in the 2nd line therapy, 22.6 months (95% CI 20.5–24.7 months) in the 3rd line therapy and 13.8 months (95% CI 12.6–14.9 months) in the 4th or higher-line therapy. The median PFS decreased from 21.0 months (95% CI 20.2–21.8 months) in the 1st line therapy to 12.4 months (95% CI 11.8–13.0 months) in the 2nd line therapy, 8.9 months (95% CI 8.4–9.5 months) in the 3rd line therapy and 5.8 months (95% CI 5.3–6.2 months) in the 4th or higher-line therapy. At 10 years after the initiation of the 1st line therapy, 84 patients were alive and without progression.

As regards the evaluation of treatment regimens, only the induction therapy (regardless of switch) was assessed. Treatment strategies up to 2010 and thereafter were compared. After 2010, bortezomib was more frequently administered in the 1st line therapy (60% increase when compared to therapies up to 2010) and less frequently in 2nd or higher-line therapies (15% decrease in the 2nd line therapy). On the other hand, an almost 30% increase was observed for lenalidomide in the 2nd line therapy; for the same drug, there was a 11.4% decrease in the 4th or higher-line therapies; pomalidomide and carfilzomib were more frequently administered in higher-line therapies. Table 2 describes treatment regimens in the 1st line (n = 2,352), 2nd line (n = 939), 3rd line (n = 426) and 4th or higher-line therapies (n = 277) initiated after 2010. As specified in the legend, drugs were classified into four categories: proteasome inhibitor (PI); immunomodulatory drug (IMiD); chemotherapy; or corticosteroids. In about half of the 1st line therapies (n = 1,198; 50.9%), PI was combined with chemotherapy and corticosteroids. From the PIs, bortezomib was the most frequently chosen drug in the 1st line therapy (n = 1,728; 73.5%). Bortezomib was combined with chemotherapy and corticosteroids in 1,168 (49.7%) patients in the 1st line therapy. Moreover, there were 33 (1.4%) 1st line therapies with carfilzomib conducted within clinical trials. From the IMiDs, thalidomide was used in 732 (31.1%) 1st line therapies; thalidomide was combined with chemotherapy and corticosteroids in 402 (17.1%) patients, with bortezomib and corticosteroids in 237 (10.1%) patients in the 1st line therapy. In the 2nd line therapy, 328 (34.9%) patients were treated with IMiD in combination with cortico-

steroids. From the IMiDs, lenalidomide was used in 428 (45.6%) patients in the 2nd line therapy; lenalidomide was combined with corticosteroids in 312 (33.2%) patients, and the same drug was combined with chemotherapy and corticosteroids in 55 (5.9%) patients. Bortezomib was administered to 282 (30.0%) patients in the 2nd line therapy. Bortezomib was combined with corticosteroids and/or chemotherapy in 209 (22.3%) patients. Thalidomide was the third most frequently used drug in the 2nd line therapy (n = 175; 18.6%). Thalidomide was combined with chemotherapy and corticosteroids in 106 (11.3%) patients in the 2nd line therapy. Thalidomide was used in combination with bortezomib in 44 (4.7%) patients in their 2nd line therapy. The majority of ixazomib therapies were administered in the 2nd line therapy (n = 36; 3.8%). A higher proportion of therapies without novel drugs was observed with the increasing number of lines of therapy. Chemotherapy and/or corticosteroids were used in 4.0%, 6.7%, 16.4% and 27.8% cases of 1st line, 2nd line, 3rd line and 4th or higher-line therapy, resp. On the other hand, we observed an increasing trend in the usage of pomalidomide and carfilzomib in higher-line therapies. From the 277 4th or higher-line therapies, 39 (14.1%) therapies included pomalidomide, and 19 (6.9%) therapies included carfilzomib.

Discussion

RMG contains data on 7,467 patients with either asymptomatic or symptomatic MM. Data for patients with SMM diagnosis are collected within a diagnostic form for MM; subsequent analyses of MM then include patients with SMM. Patients with SMM were selected using new IMWG criteria from 2014; therefore, previously high-risk SMM patients were reclassified as patients with MM [1].

Because MGUS is an asymptomatic disease, it is usually detected during a routine physician examination. Despite the assumption that MGUS precede MM in all cases, a history of MGUS was observed only in 3.7% patients in the MM cohort; rather than reflecting the prevalence, this proportion reflects an early detection of the disease. MGUS is much more common than MM and the majority of MGUS patients will never develop an active MM or a related disorder. The risk of progression from MGUS to MM or a related malignancy is stable in time, in contrast to the risk of progression from

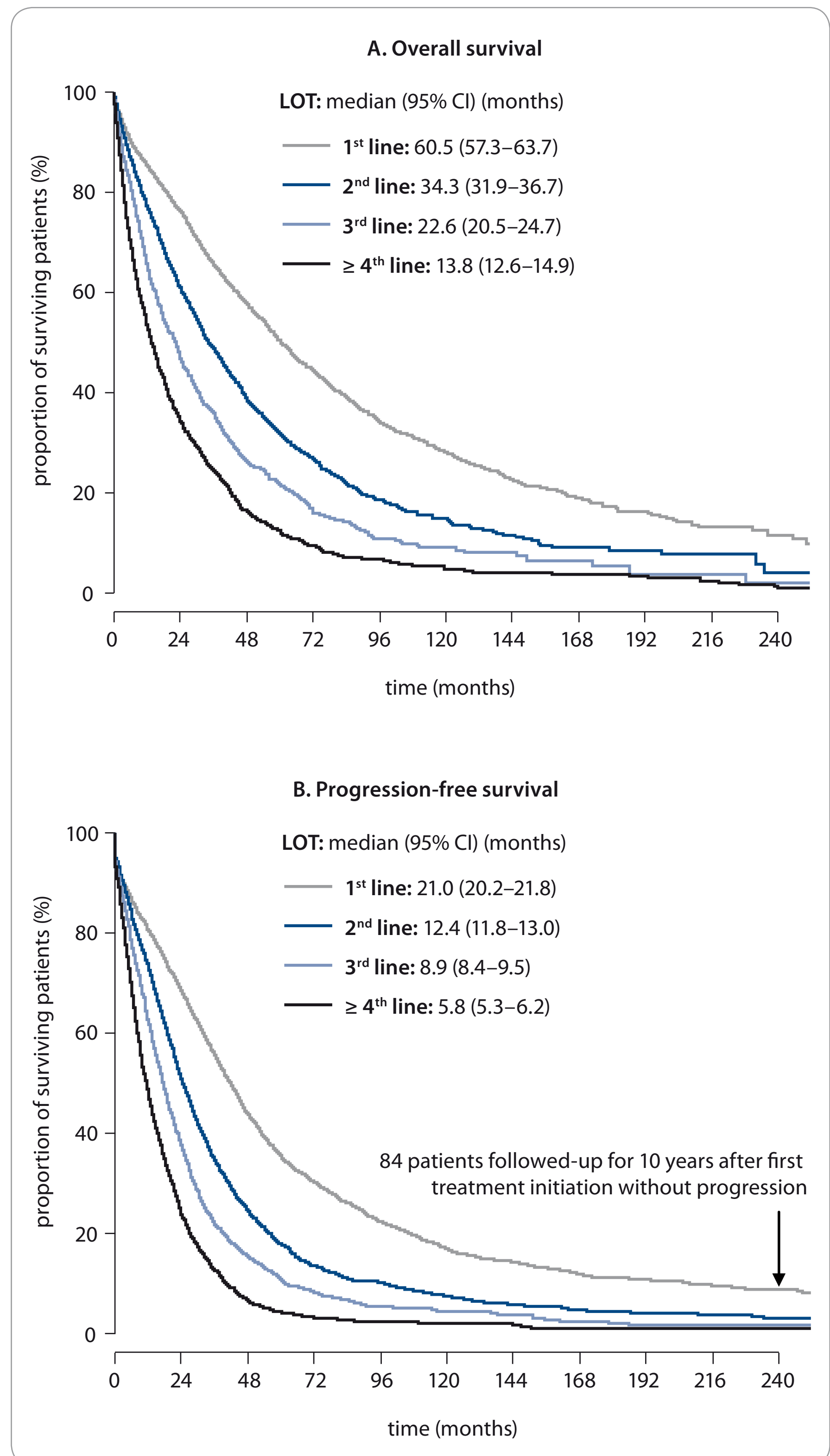


Fig. 2. Overall survival from treatment initiation (A) and progression-free survival (B) by line of therapy (LOT).

Tab. 2. Drugs used in MM therapy after 2010.

Treatment regimen[1]	1st line (n = 2,352), %	2nd line (n = 939), %	3rd line (n = 426), %	≥ 4th line (n = 277), %	Total n
Drug combinations					
PI + chemo + cort.	50.9	12.7	6.6	3.6	1,355
IMiD + chemo + cort.	17.4	17.1	13.4	12.3	661
chemo + cort.	3.2	4.8	10.8	17.7	216
IMiD + cort.	2.0	34.9	34.3	25.6	593
PI + cort.	7.7	10.6	10.8	12.6	361
PI + IMiD + cort.	10.1	7.8	8.2	7.2	366
IMiD	1.1	3.4	1.9	0.4	66
PI	2.7	2.0	2.1	0.7	94
cort.	0.6	1.4	4.5	6.1	64
chemo	0.1	0.5	1.2	4.0	24
PI + IMiD + chemo + cort.	1.6	0.6	1.4	1.8	55
PI + IMiD	1.2	0.6	1.2	0.4	40
other (low N)[2]	0.8	1.2	0.5	1.8	36
other (not specified)	0.5	2.2	3.3	5.8	63
Drug[3]					
bortezomib (PI)	73.5	30.0	26.3	18.8	2,174
thalidomide (IMiD)	31.1	18.6	22.1	18.8	1,053
lenalidomide (IMiD)	2.3	45.6	33.8	15.2	669
carfilzomib (PI)	1.4	1.6	3.1	6.9	80
pomalidomide (IMiD)	0.0	0.7	4.7	14.1	66
ixazomib (PI)	0.2	3.8	1.2	1.4	49
daratumumab	0.4	0.7	0.2	1.4	22

[1] % based on the number of patients in the respective line of therapy
[2] includes all cases of therapy with daratumumab
[3] drugs can be combined in a single line of therapy
PI (proteasome inhibitor) – bortezomib/carfilzomib/ixazomib
IMiD (immunomodulatory drug) – lenalidomide/thalidomide/pomalidomide
chemo (chemotherapy) – bendamustine/doxorubicin/cyclophosphamide/etoposide/melphalan/vincristine/idarubicin
cort. (corticosteroids) – dexamethasone/prednisone

SMM to MM: in these cases, the highest risk is observed in the first 5 years after the diagnosis and decreases thereafter. The "watch and wait" strategy is the standard of care in MGUS and SMM. Death is a competitive event of progression – death can occur before progression particularly in old patients. Almost 20% of MGUS patients are diagnosed before 50 years of age, which means that they are at a higher risk of progression due to their longer life expectancy. RMG contains data on a high number of patients with MGUS (2,506 patients were involved in the analysis); however, only seven MGUS patients have been followed-up for more than 25 years. The median OS from the time of MM diagnosis was reported in data from RMG, but variation is expected depending on the patients' characteristics (comorbidities) and disease characteristics (chromosomal abnormalities). The combination of novel drugs with chemotherapy and/or corticosteroids and ASCT (if the patient is eligible) is the gold standard of care. Depending on the availability of drugs, therapy of MM varies across different countries. Although MM remains an incurable disease, there is a small group of patients without progression or death 10 years after the diagnosis. Eventually, the power of the registry is expected to grow stronger over time – longer follow-up times will be available to see the complete risk of progression from

precancerous stages and the effects of novel drugs in MM therapy.

Conclusion

RMG is an international registry collecting clinical data about diagnosis, treatment, treatment results and survival of patients with monoclonal gammopathies in the long-term follow-up. Apart from Czech centres, data from the Slovak Republic are collected in RMG and cooperation with other countries is considered. RMG is one of the main projects of the Czech Myeloma Group [23]. Thanks to the registry, treatment response and other endpoints can be evaluated across all participating centres. The publication policy for data recorded in the registry is based on an online system for the collection of approvals from centres for all analyses. As a source of real-world data, RMG provides real-world evidence about the treatment of monoclonal gammopathies and its results on the population level.

Acknowledgements

We would like to acknowledge all physicians, data managers and researchers from both major and minor centres who contributed to the collection of data into the registry.

Conflicts of interest

I. S. has served as consultant for and received research funding from and holds membership on the board of directors or advisory committee of Celgene, Janssen-Cilag, Amgen, Bristol-Myers Squibb, and Takeda. V. M. consulted for Amgen, Bristol-Myers Squibb, Celgene, Janssen-Cilag and Takeda; received grant support from The Binding Site, honoraria from Amgen, Bristol-Myers Squibb, Celgene and Janssen-Cilag, and has been involved in advisory boards for Amgen, Bristol-Myers Squibb, Celgene, Janssen-Cilag and Takeda. R. H. has a consultant or advisory relationship and received honoraria from Amgen, Bristol-Myers Squibb, Takeda, Celgene and Janssen-Cilag; conducted a clinical research project(s) funded by Takeda, Novartis, Amgen and Janssen-Cilag. Other authors declare they have no conflicts of interest concerning drugs and other medicinal products used in the study.

Střet zájmů

I. S. je konzultantem, obdržel finanční prostředky pro výzkum a je členem předsednictva nebo poradního výboru u společností Celgene, Janssen-Cilag, Amgen, Bristol-Myers Squibb a Takeda. V. M. je konzultantem u společností Amgen, Bristol-Myers Squibb, Celgene, Janssen-Cilag a Takeda; přijal grantovou podporu od The Binding Site a honoráře od Amgen, Bristol-Myers Squibb, Celgene and Janssen-Cilag; je členem poradních výborů u společností Amgen, Bristol-Myers Squibb, Celgene, Janssen-Cilag a Takeda. R. H. je konzultantem nebo poradcem u společností Amgen, Bristol-Myers Squibb, Takeda, Celgene a Janssen-Cilag, od kterých obdržel honoráře; provádí klinické výzkumné projekty financované společnostmi Takeda, Novartis, Amgen a Janssen-Cilag. Ostatní autoři deklarují, že v souvislosti s předmětem studie ve smyslu léků a jiných léčivých přípravků nemají střet zájmů.

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Biomarkers in Immunoglobulin Light Chain Amyloidosis

Biomarkery v amyloidóze lehkého řetězce imunoglobulinů

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Summary

Immunoglobulin light chain amyloidosis (AL amyloidosis – ALA) is a monoclonal gammopathy characterized by presence of aberrant plasma cells producing amyloidogenic immunoglobulin light chains. This leads to formation of amyloid fibrils in various organs and tissues, mainly in heart and kidney, and causes their dysfunction. As amyloid depositing in target organs is irreversible, there is a big effort to identify biomarker that could help to distinguish ALA from other monoclonal gammopathies in the early stages of disease, when amyloid deposits are not fatal yet. High throughput technologies bring new opportunities to modern cancer research as they enable to study disease within its complexity. Sophisticated methods such as next generation sequencing, gene expression profiling and circulating microRNA profiling are new approaches to study aberrant plasma cells from patients with light chain amyloidosis and related diseases. While generally known mutation in multiple myeloma patients (*KRAS, NRAS, MYC, TP53*) were not found in ALA, number of mutated genes is comparable. Transcriptome of ALA patients proves to be more similar to monoclonal gammopathy of undetermined significance patients, moreover level of circulating microRNA, that are known to correlate with heart damage, is increased in ALA patients, where heart damage in ALA typical symptom.

Key words

amyloidosis – plasma cell – genome – transcriptome – microRNA

This study was supported by the Ministry of Education, Youth and Sports (Institutional Development Plan of the University of Ostrava IRP201550 and Specific university research of the Faculty of Medicine, University of Ostrava SGS10/LF/2016-2017), and by the Ministry of Health of the Czech Republic (15-29667A and DRO-FNOs/2016).

Práce vznikla za podpory Ministerstva školství, mládeže a tělovýchovy (Institucionální rozvojový plán OU IRP201550 a Specifický VŠ výzkum Lékařské fakulty OU SGS09/LF/2016-2017) a Ministerstva zdravotnictví (15-29667A a RVO-FNOs/2016).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 13. 6. 2017
Accepted/Přijato: 13. 7. 2017

doi: 10.14735/amko20172S60

Souhrn

Amyloidóza lehkých řetězců imunoglobulinů (AL amyloidosis – ALA) je monoklonální gamapatie charakteristická přítomností aberantních plazmatických buněk produkujících amyloidogenní lehké řetězce imunoglobulinů. To vede k tvorbě amyloidních fibril v cílových orgánech a tkáních, především v srdci a ledvinách, což způsobuje jejich dysfunkci. Jelikož tvorba amyloidních depozit je nezvratný proces, je kladeno velké úsilí k nalezení biomarkeru, který by odlišil ALA od ostatních monoklonálních gamapatií v časném stadiu onemocnění, kdy amyloidní depozita ještě nemají fatální následky. Vysoce výkonné technologie přinášejí nové možnosti v rámci moderního výzkumu nádorů, jelikož umožňují studovat nemoc v rámci jeho komplexnosti. Moderní metody, jako jsou sekvenování nové generace, genové expresní profilování a profilování cirkulujících mikroRNA u aberantních buněk ALA pacientů a příbuzných onemocnění patří mezi nové přístupy využívané ke studiu aberantních plazmatických buněk amyloidózy lehkých řetězců a jiných příbuzných onemocnění. Zatímco obecně známé mutace u pacientů s mnohočetným myelomem (*KRAS, NRAS, MYC, TP53*) nebyly u ALA pacientů nalezeny, počet mutovaných genů u jednotlivých diagnóz není rozdílný. Transkriptom ALA pacientů se jeví být podobnější pacientům s monoklonální gamapatií nejasného významu, a zároveň exprese cirkulujících mikroRNA, pro které je známá korelace s poškozením srdce je zvýšená právě u ALA pacientů, u nichž je poškození srdce typickým projevem.

Klíčová slova

amyloidóza – plazmatická buňka – genom – transkriptom – mikroRNA

Introduction

Immunoglobulin light chain amyloidosis (AL amyloidosis – ALA) represents the most common type of systemic amyloidosis with incidence approximately nine cases per million inhabitants per year and the average age in the time of diagnosis is 65 years [1]. ALA is incurable hematological disorder, usually present as systemic disease with multiple organ damage and dysfunction. The most common afflicted organs are heart and kidney (Fig. 1) [2]. Pathological plasma cells (PCs) in bone marrow (BM) produce amyloidogenic light chains by forming insoluble amyloid fibrils which are stored in extra-cellular matrix in involved organs and tissues [3].

High throughput technologies bring new opportunities to modern cancer research as they enable to study disease within its complexity. "Omics" aims at the collective characterization and quantification of pools of biological molecules that translate into the cell structure, function and metabolism. Unlike disorders in which the malignant clone tends to dominate the BM at diagnosis (e.g. acute leukemia), in monoclonal gammopathies target clones generally represent a very small subset of BM cells, therefore many methodological limitations have to be overcome. The aim of our study was to describe pathological PC clones in ALA patients using comprehensive approach represented by next generation sequencing (on DNA level), gene expression profiling (on protein-coding RNA level) and circulating

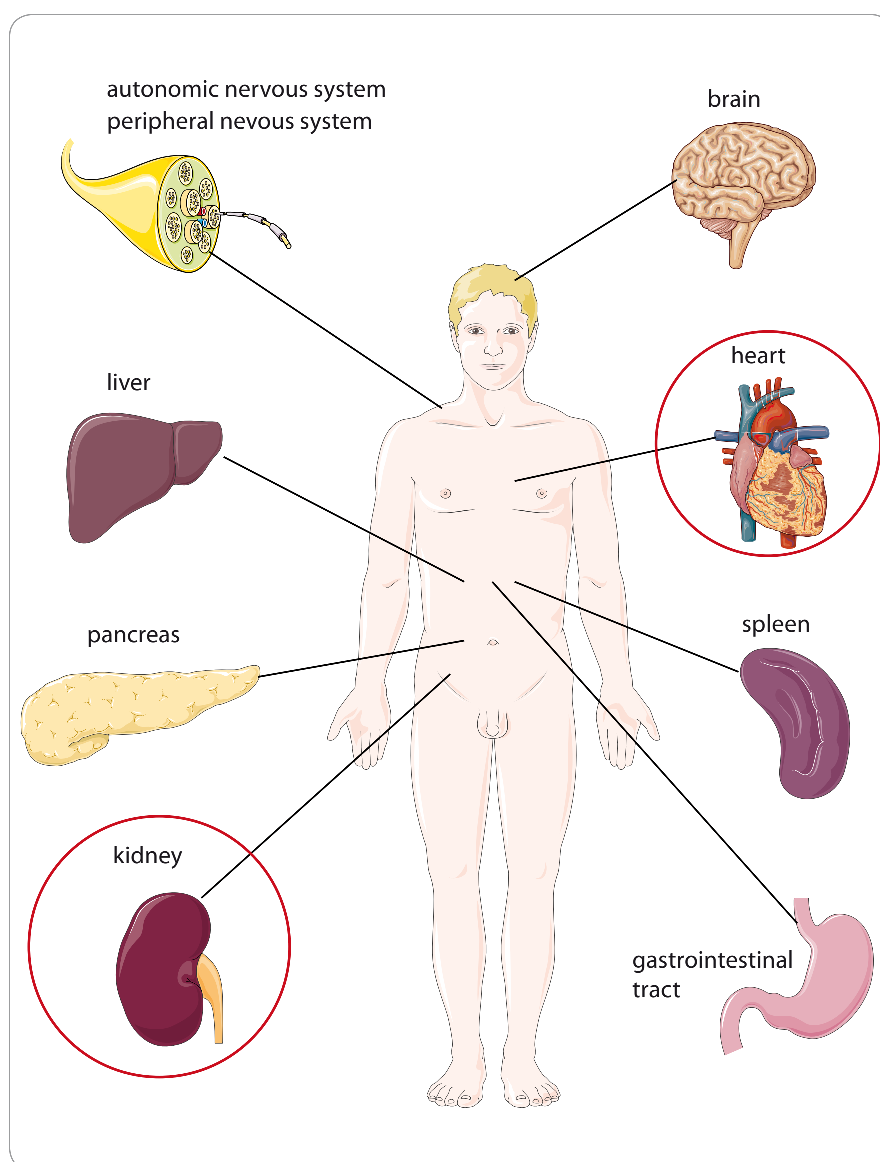


Fig. 1. Scheme of target organs for light chain deposition in AL amyloidosis.
The most frequently affected organs are highlighted with red circles.

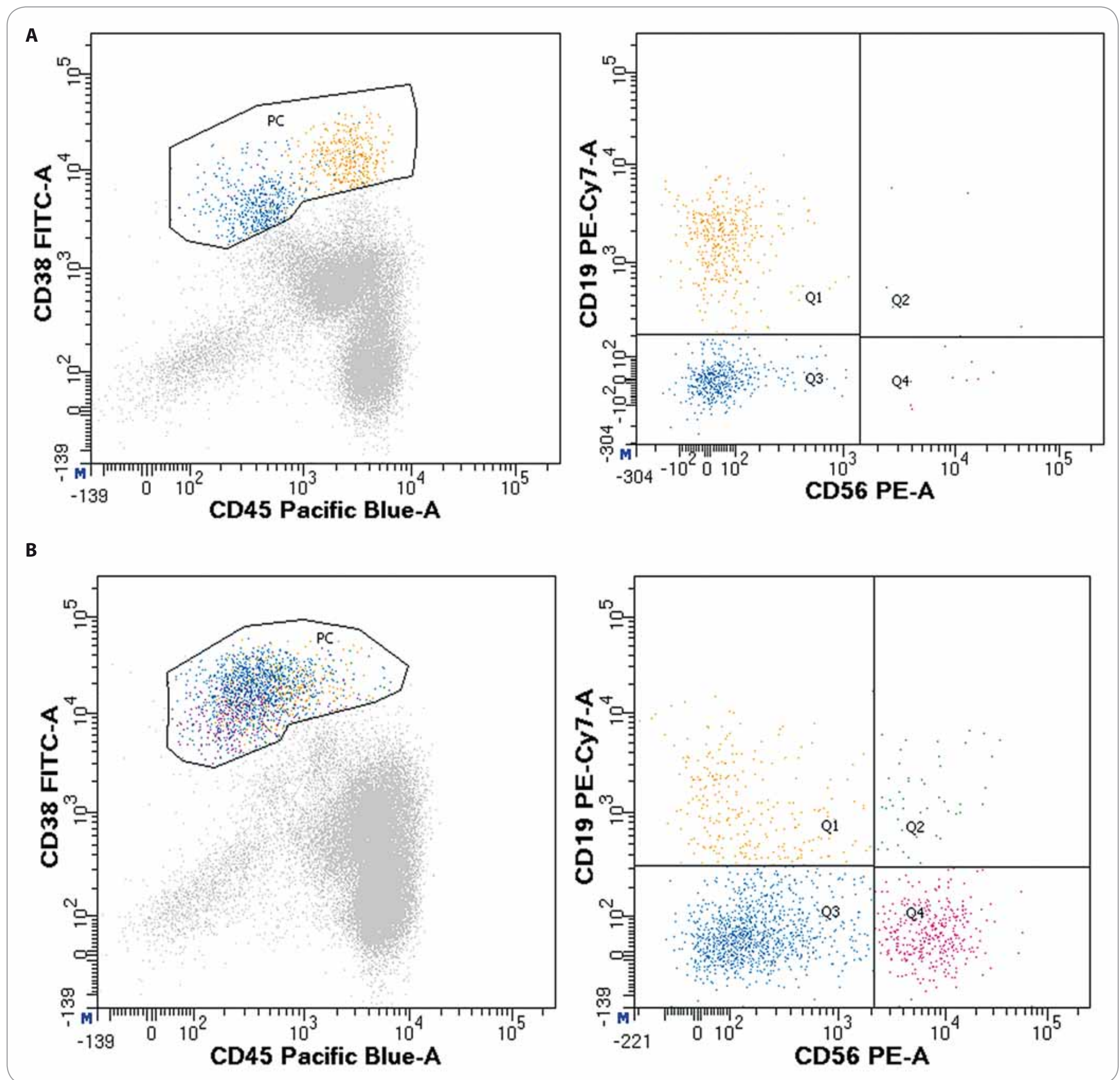


Fig. 2. Plasma cells in ALA patients show different aberrant immunophenotype.

Total plasma cells were identified according to CD45$^{+/dim}$ and CD38^{++} immunophenotypic markers. Differences between normal and aPCs were determined using CD19 and CD56. Normal plasma cells are CD19^{+}CD56^{-} while aPCs show heterogeneous expression of CD56 in different samples (A, B).

microRNA (miRNA) profiling (on non--coding RNA level).

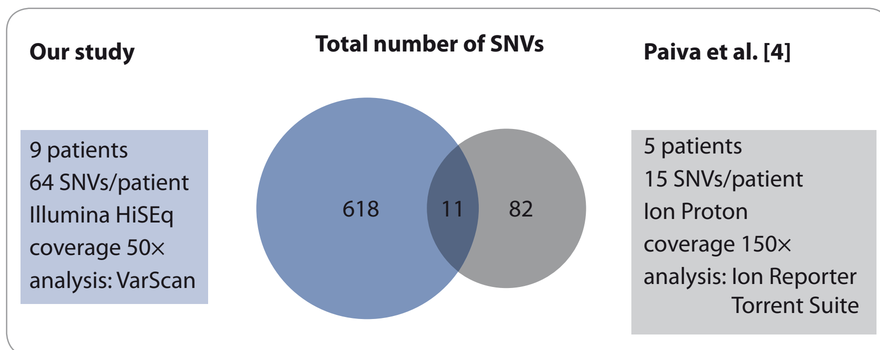
ALA is fatal and incurable disease. Detail and complex description of ALA might bring potential biomarker to distinguish ALA from other monoclonal gammopathies in early stage of disease before the amyloids will become fatal.

Methods

The samples of peripheral blood and/or BM from patients with ALA, multiple myeloma (MM), monoclonal gammopathy of undetermined significance (MGUS) and normal healthy donors (NHD) were collected between 2014 and 2016 from centers in the Czech Republic and Poland. All studies were approved by the institutional ethics review boards and all individuals provided written informed consent.

Flowcytometry and cell sorting

Clonal PCs (aPCs) were separated by fluorescence activated cell sorting (FACS) using FACS Aria III (Becton Dickinson) cell sorter. aPC were identified using



Graph 1. Comparison of the project design and sequencing results between two independent studies.

4-colors cytometry – CD45-PB, CD38-FITC, CD19-PC7 and CD56-PE (Fig. 2).

DNA and RNA isolation, whole genome amplification

Parallel nucleic acids isolation (DNA/RNA) was chosen for situation of very limited target cell amount using AllPrep DNA/RNA Micro Kit (QIAGEN). DNA concentration was measured using DNA HS Assay on Qubit® 2.0 fluorometer (Thermo Fisher Scientific) and quality was checked in 1% agarose gel. RNA concentrations and integrity numbers (RINs) were evaluated using RNA 6000 Pico kit on Bioanalyzer2100 (Agilent Technologies). In some cases, concentration was validated by RNA HS assay kit on Qubit® 2.0 fluorometer (Thermo Fisher Scientific). DNA samples were amplified using REPLI-g Midi/Mini Kit and purified by QIAquick PCR Purification Kit (both Qiagen). The DNA quantity after amplification was measured by Qubit Fluorometer 1.0 (Thermo Fisher Scientific, Waltham, MA, USA) and quality was checked in 1% agarose gel.

Exome sequencing

Parallel libraries were generated from genomic DNA derived from separated aPCs and DNA from whole peripheral blood or mononuclear cells from peripheral blood. Exome captured sequencing library was produced from Agilent's SureSelect XT Human All Exon v5 (51 MB target size) (Agilent Technologies, Santa Clara, USA). We followed the original protocol provided by Agilent (SureSelect XT Target Enrichment System for Illumina Paired-End Sequencing Library, version 1.3.1), with minor modifications based on our previous experience. Captured DNA was sequenced in Macrogen (Korea) using a HiSeq 4000 platform (Illumina, San Diego, CA, USA) with 300 bp length of insert and with paired-end reads of 100 bp for insert libraries. Expected average coverage depth was 50×.

Gene expression profiling

Total RNA obtained from separated aPCs with purity ratio 260/280 > 1.7 and integrity (RIN) > 7.5 was transcribed into cDNA (Ambion WT Expression Kit), labeled and hybridized to the Affymetrix GeneChip â Human Gene ST 1.0 array and processed through complete Affymetrix workflow (Affymetrix, Santa Clara, CA). Affymetrix power tools were used to normalize raw CEL files at the gene level. Robust multi-array averaging (RMA) normalization and complete annotation files were selected.

Circulating miRNA profiling

Sample collection

Peripheral blood serum samples from 5 ALA, 5 MM, 5 MGUS and 5 NHD were obtained for this study from centers in the Czech Republic and Poland. PB serum samples were centrifuged at 3,500 rpm/15 min/20 °C, frozen as 0.5 mL aliquots, stored at –80 °C and thawed on ice. This study was approved by institutional ethical review boards and all individuals provided written informed consent.

MiRNA extraction

MiRNA-enriched total RNA was extracted from PB serum samples using miRNeasy Serum/Plasma Kit (Qiagen). The protocol was modified for circulating miRNAs according to the manufacturer's instructions. Both quantity and quality was evaluated using NanoDrop 200 Spectrofotometer (Thermo Scientific).

Reverse transcription and quantification of miRNA

MiRNA profiling using MicroRNA Ready-to-Use PCR, Human panel I + II and ExiLENT SYBR Green (Exiqon) was performed to determine the expression level of 752 miRNAs. Protocol was modified for serum samples according to manufacturer's instruction and qPCR was performed on the LightCycler 480 Instrument II (Roche).

Statistical analyses

MiRCURY LNA profiling data were normalized using the most stably expressed miR-106a-5p. Afterwards normalized expression data were statistically assessed using IBM SPSS Statistics Kruskal-Wallis and Mann-Whitney U test to define significance. Receiver operating characteristic (ROC) analysis of chosen miRNAs was performed to describe their predictive potential.

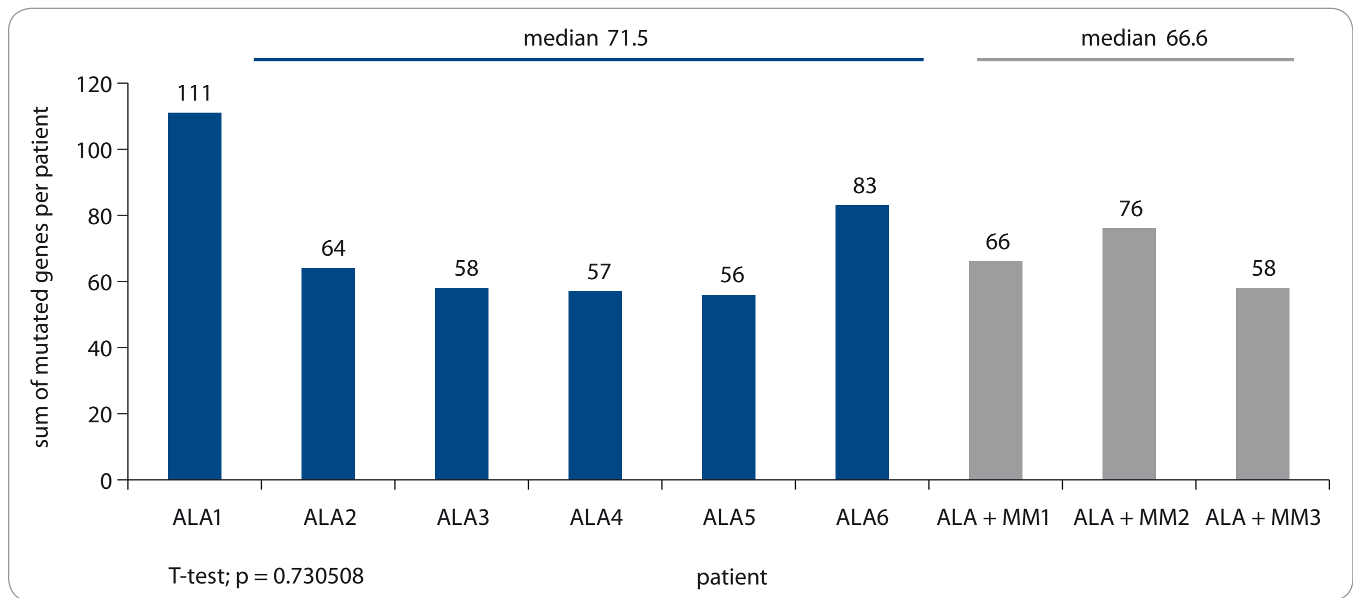
Results and discussion

Genome level

Although, knowledge about MM PC biology and pathology increased significantly in the last decade, the same cannot be said about ALA. Until this time only two studies were focused on description of genomic landscape of ALA using next generation sequencing [4] or/and array comparative genome hybridization (CGH) [4,5].

Compared to our study, work published by the Spanish group [4] analyzed 11 ALA patients and identified lower number of single nucleotide polymorphisms (SNVs) per patient (median 15 vs. 64 SNVs) (Graph 1). The discrepancy could be caused by differences in library preparation, sequencing protocol/platform and/or mutation calling algorithm.

In addition, the study of Paiva et al. [4] included high throughput genetic profiling of purified clonal PCs from the ALA patients. Copy number alterations (CNA) were detected in all tested samples with median 9 CNA per patient



Graph 2. Total number of nonsynonymous mutations per patient.

The median number of mutated genes in aberrant population was 64 (range 56–111) per patient.
ALA – light chain amyloidosis, MM – multiple myeloma

(range 1–23), which is similar to what has been described in MM (median of 12 CNA per patient). This indicates that overall pattern of CNA revealed in ALA might be CNA pattern in MM.

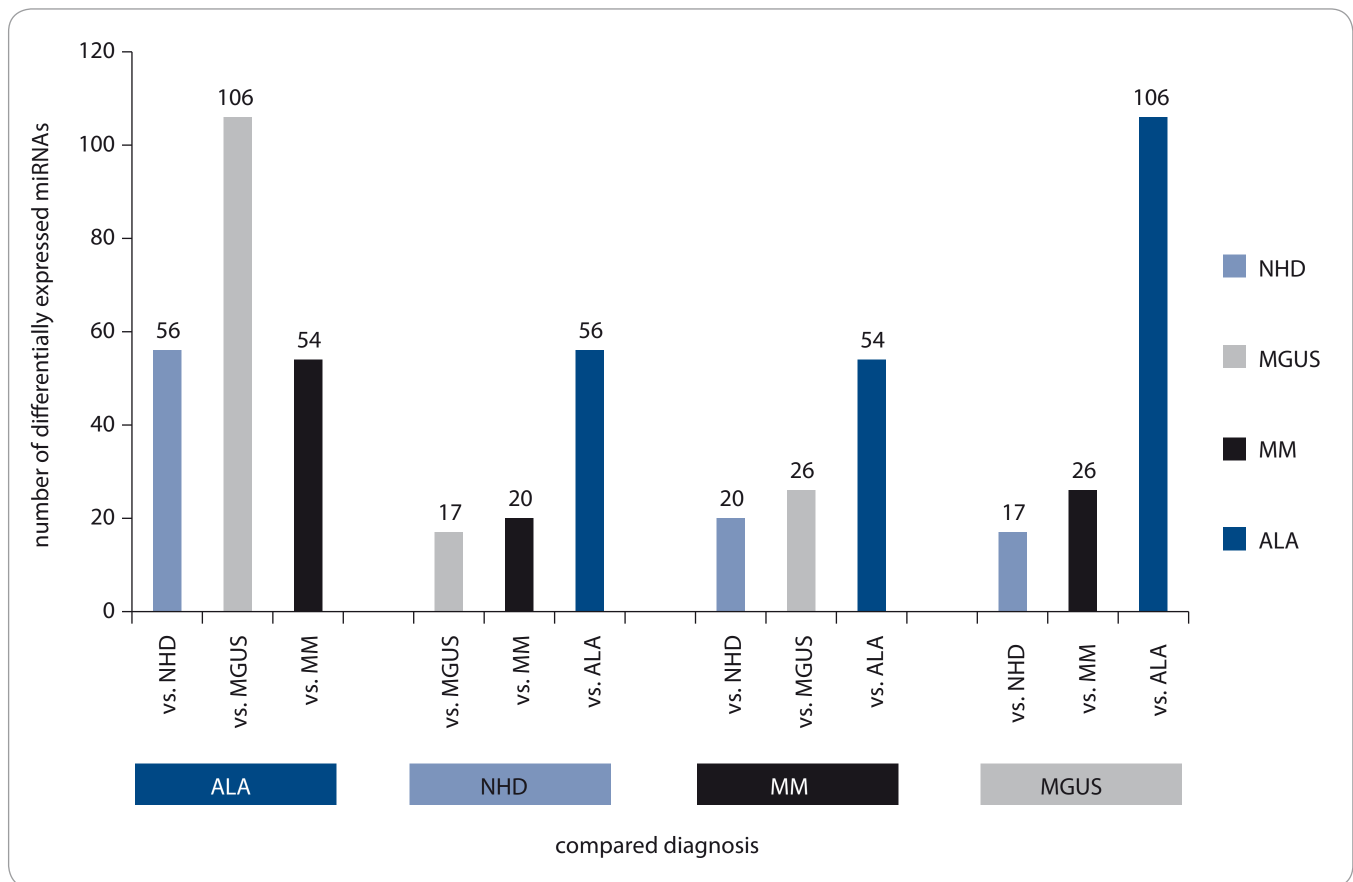
In the second study, genomic landscape of 118 ALA patients was analyzed using array CGH. In total, 5 (average) and 3 (median) aberrant chromosomes per patients were found. The most prevalent gain detected was on chromosome 1q affecting 36% patients. The most common deletion was localized on chromosome 13 in 40% of patients [5].

In our study overall nine samples of ALA were used for exome sequencing – six samples of ALA, three samples of ALA + MM. In total, 618 non-synonymous SNVs were identified with a median 64 SNVs per patient. Comparison between ALA and ALA + MM revealed median of 71.5 mutated genes for ALA and 66.6 for ALA + MM per patient (Graph 2). The difference between cohorts with different diagnosis was not found statistically significant. Ten mutated genes were shared between more than three patients, two mutated genes were common for additional nine patients. But we did not observe any unifying mutation for ALA. When comparing total number of mutations among ALA patients and ALA + MM samples there was no significant difference (F-test = 0.30452, t-test = 0.730508). Moreover, for ALA + MM samples we did not detect SNVs in any of the most common mutated genes in MM such as *KRAS, NRAS, FAM46C, DIS3* and *TP53* [6], indicating that only certain and not well defined types of MM could co-exist with ALA.

Transcriptome level

As genomic studies provide highly valued information about mutations, CNV, SNP, gene expression profiling contributes with necessary interpretation of biological relevance of these genomic chances. Recent study of ALA transcriptome comparing ALA PC, MM PC and NHD PC revealed that ALA has an transcriptome profile in between that of MM and healthy individuals [7]. Although this study presents interesting results suggesting genes involved in deregulation of protein processing and folding pathway as well as disturbance of PC maturation in ALA, the advantage of this method is untapped by the analysis of the whole $CD138^+$ cell fraction instead of analysis based on aPCs only. The $CD138^+$ marker is expressed by both aberrant ($CD138^+/CD19^-/CD56^{-/+}$) and normal ($CD138^+/CD19^+/CD56^-$) immunophenotypes [8] and this leads to makes impossible to distinguish the expression profiles of these two cell subpopulations [7]. However, recent study comparing gene expression of ALA aPC purified according to patient specific immunophenotype confirmed less deregulated expression profile in ALA compared to MM, in other words ALA aPCs have transcriptome remarkably similar to normal PCs [4].

The objective of our study was to compare gene expression profile of two malignant disease entities (ALA and MM) and their pre-malignant condition (MGUS). We hypothesized that processes shared between ALA and MM but not present in MGUS will help to identify events important for PC malignant transformation during disease development. To test our initial hypothesis, we analyzed gene expression profile of PC from ALA, MM and MGUS patients. Our data confirmed expected overexpression of immunoglobulin light chain genes in ALA. Pathway analysis of gene expression profile did not show any significantly deregulated pathway. Cluster analysis indicated more similar profile of ALA patient to its precancerosis MGUS than in MM, which is in agreement with previously published data [7].



Graph 3. Monoclonal gammopathies pairwise comparison showing numbers of differentially expressed circulating serum miRNAs.
The chart shows number of differentially expressed circulating miRNA in ALA vs. NHD/MGUS/MM. Digits indicate numbers of miRNAs in each pairwise comparison.
ALA – light chain amyloidosis, NHD – normal healthy donor, MGUS – monoclonal gammopathy of undetermined significance, MM – multiple myeloma

MiRNome level

MiRNA are highly conserved, single--stranded, small (21–23 nucleotides long), non-coding RNA regulating gene expression and protein synthesis and playing a key role in fundamental biological processes, pathological events and tumorigenesis [9]. Moreover, miRNA were described as important diagnostic and prognostic markers in oncology, since their expression profiles were able to stratify patients and predict clinical outcome [10]. MiRNA were repeatedly proven to play an important role in the pathogenesis of MM, including different development stages from MGUS till extramedullary myeloma [11–15]. Nevertheless, there is a complete lack of knowledge about circulating miRNA in ALA.

Tissue specificity of many miRNA [16] was the reason for focusing of clinical studies on the quantification of specific miRNA expression extracted from defined cellular material. Despite this fact, free circulating miRNAs are present in various body fluids such as blood serum, plasma, urine or saliva [17]. The source of circulating miRNA could be normal healthy as well as aberrant cells. Circulating miRNAs are mostly associated with RNA-binding proteins or membranous vesicles, which prevent their degradation [18].

Since monitoring of predictive and prognostic markers from BM (which is a standard nowadays) is complicated and invasive, stable markers from peripheral blood are easily available and ideal for frequent monitoring. Circulating serum miRNA may become a new, easily obtainable and stable disease marker, which can be examined as diagnostic in patients with ALA predisposition or as predictive during disease progression.

Studying the presence of circulating miRNA in peripheral blood of ALA patients allow us to distinguish this disease from other monoclonal gammopathies such as MM or premalignant stage MGUS thus have a big potential in clinical diagnosis. Comparison of circulating miRNA profiles of MM, MGUS and ALA patients with NHD and with each other could uncover specific circulating miRNA as biomarker for ALA and facilitate choice of right therapy. It is also important to define specific circulating serum miRNA deregulated in ALA in context of clinically important parameters and cytogenetics.

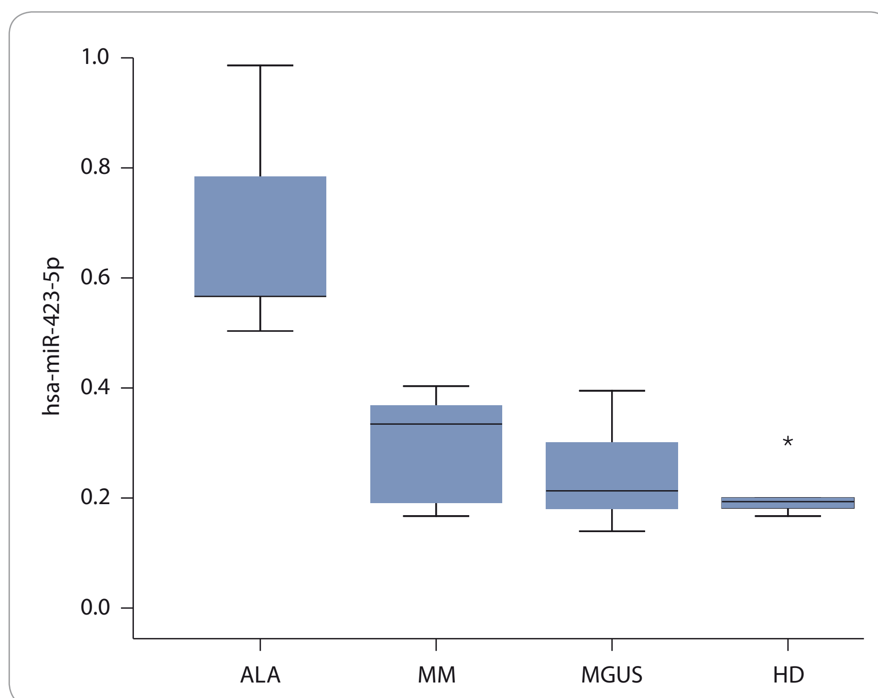
Numbers of methods suitable for measurement of miRNA expression level were developed including northern blot, microarray, quantitative real-time

PCR (qRT-PCR) and deep sequencing. For detection of miRNA expression in peripheral blood considering high sensitivity, specificity, reproducibility and also financial aspect is qRT-PCR is the most available method. Besides other mentioned advantages, qRT-PCR is adapted to multi-well plates (384, 768 or 1,538) that allows screening of almost all known circulating miRNAs in one run [19].

Although all previously mentioned methods are well validated, published studies concerning circulating miRNAs in MM show heterogeneous results [12,20–26]. There are various potential reasons for this discrepancy. First, correct sample processing is essential, as contamination of cellular material caused by hemolysis could misrepresent results. E.g. expression level of several miRNA (e.g. miR-92a-3p and miR-451) is deregulated depending on hemolysis [27]. Secondly, various types of anticoagulants have an impact on PCR-based quantification used for miRNA profiling. Moreover, time delay between sample collection and further processing may introduce bias into study [28] and different normalization approaches have impact on final results of mentioned studies, too. On the other hand, heterogeneity of MM patients could also be the potential explanation for results discrepancy.

Analysis of 752 circulating miRNAs in ALA, MM, MGUS a NHD demonstrates dissimilarity of miRNA expression profile in diverse diagnosis. Results show, that circulating miRNA profile in ALA patient is the most diverse compared to other diagnosis. According to miRNA profile the highest number of differently expressed miRNAs was found in expression profile between ALA and MGUS (106 miRNAs) and the most similar groups are MGUS and NHD (18 miRNAs) (Graph 3). This suggests that miRNA expression profile of ALA is more similar to MM than to MGUS, which is in contrary to overall gene expression profile analyzed by gene expression profiling.

Significantly higher expression of circulating miR-423-5p was detected in ALA patients (p = 0.009) contrary to other analyzed diagnosis (Graph 4). Recent evidence has shown that the circulating miR-423-5p displays increased levels during the heart failure and can be used as a biomarker. In other words, it was observed that the increase of miR-423-5p was a strong diagnosis predictor of heart failure [29]. Moreover, positive correlation between the levels of miR-423-5p and NT-proBNP was described [30]. All MM and MGUS patients, included in miRNA profiling study, do not suffer from heart failure, which is supported by the fact that the expression of miR-423-5p was significantly higher in ALA patients. There is no difference in expression of miR-423-5p between NHD and MGUS or MM.



Graph 4. Expression level of hsa-miR-423-5p.

MiR-423-5p is significantly higher in ALA vs. NHD/MGUS/MM.
ALA – light chain amyloidosis, NHD – normal healthy donor, MGUS – monoclonal gammopathy of undetermined significance, MM – multiple myeloma

Conclusion

Our preliminary results did not reveal any mutations common for all ALA patients. Nevertheless, larger cohort of patients and further bioinformatical analyses might be useful to discover potential ALA biomarker. Expression profile of protein coding genes supports the idea of close relationship of ALA and MGUS and correlates with ALA immunophenotype as well as non-coding miRNA results may be associated with ALA symptoms (heart damage). Modern research has revealed that important insights can be found not only within the individual levels (at the genomic, epigenomic, transcriptomic, proteomic, post-translational modifications, and metabolic) but also through understanding interactions between these levels.

Acknowledgments

We would like to thank to Biobank Ostrava (FN Ostrava) for providing bone marrow and peripheral blood samples. Figures were created using http://www.servier.com/Powerpoint-image-bank.

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CRISPR in Research and Treatment of Multiple Myeloma

CRISPR ve výzkumu a léčbě mnohočetného myelomu

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Summary

In the recent years, there was a remarkable advance in research and clinical implementation of the genome editing technologies. The most remarkable was a discovery of the bacterial adaptive immune system called CRISPR and its rapid transformation into a robust and broadly applicable technology that completely revolutionized both basic and applied biomedical research. Implementation of CRISPR makes genome modification easier, faster and significantly cheaper compare to any other currently available technology. It also offers a tremendous potential for desiging novel research approaches and future treatment options for various genetic diseases including multiple myeloma. The hightroughput use of CRISPR in pooled screen formats promises faster identification and validation of valuable drug targets together with revealing high-confidence biomarkers and unknown resistance mechanisms. This can provide clinicians with new diagnostic and prognostic tolls and ultimately allow more accurate patient stratification for personalised treatment with better eficacy. In this review, we summarize current knowledge about the CRISPR technology and focus especially on its impact in exploring gene functions, screening for novel drug targets, diagnostic markers and genes involved in resistance to commonly used drug in the treatment of multiple myeloma. Finally, we also highlight a potential future use of CRISPR in actual clinical practise.

Key words

multiple myeloma – CRISPR – therapeutics

Souhrn

V posledních letech došlo ke značnému pokroku v oblasti vývoje technik editace genomu a možnosti jejich klinického využití. Především objev adaptivního imunitního systému bakterií známého jako CRISPR a jeho rychlá implementace jako široce využitelné technologie způsobila zásadní převrat jak v základním, tak v aplikovaném biomedicínském výzkumu. Technologie CRISPR umožňuje editovat genom snadněji, rychleji a výrazně levněji než jakákoli jiná v současnosti dostupná technologie. Tímto se nabízí obrovský potenciál pro realizaci nových výzkumných přístupů a budoucí možnosti léčby nejrůznějších genetických onemocnění, vč. mnohočetného myelomu. Robustní využití CRISPR technologie v rámci genetických screeningů slibuje rychlejší identifikaci důležitých terapeutických cílů a současné odhalení biomarkerů s vysokou prediktivní hodnotou a doposud neznámých mechanizmů lékové rezistence. Výsledky takto směřovaného výzkumu tak mohou poskytnout nové diagnostické a prognostické přístupy, které umožňují přesnější stratifikaci pacientů pro personalizovanou léčbu s vyšší účinností. V tomto přehledném článku shrnujeme dosavadní znalosti technologie CRISPR s důrazem na její uplatnění při hledání nových terapeutických cílů, diagnostických markerů a genů zapojených do mechanizmů resistence na běžně používanou léčbu u mnohočetného myelomu. Závěrem prezentujeme potenciální budoucí využití technologie CRISPR v klinické praxi.

Klíčová slova

mnohočetný myelom – CRISPR – léčiva

This study was supported by the Ministry of Education, Youth and Sports (Institutional Development Plan of the University of Ostrava IRP201550 and Specific university research of the Faculty of Medicine, University of Ostrava SGS03/LF/2017-2018), project: Strengthening international cooperation in science, research and education 01211/2016/RRC) and by the Ministry of Health (DRO-FNOs/2017).

Tato studie byla podpořena Ministerstvem školství, mládeže a tělovýchovy (Institucionální Rozvojový Plán Ostravské univerzity IRP201550 a Studentskou grantovou soutěží Lékařské fakulty, Ostravské univerzity SGS03/LF/2017–2018), projektem: Posílení mezinárodní spolupráce ve vědě, výzkumu a vzdělávání 01211/2016/RRC) a Ministerstvem zdravotnictví (RVO-FNOs/2017).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 1. 6. 2017
Accepted/Přijato: 22. 6. 2017

doi: 10.14735/amko20172S68

Introduction

In the last decade we have seen tremendous improvements in analysing human genome. Genomic methods such as next generation sequencing (NGS) and gene expression profiling (GEP) are gradually moving from the research laboratories to rutine use in everyday clinical practise. Although powerfull, these techniques are not used in the actuall treatment processes and serve rather as important dianostic and prognostic tools. The real currative potential for genetic and oncological diseases including mutliple myeloma (MM) comes with the gene editing technologies.

Genome editing technologies

Adenoviral vector-based approach was the first gene therapy tool used in childrens the ADA-SCID syndrom. It was also considered as potential treatment option for MM patients [1], but due to the early clinical failures – imprecise virus insertion resulting in the activation of oncogenes and development of leukemia – this approach was withdrawn from the clinic [2]. To overcome these issues novel gene editing techniques implemented additional step – generation of double stranded brakes in precisely defined positions in genomic DNA. This increased both specificity and efficacy of the gene targeting.

Initial, highly specific gene modifying techniques were based on engineered zinc finger nucleases (ZFN) or transcriptional activator-like nucleases (TALEN) that are designed to cleave in predefined sites in genomic DNA. Both ZFN and TALEN are modular proteins, where one module is set to bind to a desired DNA sequence and the other generates double-strand breaks near the binding site (Fig. 1A, B) [3,4]. Even through they achieve higher specificity and efficacy than adenoviral vectors, these techniques are still too complex and very difficult to use laboratory research, and it would be even more complicated to transfer them into actual clinical practise.

The recently described method called clustered regularly interspersed short palindromic repeats (CRISPR) is the most powerfull and versatile gene editing tool that ever existed. It was originally described in bacteria, where it functions as an adaptive immune system fighting againts invaiding bacteriophages and other DNA or RNA-based infection elements [5]. Since the 2012, CRISPR reached a spectacular level of interest both in academia, biotech companies and even several clinical trials using this technique will be initiated soon (see below). All that came with masive investments into further optimalisations for transfer of CRISPR-based technologies into clinical practise together with a remarkable engagement of public media making CRISPR well know technology with a huge potential [6].

CRISPR basic

CRISPR is composed of two components – the protein Cas9 and the short non-conding small guide RNA (sgRNA). Cas9 is an ATP-independent endonuclease, originially found in *Streptococcus pyogenes*, that cleaves DNA in precisely definded positions generating double

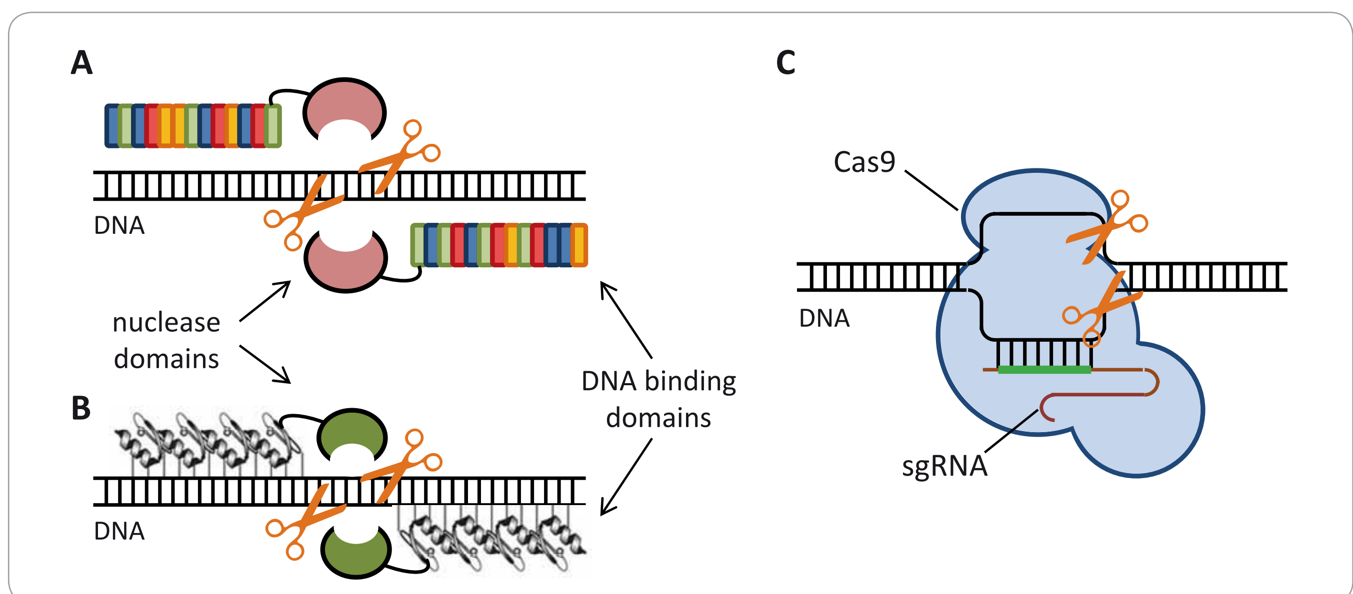

Fig. 1. Genome editing tools.
A. TALEN – Each TALE domain (coloured boxes) binds to one of the four DNA nucleotides. Combining these domain creates protein binding to defined DNAsequences. TALE domains are further fused to the FOK1 nuclease that acts only as a dimer, therefore tandem binding of TALENs is required for DNA cleavage.
B. ZFN – Each zinc finger domain binds to three nucleotides. There are multiple tri-nucleotide specific zinc fingers that can be combined and fused to FOK1.
C. CRISPR – RNA-guided DNA cleavage by the *S. pyogenes* Cas9 endonuclease – Cas9-associated sgRNA contains unique ~20nucleotide "seed" sequence (green) defining binding site in target DNA. Once boud, monomeric Cas9 cleaves simultaneously both DNA strands. The sgRNA seed sequence can be engineered to target Cas9 to any site in human genome.

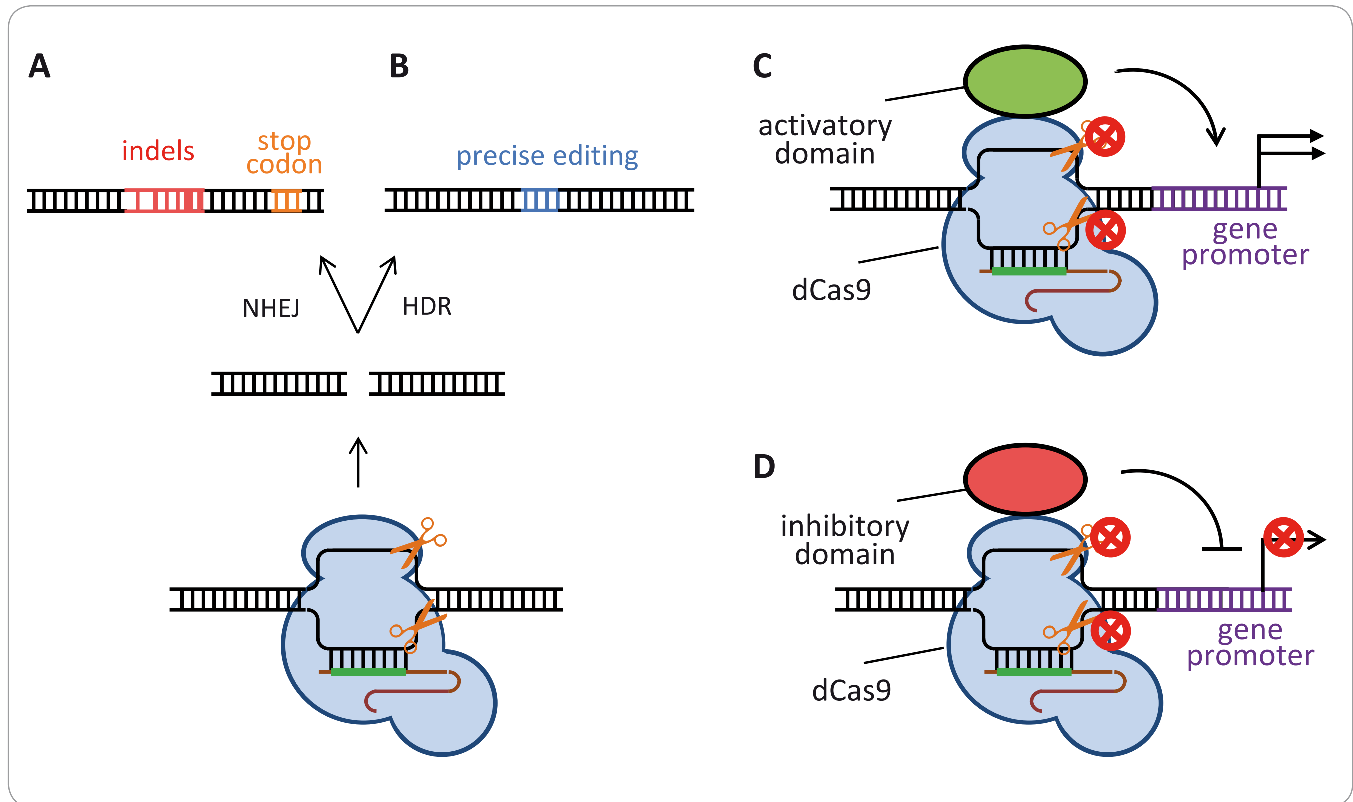

Fig. 2. Gene targeting by CRISPR.
A. Gene knock out – NHEJ repair leads to generation of indels (insertion/deletion) resulting in a premature stop codon abolishing the gene expression.
B. Gene editing – HDR repair can be used to intentionally modify desired genomic sequence (e.g. removing disease/oncogenic mutantions).
C. Activation of gene expression – catalyticaly dead Cas9 (dCas9) fused to transcription activatory domain promotes expression of targeted gene.
D. Inhibition gene expression – dCas9 fused to transcription inhibitory domain downregulates expression of targeted gene.

stranded brakes. To achieve site-specific recognition and cleavage, Cas9 must be complexed with the sequence-specific sgRNA that guides Cas9 to unique DNA motifs on basis of nucleotide complementarity between the 20 nucleotide long sgRNA seed sequence and the target site in genomic DNA (Fig. 1C) [7]. The binding to and cleaving in a particular genomic locus by CRISPR is extremelly specific with either very low or often no detectable off targets [8].

Once, the double stranded brake is made, cells have several ways to repair the damage. The predominant repair mechanism to fix these cytotoxic DNA lesions is the error prone non-homologous end joining (NHEJ). This type of repair is usually inaccurate and frequently leads to insertions and/or deletions (indels) of multiple nucleotides in the vicinity of the cleavage site. If this occurs in the gene coding sequence, the consequences are often frameshift mutations leading to the creation of a premature stop codon, which then results in inactivation of the targeted gene (gene knock out – KO) (Fig. 2A). This can be used in many different applications from switching off a single oncogene in cancer cell to choping off large chomosomal regions generated during aberrant translocation events.

By contrast, the alternative repair mechanism, homology directed repair (HDR), is using the second allele or externally provided homologous DNA as a repair template. Thus, HDR can be applied to intentionally generate precise and specific alterations to desired genomic locus, e.g. mutating the oncogenic variant to the wild type version or inserting a new DNA sequence (Fig. 2B). This phenomenon could be used in treatment of heritable or somatic genetic diseases. Pilot experiments with mouse models using CRISPR to repair the CFTR gene mutated in cystic fibrosis [9] or inactivating the NRL gene involved in retinitis pigmentosa [10] in adult mice are suggesting that succefull and widely applicable gene therapy that was for long time only science fiction might soon becam a reality.

To further extend the genome editing possibilities, CRISPR can be also employed to activate expression of particular genes. When using the nuclease-dead version of Cas9 (dCas9) fused to transcription activator complexes, dCas9 that is no longer active as DNA cleaving enzyme can serve as a hook delivering the transcription activatory domain to the

gene promotor region ultimatively triggering expression of the selected gene. Similarly, this approach can be also used to temporarily and specificaly inactivate transcription (Fig. 2C, D) [5,6]. This offers a possiblitiy of fine tuning the gene expression in a timely dependent manner, especially when inducible version of dCas9 is used. In other words, one can switch on and off expression of particular gene(s) to a certain level only when the cell is exposed to specific stimuli, avoiding unwanted side effects exerted by general transcription modulators such as dexamethasone.

As a research tool, the CRISPR technology enables more efficient modifications of DNA sequences, repression and activation of single or multiple genes at once and it also greatly improves and simplifies creation of complex animal models of many genetic diseases. Therefore, identification of genes, signaling pathways and genetic interactions essential for specific phenotypic changes and disease pathologies is now significantly easier than ever before and it is only a matter of time, when the fundamental findings will be translated into clinically relevant applications.

CRISPR in search for novel drug targets

In a basic research of MM, CRISPR already helped to identify multiple novel players affecting the disease development, progression and offered several novel therapeutic targets. CRISPR-based KO cells are extremelly valuable research tool in revealing the drug specificity. By simple comparison of the wild type cells and the cells with specifically inactivated gene, one can easily distinguish, whether the drug targets given protein or whether the effect is more pleiotropic. In this section, we will describe several recent studies utilizing CRISPR in the identification of new target genes crutial for survial of MM and show how CRISPR can be adopted in testing and validation of the target specificity of novel therapeutic compounds.

It is well established, that expression of MYC oncoprotein is required for survival of MM and many other malignancies. However, very little is known about the regulation of MYC expression in MM cells. Two recent studies used CRISPR to identify and validate novel key drivers of MYC expression. First, knocking out MUC1-C gene by CRISPR or its inactivation by GO-203 inhibitor was associated with a drop in expression of the MYC target genes, including CCND2, hTERT and GCLC [11]. Secondly, CRISPR-engineered cells suggested that let-7, endogenous inhibitor of the translational regulator LIN28, specifically affects MYC expression and cell cycle pathways in MM [12]. Together, these studies provided new pharmacological targets that can be utilised for suppressing MYC in MM.

With the CRISPR technology researchers have already demonstrated the ability to more specifically target genes of the Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3) in MM. IKZF1 and IKZF3 are the primary targets of Cereblon, which activity can be blocked by thalidomide and its derivates including lenalidomide. Analysis of MM cell lines revealed that the loss of IKZF1 and IKZF3 is both necessary and sufficient for lenalidomide's therapeutic effect [13]. This work further inidicated that the anti-tumor and teratogenic activities of thalidomide-like drugs are dissociable providing a completely new perspective of how we look at the Cereblon inhbitors.

MM is charecterised by high genome instability that might result from aberrant function of chromatin regulatory proteins such as histon modifiers. Recent study used CRISPR to delineated the role of histone demethylase KDM6B in MM. Knocking out the KDM6B gene by CRISPR signigicantly abrogated growth and survival of MM cells. The authors also linked KDM6B functions with the NF-κB and MAPK signaling pathway, highligting another drugable target in the treatment of MM [14].

Finally, in a search for the bortezomib-based therapy with a lower toxicity, fewer side effects, higher dose limits and limited resistancy research focused on the identification of compounds that would inhibit proteasome function in MM without blocking the general proteasome activity in the other cell types. CRISPR-based KO of the proteasomal regulatory subunit RPN13 demonstrated that the drug known as RA190 can decrease viability of the MM cell lines and primary cells isolated from MM patients. Importantly, RA190 inhibits proliferation of the MM cells even in the presence of bone marrow stroma and can overcame acquired bortezomib resistance [15].

Identification of the MM resistance genes by CRISPR screens

Although the treatment options of MM have markedly improved especially due to the development of numerous new compounds in the last decade, the recurrent problem in the treatment is disease relaps and fast progression into resistant and refractory stage. The large genomic studies using MM patient samples gave us better insights into the mutation signature in the relapsed MM patients [16–18]. But we still lack precise information, which genes and mutations are the crucial players is development of resistance to certain drugs. Once we will have a solid and validated panel of such genes in MM patients, we might use it as a predictor to stratify patients for a specific type of treatment to enhance its efficacy and avoid resistance. Basically, apply the principles of personalised medicine.

CRISPR technology brings new, exciting possibilities for identification of new genes and their combinations that are responsible for resistance and disease relaps. In so called CRISPR screens, the entire human genome can be profiled to find a particular set of genes that – when altered – might contribute to a loss of sensitivity to a given compound. To systematicaly analyse whole genomes, the CRISPR-based gene profiling (CRISPR screen) is usually done in a high-throughput manner and can be applied on both *in vitro* and *in vivo* systems. In the next section, we will describe the theoretical basis of the CRISPR screens and provide general and simplified protocol how to perform genome-wide screen to identify genes involved in drug resistance.

CRISPR screen methodology

The main part of the CRISPR screen is the CRISPR library, which is a mixture of

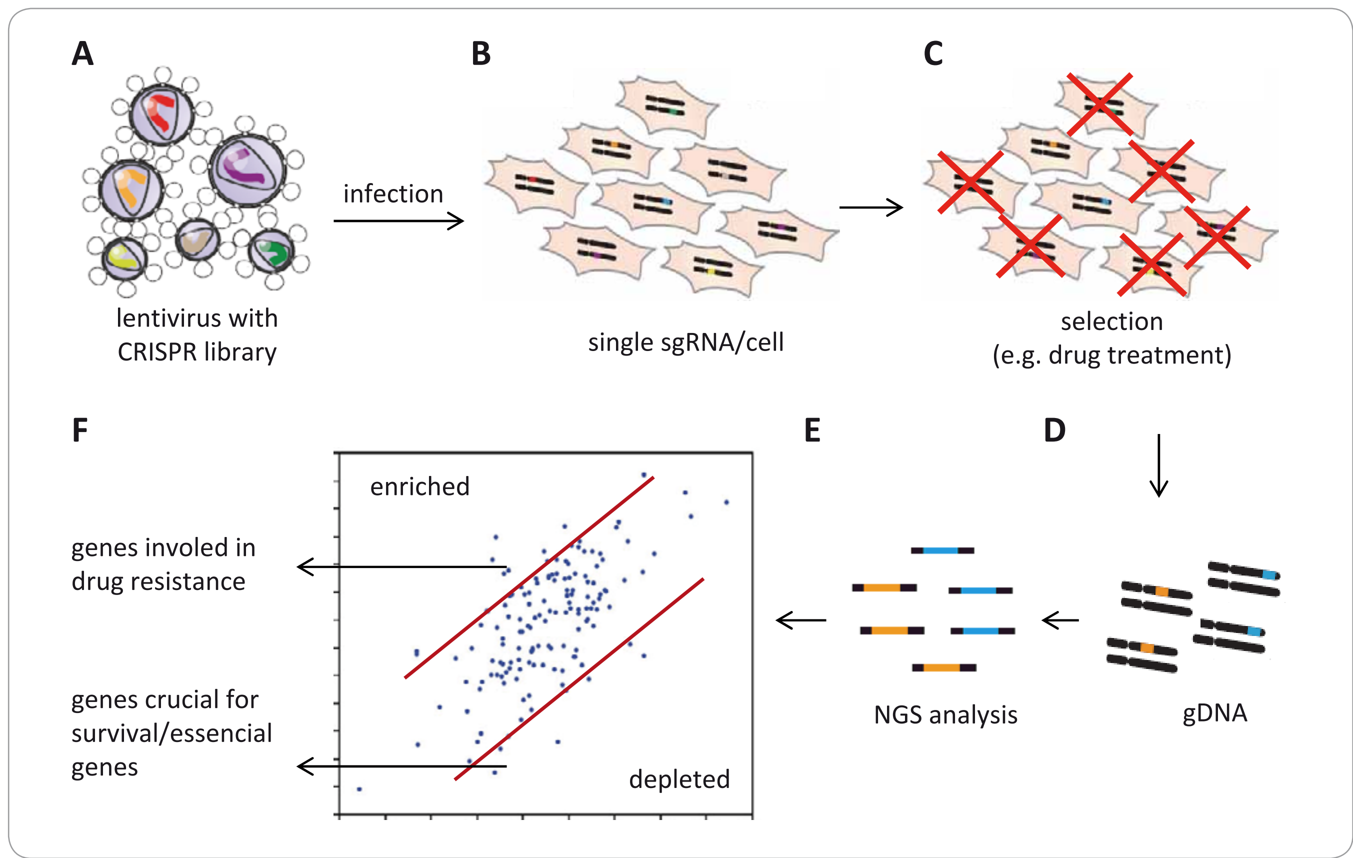

Fig. 3. CRISPR screen – experimental workflow.
A. Lentiviral vectors containe CRISPR components (Cas9 and sgRNA) targeting defined set of genes.
B. Cell line infected with the final rate – 1 cell = 1 sgRNA = 1 targeted gene.
C. Selection pressure is applied on the cell line library (+ control population without selection).
D. Isolation of genomic DNA.
E. PCR amplification and NGS analysis of barcoded sgRNA.
F. Identification of enriched/depleted sgRNA – comparism to control/non-treated population.

plasmids expressing the CRISPR components – sgRNA and Cas9. There are two types of CRISPR libraries – activatory or inhibitory. The first one is used to promote expression of particular genes and the other to knock out (permanently inactivate) selected genes. The library can be either small, focused only on a limited, pre-selected group of specific genes or large, covering every gene in the human genome. The whole genome library contains at least 19,000 different plasmids (small, circular DNA possesing genes to be transfered and expressed in the recipient cells) each containing different sgRNA to cover every coding sequence in the human genome. In most set ups, however, each gene is targted by multiple different sgRNAs to increase the robustness and limit the potential off-target effects.

In most scenarios the plasmids are introduced into the *in vitro* cultured cells usually via viral infection. The screening experiment is designed in a way that every cell will receive only single sgRNA targeting just one specfic gene. Thus, each cell in population will have only one gene activated or knocked out (Fig. 3A, B). After establishing such library of geneticaly modified cells, the selection pressure is applied. Next steps depend on the type of screening and the seeked phenotype.

When performing the resistance studies, cells are treated with a drug of interest. After certain time, only the resistant cells survive and their genomic DNA is collected and analysed by NGS. Abundance (enrichment/depletion) of all sgRNAs is then compared to the control/non-treated population. Because every sgRNA targets only one, unique genomic sequence the hits can be easily assigned to specific genes and their contribution to the cell survival under the tested stress condition is revealed (Fig. 3C–F).

Safety constraints for viral vectors

As already mentioned, in the experimental laboratory settings, the plasmids coding sgRNA and Cas9 are introduced into the cells mostly via infection with retroviral or lentiviral particles. Lentivirus-based systems are more commonly used due to their ability to infect both non-dividing and diving cells. The viral partical is made and delivered in a from of pseudovirus, from which all virulent genes were removed and the virus is unable to replicate in the host cell. To comply with the safety restrictions, production of the

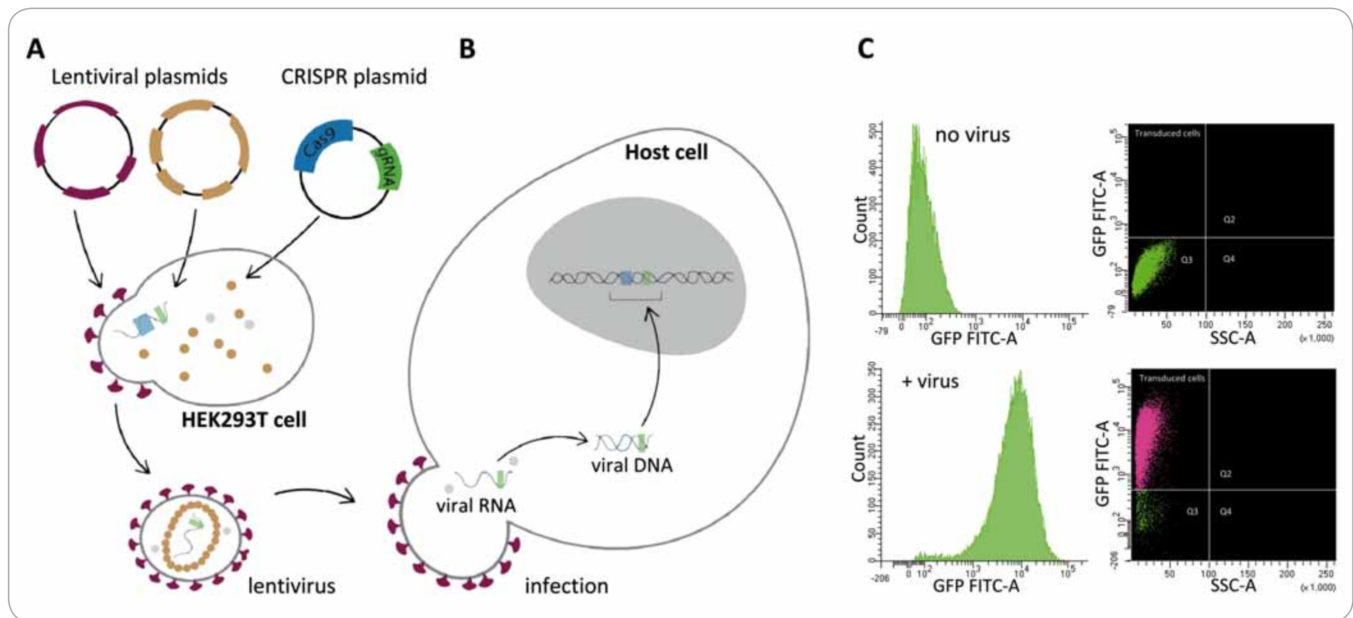

Fig. 4. Generation of pseudo-lentivirus.

A. Virus production – plasmids coding capsid proteins and enzymes required for virus integration are introduced into the virus producing cell line (usually HEK293T).

B. Infection of the target cell – viral RNA is reverted into DNA and intergrated into the host genome.

C. Example experiment – infection with lentivirus coding GFP. The non-infected cells do not show any fluorescence in the GFP/FITC channel analysed by flow cytometry, while > 95% of cells infected with GFP-coding virus are positive.

pseudoviral particles belongs to the biohazard class II and can be done only in the laboratories with dedicated cell culture rooms that are equipped with the appropriate laminar flow hoods containing HEPA filters. Other safety rules including adequate waste managment must be also fulfilled. An official approval for the work with geneticaly modified organisms of class I and II is also needed [19].

To ensure safety of the viral system, genes encoding components for lentivirus are splitted into multiple plasmids. Components of the viral capsid and enzymes required for virus integration in the host cell are usually expressed from two (sometimes even three) plasmids. The third (fourth) plasmid then possess the actuall transgenes – Cas9, sgRNA and selection marker to monitor and enrich the infected cells. To generate the virus all plasmids need to be first transfected into the virus producing cells. In the laboratory settings, the viral particles are made in the specialised HEK293T cells that express the SV40 Large T antigen that is required for the viral genome replication and ensures high virus titers. The finally assembled viral particals are then secreted into the culture media, which can be either directly used to infect the host cells or further concentrated to increase viral titer and improve infection efficacy. Selection of infected cells is mostly based on expression of fluorescent protein (GFP, RFP, etc.) or antibiotic resistance gene (puromycine, blastocidin, etc.) in addition to introduced transgene (Fig. 4A–C).

Successfull CRISPR screens in MM

The first CRISPR screens done with MM cell lines searched for genes responsible for resistance to bortezomib, carfilzomib and lenaliomide [20,21]. The first study identified proteasomal regulatory subunit PSMC6 as the gene confering bortezomib and carfilzomib resistance in the RPMI8266 and KMS11 cell lines. However, the work failed to correlate PSMC6 expression levels in the primary MM samples with the clinical outcome of the bortezomib treated patients. The study currently continues by investigating the PSMC6 mutation rate in the relapsed MM patients after bortezomib treatment. It is likely that also other factors including mutational status of PSMC6 and especially contribution of tumor microenviroment will have an impact on the resistance development.

Another CRISPR whole genome screen in MM cell lines focused on lenalidomide, which primary target is the ubiquitin E3 ligase Cereblon [21]. Lenalidomide is the thalidomide derivate and by inhibiting function of Cereblon it facilitates degradation of the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3). Interestingly, the precise mechanism of action and consequently the therapeutic effects of lenalidomide are different compare to thalidomide even through they both target the same molecule [22]. The study utilised CRISPR screen in the lenalidomide sensitive MM cell line MM1S and highlighted the ubiquitin conjugating enzymes UBE2D3 and UBE2G1 as important players in the lenalidome-mediated regulation of Cereblon function [21]. Further follow-up experiments demonstrated distinct functional roles for both enzymes and provided additional hits for the targeted drug design in the treatment of MM with a potential to avoid development of resistance and lower the side effects exerted by lenalidomide.

A smaller, focused CRISPR screen targteting only 20 critical MM genes was used in an elegant study that examinated clonal evolution of MM cells and development of metastasis in the *in vivo* settings. The study defined specific plasma cell subclones with a high metastatic potential and found unique metastatic gene signature together with two genes acting as potential regulators of metastasis formation in MM [23]. The same group also established a platform for a genome wide *in vivo* CRISPR screening in the xenograft mouse models to investigate genes regulating metastasis dissemination and specific organ colonization in progression of MM. The authors also investigated contribution of multiple genes on several stages of MM development – early primary tumor, late primary tumor, and bone marrow metastasis. As crucial genes for both early and late developmental stages were found genes involved in mTORC1 and DNA repair pathways, many of which are regulated by MYC and cell cycle related targets of E2F transcription factors [24].

The *in vivo* CRISPR screening could be also used to explore essential genes in response to targeted drug therapies and/or immunotherapies. Thus, the CRISPR-based *in vivo* screening offers a very powerful tool for functional genomics and promise many exciting discoveries.

CRISPR in MM therapy

MM takes a reputed privilage as one of the first human diseases for which CRISPR will be used as a method applied directly in the treatment process. The last year (2016), scientists at the University of Pennsylvania announced a launch of a pioneer study using geneticaly modified patient T-cells targeted against cancer cells including MM, melanoma and sarcomas [25,26].

Their strategy is following up on the fingings of high NY-ESO1 expression in plasma cells of MM patients with a poor-prognosis. First, the already established technology of the cell therapy based on the chimeric antigen receptor (CAR) will be used to generate geneticaly modified T-cells (CAR T-cells) specifically targeting cells with increased expression of NY-ESO1 [27]. Secondly, the PD-1 receptor, which is expressed on the surface of antigen-activated and exhausted T-cells, will be deleted by CRISPR. The engagement of PD-1 with its ligands PD-1L and PD-2L, that are highly expressed on cancer cells including MM, leads to temporal down-regulation of T-cells functions and increases their susceptibility to apoptosis [28]. Therefore, removing PD-1 from autologous T-cells is a viable and promising strategy to preserve and activate the tumor specific T-cells (CAR T-cells) that will likely promote specific targeting of patient MM cells.

Conclusion

Application of CRISPR will have a profound effect on our understanding of the development of many hematological malignancies including monoclonal gammapaties and specifically MM. CRISPR-based genome editing offers an unprecedented ability to define novel MM relevant genes and mutations, provide rational targets for new drug development, reveal resistance mechanisms and most importantly it moves the field of genetic manipulation to the next level – a real personalised medicine. In summary, extreme simplicity, broad versatility and aplicability, very high efficacy together with low cost compare to all previous genome modifying methods makes CRISPR the gene eiditing tool of choice in research and clinic with a great potential. Thus, CRISPR technology will undoubtedly transform the way we conduct both medical research and the actual treatment of genetic diseases in the near future.

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Whole Exome Sequencing of Aberrant Plasma Cells in a Patient with Multiple Myeloma Minimal Residual Disease

Celoexomové sekvenování aberantních plazmatických buněk pacienta s minimální reziduální chorobou u mnohočetného myelomu

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Summary

Multiple myeloma is a plasma cell dyscrasia. It is the second most common hematological malignancy which is characterized by proliferation of clonal plasma cells producing harmful monoclonal immunoglobulin. Despite treatment modalities greatly evolved during the last decade, small amount of aberrant residual cells reside in patients after therapy and can cause relapse of the disease. Characterization of the residual, resistant clones can help to reveal important therapeutic targets for application of effective and precious treatment. We use CD38, CD45, CD56 and CD19 sorted aberrant plasma cells to perform next generation sequencing of their exome. Among the 213 genes in which at least one variant was present, the most interesting was found gene *NRAS*, one of the most often mutated gene in multiple myeloma, and homologs of 88 gene panel previously used for multiple myeloma sequencing among which was a gene previously identified as gene meaningful in bortezomib resistance. Nevertheless, the results of next generation exome sequencing need to be interpreted with caution, since they rely on bioinformatical analysis, which is still being optimized. The results of next generation sequencing will also have to be confirmed by Sanger sequencing. Final results supported by larger cohort of patients will be published soon.

Key words

multiple myeloma – minimal residual disease – exome – next generation sequencing

Supported by The Ministry of Education, Youth and Sports (Specific university research of the Faculty of Science, University of Ostrava; project No. SGS18/PřF/2017-2018 and The Institutional Development Plan of University of Ostrava, project No. IRP201550) and by Ministry of Health (CZ – DRO – FNOs/2016 and 17-30089A).

Práce vznikla za podpory Ministerstva školství, mládeže a tělovýchovy (Specifický VŠ výzkum Přírodovědecké fakulty, Ostravské university v Ostravě pod grantovým číslem SGS18/PřF/2017-2018 a Institucionálního rozvojového plánu Ostravské univerzity; projekt č. IRP201550) a Ministerstva zdravotnictví ČR (ČR – RVO – FNOs/2016, 17-30089A).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 27. 6. 2017
Accepted/Přijato: 30. 6. 2017

doi: 10.14735/amko20172S75

Souhrn

Mnohočetný myelom je dyskrazie plazmatických buněk. Je to druhá nejčastější hematologická malignita, která je charakterizována proliferací klonálních plazmatických buněk produkujících škodlivý monoklonální imunoglobulin. Navzdory možnostem léčby, která se v posledním desetiletí velmi vyvinula, se i u pacientů po terapii často nachází malé množství abnormálních reziduálních buněk, které mohou způsobit návrat onemocnění. Charakterizace reziduálních rezistentních klonů může pomoci odhalit důležité terapeutické cíle pro aplikaci účinné personalizované léčby. Pro tento výzkum používáme aberantní plazmatické buňky vysortované podle markerů CD38, CD45, CD56 a CD19, následované sekvenováním nové generace jejich exomu. Ze seznamu 213 genů, ve kterých byla přítomna alespoň jedna varianta, byl přítomen zajímavý gen *NRAS*, který je jedním z nejčastěji mutovaných genů u mnohočetného myelomu, homology genů z panelu 88 genů dříve použitých pro sekvenování nové generace u mnohočetného myelomu, mezi kterými byl i gen identifikovaný jako významný při rezistenci na bortezomib. Přes to, že jsou výsledky exomového sekvenování prvního pacienta zajímavé, jsou pouze předběžné a při jejich interpretaci je potřeba být obezřetný, neboť závisí na optimalizaci bioinformatické analýzy, která stále probíhá. Získané výsledky je také potřeba validovat Sangerovým sekvenováním. Brzy budou prezentovány definitivní výsledky podpořené větší kohortou pacientů.

Klíčová slova

mnohočetný myelom – minimální reziduální choroba – exom – sekvenování nové generace

Introduction

Multiple myeloma (MM) is a plasma cell (PC) malignancy which belongs to a group of diseases called monoclonal gammopathies. Common feature of MM is extensive proliferation and accumulation of clonal PCs (aPCs) in bone marrow, connected with increased production of aberrant monoclonal immunoglobulin (M-protein). The M-protein can be detected by protein electrophoresis in gamma globulin fraction in blood serum and/or urine samples [1]. The most common symptoms of MM are hypercalcaemia, renal insufficiency, anaemia and bone lesions (CRAB) but also non-CRAB features, for example neuropathy, can be present [2]. Due to high genetic heterogeneity (around 5–6 clones per tumour) treatment of MM is problematic [3].

Thanks to the availability of a number of effective drugs and strategies like combination of proteasome inhibitors and immunomodulatory drugs followed by autologous transplantation, patients with MM now achieve complete response more often compared to the past (up to 75% reaching a near-complete or complete response) [4,5]. In both groups of patients (minimal residual disease (MRD) and residual disease) persist residual cells that can cause relapse of the disease [6,7]. Therefore, characterisation of residual cells resistant to a frontline therapy will become an important research aim in near future. The reason for increased interest in properties of residual cells is the fact that today there is a range of targeted drugs that can be used to eliminate a well-defined residual cell population [8,9].

We use method of whole genome amplification followed by next generation exome sequencing to study mutation spectra in aberrant PCs (A-PCs) residing in myeloma patients after treatment. The complex analysis will require mapping of single nucleotide polymorphism, identification of mutated genes and copy number evaluation, pathway analysis and other related bioinformatical analyses. Here we report preliminary results of exome sequencing from our pilot patient with MM MRD.

Characterization of residual cells in MM MRD

Detection of residual cells is an essential starting point for its fluorescence-activated cell sorting and subsequent molecular characterization. Nowadays, aberrant PCs (A-PCs) in MRD can be detected by many techniques – multiparameter flow cytometry (MFC) with standardized second generation Euroflow protocol and/or Euroflow-based next generation flow (NGF) approach [10], allele-specific oligonucleotide qPCR (ASO-qPCR) [11], fluorescent polymerase chain reaction [12], next generation sequencing (NGS) of immunoglobulin gene segments [13], whole-body magnetic resonance imaging (WB-MRI) [14] or positron emission tomography/computed tomography (PET/CT) [15]. PET/CT and WB-MRI are not used very often. Each technique has its own limits of malignant cell population assessment. We selected reliable, relatively fast and cheap, universally applicable and high sensitive (10^{-5}–10^{-6}) [16] method MFC, which is based on identification of surface cell markers (proteins) which in combination characterizes specific type of cells. Detection of cells by MFC is also used for fluorescence activated cell sorting [17].

Detection of A-PCs by flow cytometry

Markers CD38 and CD138 are used as markers for PC identification. There is no single immunophenotypic marker for identification of A-PCs, therefore combination of several important markers are used. Combination of CD38 and CD45 should be used for identification of myeloma PCs (their expression is very frequently down regulated) together with CD19 (negative on aPCs) and CD56 (positive on aPCs in 60–75% of all MM patients) assessment. There are also several markers that show aberrant pattern in myeloma cells like expression of CD117, CD28 or decreased/missing expression of CD27 compared to normal PCs (N-PCs) [18].

NGS of exome or genome in MM

MM is a genetically heterogeneous disease [3]. Recent study identified 11 "the most often" mutated genes (*KRAS* – 23%, *NRAS* – 20%, *DIS3* – 11%, *FAM46C* – 11%, *TP53* – 8%, *BRAF* – 6%, *TRAF3* – 5%, *PRDM1* – 5%, *RB1* – 3%, *CYLD* – 2%, *ACTG1* – 2%) in 203 untreated and previously treated patients [3]. Most of these mu-

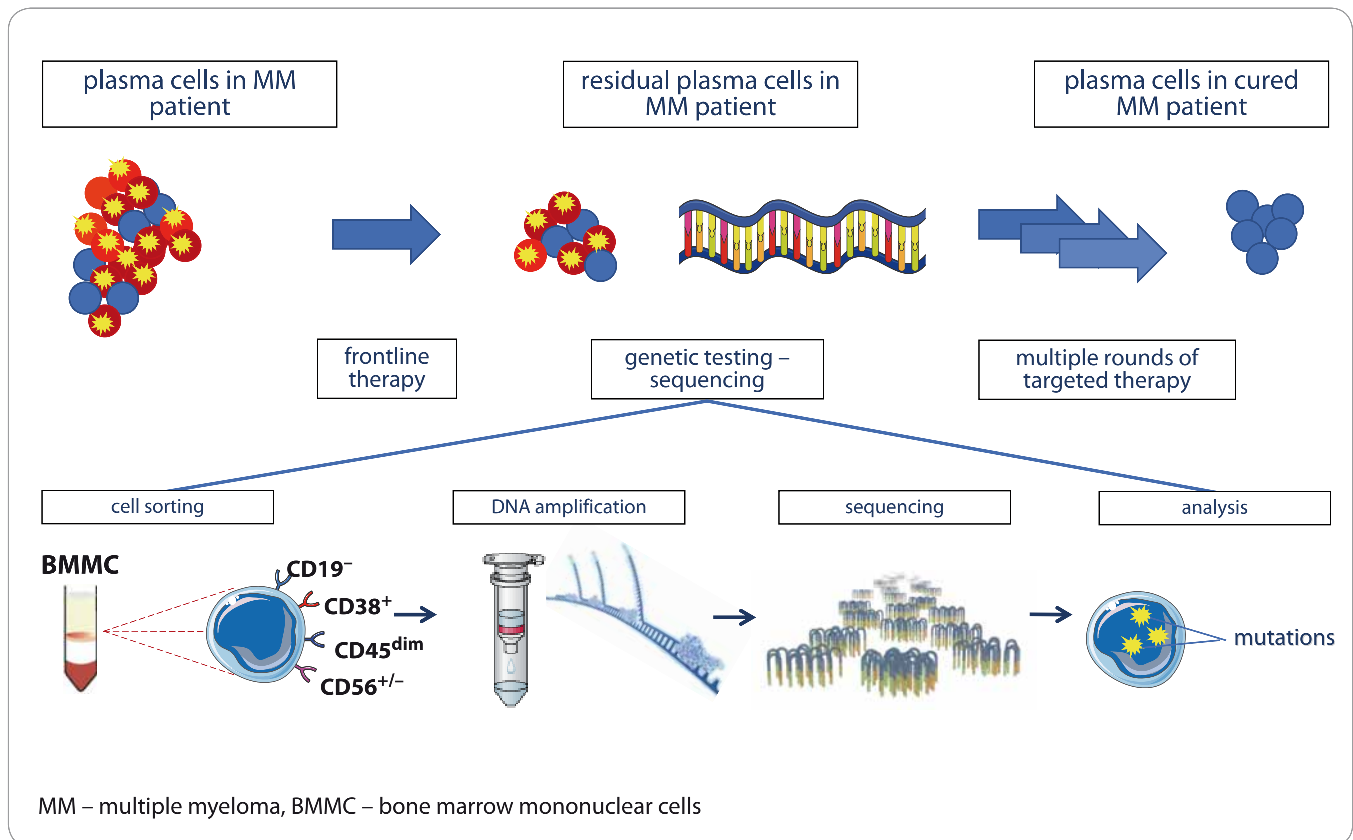


Fig. 1. Schematic overview of MRD genetic analysis.

After frontline therapy number of aPCs decreases, residual mutations can be studied and targeted therapy eliminating those mutations can be applied. Heterogeneous population of PCs is depicted as coloured circles, A-PCs are highlighted with asterisks.

tations were present in higher frequencies across the cohort of patients with refractory MM (*KRAS* – 32%, *NRAS* – 26%, *TP53* – 26%, *BRAF* – 18%, *CRBN* – 12%, *FAM46C* – 12%, *ATM* – 10%) [19].

Kortum et al. [19] created a MM mutation panel (M^{3}P) for targeted NGS, actually containing 88 genes, which are generally expressed in MM and mutated in > 2% of patients together with genes involved in MM important pathways (MAPK, nuclear factor κB, interleukin-6, cell cycle, MYC). Until now, there was not detected any unifying mutation common for all patients, as it is known for example for hairy cell leukemia (*BRAF*) [20] or Waldenström's macroglobulinemia (*MYD88*) [21].

In MM, intratumor heterogeneity was reported for aberrant cell population. Molecular analysis of tumour samples revealed that at least five subclones are present in patient. However, this number may depend on the sampling from specific bone marrow sites and the sensitivity of currently used detection methods [3]. Moreover, different myeloma clones can undergo several ways of evolution leading to linear, differential or branching development. In addition there are also clones that do not change its composition during the time of treatment [22].

Methods

Strategy of Work flow of our project is summarized in Fig. 1. The process of its methodological optimizing was published before [23].

Sampling

We are thankful for established collaboration and sample collection with centres in Ostrava, Hradec Králové, Olomouc, Brno, Bratislava and Pilsen. Basic overview of actual state of samples is shown in results. For the first patient in VGPR, samples of bone marrow and peripheral blood were taken after signing of informed consent. The material was collected in EDTA-anticoagulated tubes and further processed within 24 hours. Bone marrow of the patient was sampled into two tubes. The first one with more concentrated bone marrow was sent for investigation of MRD assessment in University Hospital Brno. The second tube was used for sorting and other processing of A-PC and N-PC population in University Hospital Ostrava.

A-PCs sorting

Presence of MRD was assessed by 8-color MFC (CD38-PB/CD45-PO/CD56-FITC/CD27-PE/CD138-PerCP/CD19-PC7/CD117-APC/CD81-APCH7) on BD FACSCanto II (BD Biosciences). A-PCs and N-PCs were identified and separated by fluorescence activated cell sorting (FACS) using 4-color MFC. CD45-PB, CD38-FITC, CD19-PC7 and CD56-PE (Fig. 2) on FACS Aria III (Becton Dickinson) cell sorter. The threshold for sorting was set as 100 aberrant cells in 10^6 and total amount of them needed

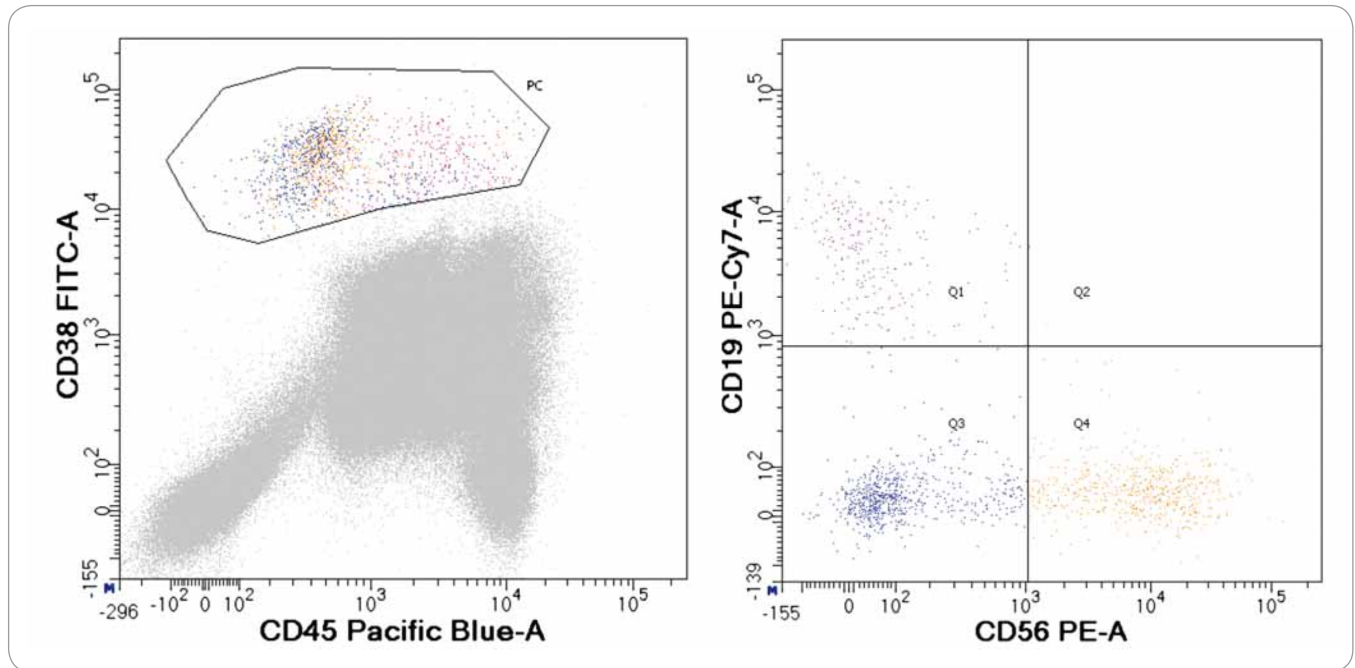
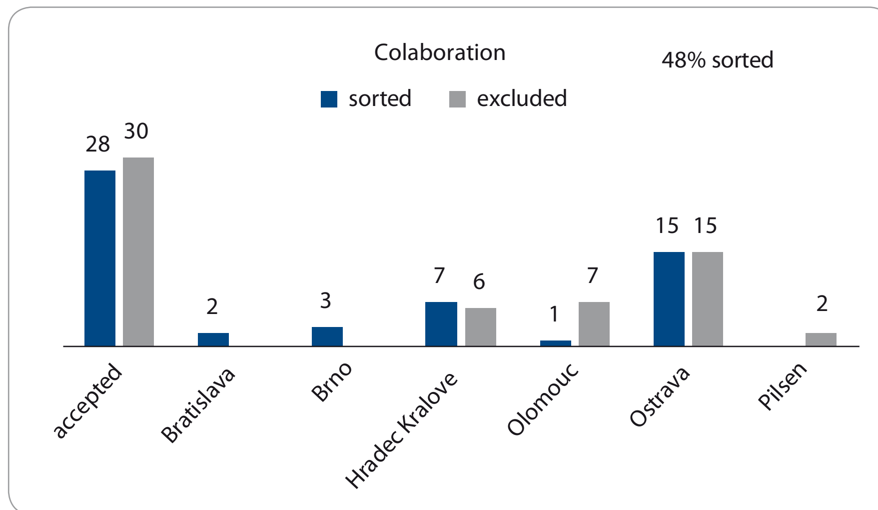


Fig. 2. Separation of MRD A-PCs.

A-PCs were identified using specific antigens: CD45 and CD38 for identification of total PCs, CD19 and CD56 for detection of A-PC. In this case specific aberrant immunophenotype is $CD45^{dim}$, $CD38^{++}$, $CD19^{-}$, $CD56^{+/-}$. While N-PCs are characterized by expression of CD19 and higher expression of CD45 compared to A-PCs.



Graph 1. Overview of samples sorted acording to colaborating centres.

to be at least 500 sorted aberrant cells from whole sample, which was established as a minimum for right working of amplification [23].

DNA isolation and exome sequencing

DNA was amplified directly from 2,000 cell aliquots of A-PCs and N-PCs by REPLI-g Single Cell Kit (QIAGEN, Hilden, Germany). Amplified DNA was purified by QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Sequencing libraries with 300 bp inserts were prepared using Sure Select XT Human All Exon v5 Kit (Agilent Technologies, Santa Clara, USA) target size 51 MB and exome pair-end sequencing was performed in Macrogen company using HiSeq4000 platform (Illumina, San Diego, CA, USA) with 100 bp length. Expected coverage depth was set on 100×.

Bioinformatics

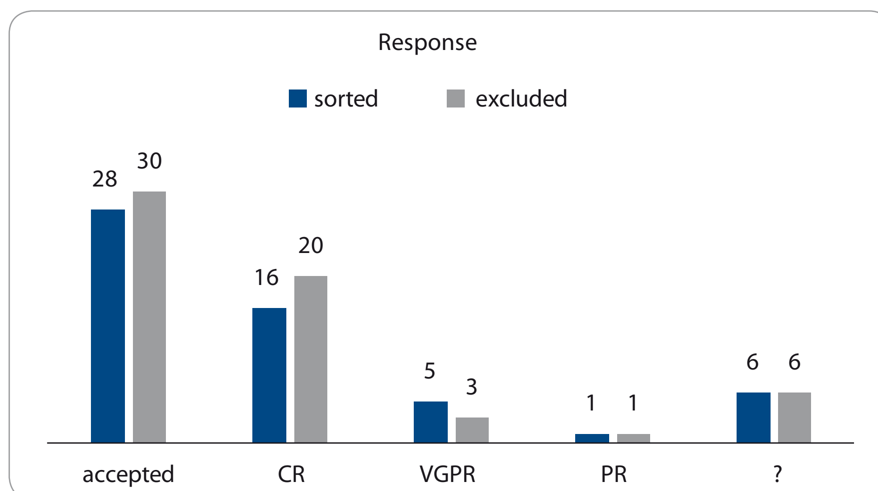
Trimming of raw fastq files was performed by Trimmomatic software [24]. BWA MEM algorithm [25] was used for aligning reads to the ensembl GRCH38 reference genome by. Low mapping Phred quality (MQ < 10) reads were removed, the alignment files were sorted and converted to mpileup format. Editing and format conversion of sequence alignment files was done by SAMtools [26]. VarScan v 2.0 [27] was used for variant calling from mpileups (minimal coverage – 10, minimal supporting reads – 4, frequency threshold – 0.3) (VCF format). In-house scripts were used for annotation of VCF files against human reference genome GRCH38 with relevant GTF file (v 83). Subsequently, all variants were compared with dbSNP (build 149) database (ncbi.nlm.nih.gov) and functional predictions of nonsynonymous mutations were obtained from dbNSFP [28], MutTaster and Provean [29,30].

Preliminary results

Sampling

Amount of samples obtained from all centres and their suitability for cell sort-

ing are shown in Graph 1. In general, the sorting rate was 48%. Until 20. 6. 2017, we processed 58 patient samples of bone marrow. In 29 of them was present enough amount of material for cell sorting. Majority of samples (36) was obtained from patients in complete remission (CR). We finally sorted 16 (44%) of them. Eight samples were from patients with very good partial response (VGPR) and sorting was successful for five of them (63%). From two samples of patients with partial response (PR), we were able to sort one case (Graph 2). In 12 cases we are missing final assessment of patient response.



Graph 2. Overview of samples acording to treatment response.
CR – complete remission, VGPR – very good partial response, PR – partial response

Case report

In whole study we plan to analyze 50–70 samples of bone marrow from patients with MRD. Here we report the case of first pilot patient, from which we obtained preliminary results of exome sequencing. In whole exome of A-PC population were found 2,632 variants and 36% (938) of them was in coding sequences (CDS)/splice-site. In CDS, the most commonly found variants were single nucleotide variants (SNVs) (77%). Ratio of synonymous and non-synonymous SNVs was balanced (37% and 40%). Insertions were more common (22%) then deletions (1%). Different variants of splice-sites were found in six cases (three SNPs and three deletions).

At least one variant was found in 213 genes after filtering of common single nucleotide polymorphisms in the population and benign predictions for function of protein. These genes were compared with records in The drug gene interaction database (http://dgidb.genome.wustl.edu/). According to this database, interaction with some drugs is already known for 30 out of those 213 genes. In two of those 30 genes was previously described interaction with bortezomib and carfilzomib. One of them possibly causes resistance on bortezomib and our patient was really treated with this drug.

We also compared those 213 genes with at least one variant with panel of 88 MM specific genes (M^3P) used for sequencing of MM before [19]. Surprisingly, *NRAS* was the only gene that completely fit to the list.

Small amount of genes found with a variant in our data were homologs of genes included in the M^3P panel. Complete results supported by enlarged cohort of MRD patients will be published soon.

Conclusion

NGS is a robust method for identification of variants in the genome. Context of variants and interactions in genome is not well understood yet. Problems of residual disease lies in the fact that it leads to relapse and resistance to the treatment [19]. Better understanding of genetic changes that occur in residual malignant cells may help to find a way how to eliminate them and completely treat the patient.

Despite preliminary exome sequencing results are promising, the careful interpretation is needed, because process of its bioinformatical assessment is still in process and the results require validation by Sanger sequencing.

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Diagnostic Tools of Waldenström's Macroglobulinemia – Best Possibilities for Non-invasive and Long-term Disease Monitoring

Diagnostické přístupy u Waldenströmovy makroglobulinemie – nejvhodnější dostupné možnosti neinvazivního a dlouhodobého monitorování nemoci

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Summary

Waldenström's macroglobulinemia (WM) is a B-cell malignancy characterized by high level of monoclonal immunoglobulin M (IgM) paraprotein in blood serum and associated with the bone marrow infiltration by malignant cells with lymphoplasmacytic differentiation. WM remains incurable advances in therapy. Most of WM cases are associated with a somatic point mutation L265P in *MYD88*. Significantly higher risk of progression from the IgM monoclonal gammopathy of undetermined significance (IgM MGUS) to WM for patients with mutated *MYD88* gene suggests that this mutation is an early oncogenic event and plays a central role in development of malignant clones. The second, most prevalent mutation in WM is found in the *CXCR4* gene and is often associated with drug resistance and aggressive disease presentation. Therefore, detection of these mutations (*MYD88*^{L265P} and *CXCR4*^{S338X}) could be useful diagnostic and prognostic tool for the patients with WM. While detection of these mutations in bone marrow sample is common, the aim of our study was to compare sensitivity of detection of mutation from different cell fraction from peripheral blood and bone marrow. The results show possibility to describe *MYD88* and *CXCR4* mutation status even from peripheral blood sample (sensitivity for *MYD88*^{L265P} was 100%, for *CXCR4*^{S338X} 91%), which significantly facilitate material collection. Moreover, comparable detection sensitivity of these mutations in bone marrow and peripheral blood samples examined before and during the therapy offers a promising tool for more routine diagnostic and monitoring of disease progression.

Key words

Waldenström macroglobulinemia – hematology – neoplasms – lymphoma – mutation – *MYD88* – *CXCR4*

This study was supported by the Ministry of Education, Youth and Sports (Institutional Development Plan of the University of Ostrava IRP201550 and Specific university research of the Faculty of Medicine, University of Ostrava SGS09/LF/2016-2017), and by the Ministry of Health (DRO-FNOs/2015).

Práce vznikla za podpory Ministerstva školství, mládeže a tělovýchovy (Institucionální rozvojový plan OU IRP201550 a Specifický VŠ výzkum Lékařské fakulty OU SGS09/LF/2016-2017) a Ministerstva zdravotnictví (RVO-FNOs/2015).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE recommendation for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Obdrženo: 9. 6. 2017
Accepted/Přijato: 10. 7. 2017

doi: 10.14735/amko20172S81

Souhrn

Waldenströmova makroglobulinemie (WM) je B buněčné maligní onemocnění charakteristické vysokou hladinou monoklonálního imunoglobulinu M (IgM) v krevním séru a je spojeno s infiltrací kostní dřeně lymfoproliferativní populací maligních buněk. Navzdory pokrokům v léčbě zůstává toto vzácné onemocnění stále nevyléčitelné. Téměř všechny případy WM souvisí s přítomností somatické bodové mutace L265P v proteinu MYD88. Pacienti s touto mutací mají signifikantně vyšší riziko progrese z IgM monoklonální gamapatie nejistého významu (IgM MGUS) do WM, což nasvědčuje významnou roli této mutace v nádorové transformaci. Druhá nejčastější mutace v genu pro chemokinový receptor CXCR4 je spojena s klinickým významem, konkrétně ovlivňuje rezistenci k léčbě. Detekce zmíněných mutací (*MYD88*^{L265P} a *CXCR4*^{S338X}) se tudíž jeví být užitečným diagnostickým a prognostickým nástrojem u pacientů s WM. Zatímco vyšetření mutací z kostní dřeně je běžné, naším cílem bylo porovnat senzitivitu záchytu mutací z odlišných buněčných frakcí periferní krve a kostní dřeně. Výsledky ukazují na možnost vyšetření mutačního stavu i z periferní krve (senzitivita pro *MYD88*^{L265P} je 100 %, pro *CXCR4*^{S338X} je 91 %), což výrazně usnadňuje odběr potřebného materiálu. Navíc obdobná senzitivita záchytu mutací ze vzorků kostní dřeně a neinvazivního vyšetření periferní krve během progrese onemocnění a léčby se zdá být příslibem pro rutinní diagnostiku a monitorování onemocnění.

Klíčová slova

Waldenströmova makroglobulinemie – hematologie – neoplazmy – lymfom – mutace – *MYD88* – *CXCR4*

Introduction

Waldenström macroglobulinemia (WM) is defined as a lymphoplasmacytic lymphoma characterized by uncontrolled bone marrow (BM) infiltration with malignant cells characterised by plasmacytic differentiation and increased production of monoclonal immunoglobulin M (IgM) [1]. WM accounts for approximately 2% of hematologic neoplasms with nearly 2× higher incidence in men (7.3 in men and 4.2 in women per million in the Europe) and with a prevalence in elderly people of median age about 63–68 years in Caucasians population. Overall survival (OS) ranges from 5 to 10 years. Among the main causes of death belong disease progression, treatment complications or transformation to high-grade lymphoma [2].

Clinical course of WM is typically indolent, but there are district differences in OS [3]. Asymptomatic patients require no treatment and their survival is not different from healthy population [4]. WM patients with cytopenias, organomegaly, lymphadenopathy and IgM related complications as hyperviscosity, cryoglobulinemia, cold agglutinin in disease, amyloidosis and progressive neuropathy, require a treatment [5]. Twenty-five percent of patients with WM show familial predisposition documented by the presence of B-cell malignancies or multiple cases of WM or IgM type of monoclonal gammopathy of undetermined significance (IgM MGUS) in the family [6–8].

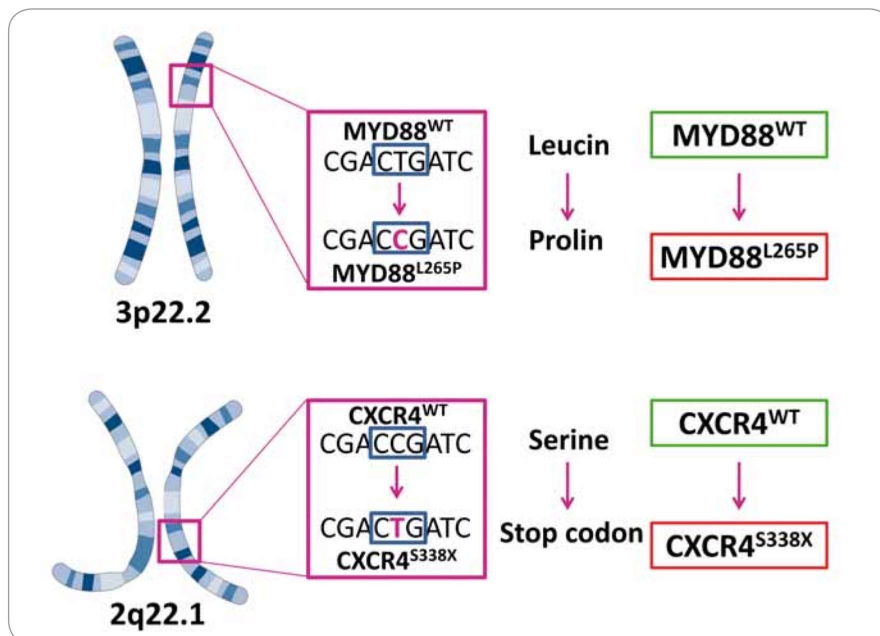

Fig. 1. Mutation *MYD88*^{L265P} and *CXCR4*^{S333X} in WM.

Two most common mutations in WM cells, *MYD88*^{L265P} and *CXCR4*^{S333X}, are presented. Figure shows position, nucleotide change and amino acid change of both mutations. Single nucleotide substitution T→C at position 3p22.2 resulting switch of leucine to proline at amino acid position 265 (L265P) and leading to constitutive activation of MYD88. Single nucleotide substitution C→T at position 2q22.1 resulting in stop codon (S338X) and leading to decrease CXCR4 internalization.

IgM MGUS is a pre-malignant disorder preceding development of WM and other lymphomas. MGUS is defined as a pathological state, when BM is infiltrated by less than 10% of aberrant cells and M-protein level (IgM in case of IgM MGUS) of less than 30 g/L. Overall MGUS affects approximately 3.5% of the population over 50 years of age, the median age of patients at diagnosis is 62 years. MGUS represents a risk factor for the development of symptomatic WM, various B-cell non-Hodgkins lymphoma, multiple myeloma or primary amyloidosis (AL) with total incidence 1% per year. The potential risk of IgM MGUS lies also in the production of aberrant monoclonal IgM which may be responsible for organ damage due to the targeting of endogenous antigens or antibody deposition in tissues [9,10]. Treatment of asymptomatic IgM MGUS patients is not indicated [11].

The transition stage between IgM MGUS and WM is known as smoldering WM (SWM). SWM is a poorly de-

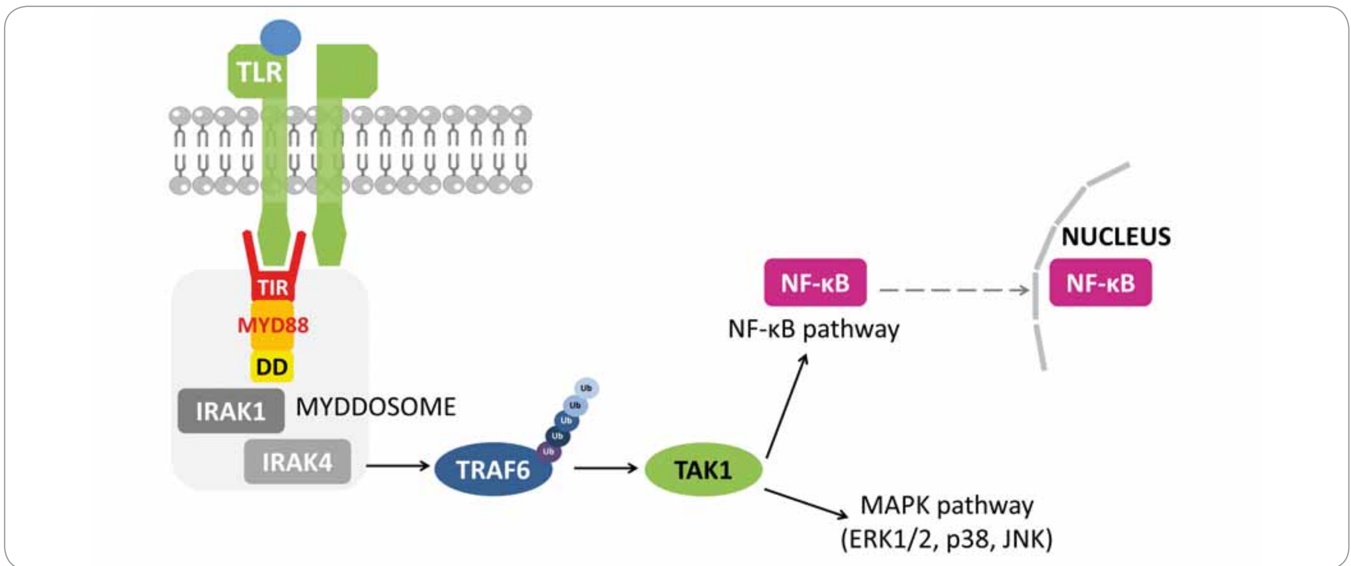

Fig. 2. MYD88 signalling pathway.

MYD88 is an adaptor protein for Toll-like receptors (TLR). Its modular structure consists of a Toll/IL-1R (TIR) domain at COOH terminus and a death domain (DD) at NH2 terminus. MYD88 binds the serine-threonine kinases IRAK and together create myddosome, responsible for polyubiquitination of TRAF6 and activation of the TAK1 protein kinase complex. TAK1 lead to activation of two different pathways, NF-κB and MAPK (ERK1/2, p38, JNK).

scribed asymptomatic phase with an increased risk of progression to symptomatic WM. A presence of a serum IgM value ≥ 3 g/dL and/or ≥ 10% BM lymphoplasmacytic infiltration with no evidence of end-organ damage (anaemia, hyperviscosity syndrome, lymphadenopathy, hepatosplenomegaly etc.) is typical for SWM. The median age at time of diagnosis is 63 years. The probability of progression to symptomatic WM is 12%, which is slightly higher than risk for IgM MGUS. Because both entities, SWM and IgM MGUS, represent a possible risk of developing the symptomatic stage, we could speculate that most WM patients have event. gone through the benign stages of IgM MGUS and SWM before developing clinical symptoms [12,13].

A broad spectrum of novel drugs is currently available for the therapy, including monoclonal antibodies, proteasome inhibitors and Bruton tyrosine kinase inhibitors. Treatment should be tailored to the individual patient while covering many clinical factors [8].

Myeloid differentiation factor 88

A highly recurrent somatic mutation in gene *MYD88* (T>C) at the position 38182641 in chromosome 3p22.2 (Fig. 1) results in amino acid change from leu-

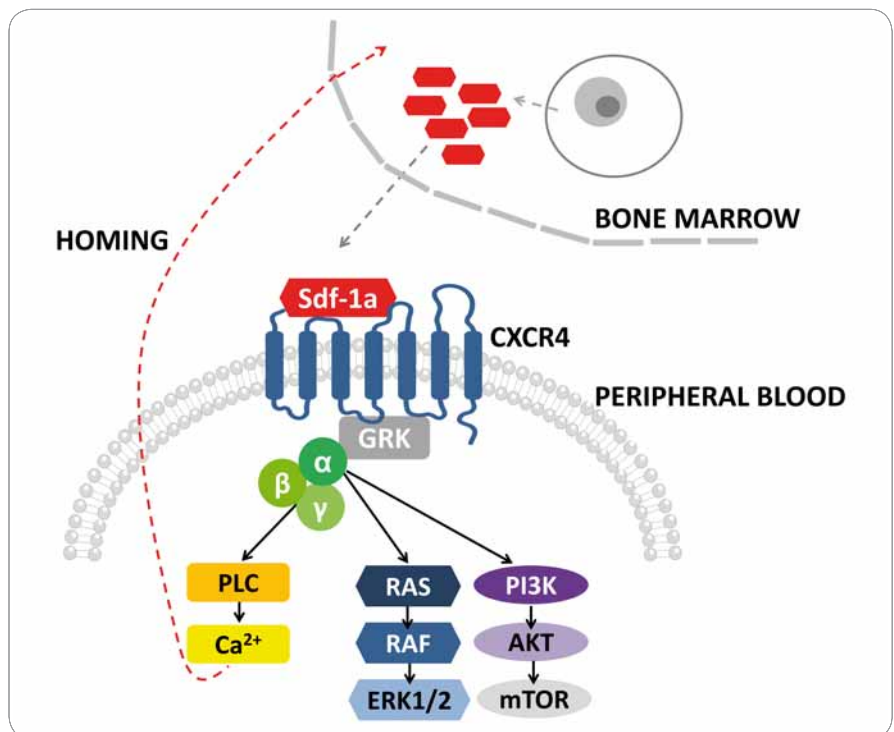

Fig. 3. CXCR4 signalling pathway.

C-X-C chemokine receptor type 4 (CXCR4) is expressed on peripheral blood cells (PB cells) – monocytes, B-cells, and naive T-cells. Stromal derived factor 1a (SDF-1a/CXCL12), ligand for CXCR4, is released by bone marrow stromal cells and binds to extracellular loops of CXCR4. G protein-coupled receptor kinase (GRK) interacts with intracellular loops of CXCR4 and diverse conformations of CXCR4 complex subunits regulate different signals. Dimer of Gβ and γ subunits regulates intracellular calcium mobilization through phospholipase C (PLC), whereas Gαtriggers PI3K/AKT/mTOR and ERK1/2 signalling therefore regulates cell survival and proliferation.

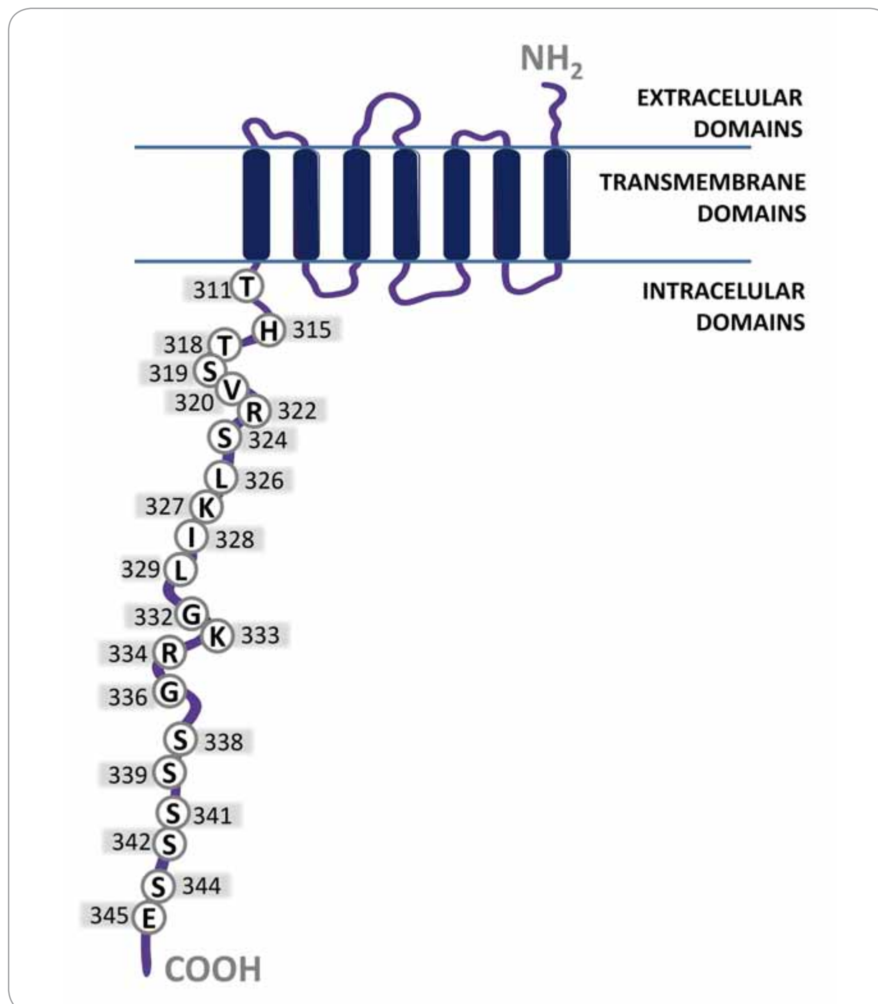

Fig. 4. Familiar mutation in CXCR4.

The table shows *CXCR4* mutation detected by Sanger sequencing in WM patients. Mutated amino acid sites at the C-terminal domain are depicted by white balls labelled with number of position and name of amino acid.

cine to proline (L265P). This mutation was first identified in WM patients by Treon et al. using whole-genome sequencing of paired tumor and normal samples [14]. The results were confirmed by multiple groups using Sanger sequencing or allele-specific polymerase chain reaction [14,15].

Myeloid differentiation factor 88 (MYD88) is recurrently mutated in 95% of WM and 50% of patients with IgM MGUS [13]. MYD88 is an essential adaptor protein in the Toll-like receptors (TLRs), interleukin (IL) -1 and IL-18 signalling pathways (Fig. 2). Modular structure of MYD88 consists of the Toll/IL-1R (TIR) domain at the C-terminus and the death domain at the N-terminus. MYD88 binds the serine-threonine kinases IRAK1 and IRAK2. After engagement of TLR receptor, IRAK1 is activated and associates with the E3 ubiquitin ligase TRAF6, which autoubiquitinates itself and the TAK1 protein kinase complex. TAK1 (MAPKKK family member) forms a complex interacting with polyubiquitin chains and lead to the TAK1 activation. Once active, TAK1 triggers two separate signalling cascades – the NF-κB and MAPK pathway. In the NF-κB axis, the inhibitory protein IκBα is phosphorylated by the IKK complex and undergoes proteasome degradation. This step allows NF-κB translocation into the nucleus and induces expression of pro-inflammatory genes. In the second scenario, the MAPK family members (ERK1/2, p38, JNK) together with activated TAK1 regulate other parts of the inflammatory response. Taken together MYD88 is essential for induction of NF-κB and MAPK signalling pathways in reaction to IL-1, IL-18, lipopolysacharide (LPS) and stimuli engaging some of the TLR receptors [16–18].

Overexpression of $MYD88^{L265P}$ is supportive for survival and growth of WM cells [18]. *MYD88* mutation status can also affect treatment response. This is supported by outcomes of the clinical trial using ibrutinib in relapsed patients with WM. The trial showed major responses and fewer OS among patients with wild type MYD88 and C-X-C chemokine receptor type 4 (CXCR4) compare to patients with aberrantly activated $MYD88^{L265P}$ and either wild type CXCR4 or mutated CXCR4 (see below) [19].

Presence of overlapping pathological symptoms complicates discrimination of WM from other B-cells malignancies such as marginal zone lymphomas, IgM-secreting myeloma or follicular lymphoma. Thus, screening for *MYD88* mutational status in BM or more routinely in peripheral blood samples would greatly facilitate disease diagnosis. In patients with IgM MGUS, *MYD88* gene should be analysed to assess the risk of progression to WM. Quantitative assessment of *MYD88*^{L265P} may provide sensitive and inexpensive method to identify residual clones in WM patients after treatment and monitor the disease progression. [17,18]. Present knowledge suggests that *MYD88*^{L265P} plays role as an unifying event in the pathogenesis of WM but only a single mutation is unlikely to explain the malignant transformation from the premalignant to symptomatic stage [13]. In future, we will need to extend our knowledge of WM pathogenesis and aim our interest on translational research that can provide novel tools for direct use in clinic.

C-X-C motif chemokine receptor 4

While *MYD88*^{L265P} might be considered as the founder mutation, C-X-C motif chemokine receptor 4 (CXCR4) could be one the later events that accelerate WM progression. The *CXCR4* gene is located at

chromosome 2q22.1 (Fig. 1) and encodes the G-coupled receptor for the C-X-C chemokine. It is expressed on monocytes, B-cells, and naive T-cells in PB [20,21]. The CXCR4 pathway plays a role in chemotactic activity of lymphocytes, cancer cell homing and metastasis [22], it can also promote tumor vascularization and act as a survival or growth factor [23]. The ligand for CXCR4, stromal derived factor 1a (SDF-1a/CXCL12), is released by BM stromal cells. Binding of SDF-1a to CXCR4 induces increase in the intracellular Ca^{2+} level and triggers aberrant cells to home in BM [24]. The N-terminal domain of SDF-1a binds to the extracellular domain of CXCR4 resulting in the activation of intracellular signalling pathways. The intracellular part of CXCR4 interacts with G protein-coupled receptor kinase (GRK) translating the input into multiple signalling cascades including PI3K/AKT/mTOR and ERK1/2 that play important role in cell survival and proliferation (Fig. 3) [25,26].

Somatic mutations in *CXCR4* were initially associated with cancer by study identifying nucleotide changes in WM patients [27]. This receptor is the second most commonly mutated gene in the WM cells with more than 40 mutations mostly located in the C-terminal domain (Fig. 4). About 30% of WM patients have frameshift or nonsense mutations in the *CXCR4* gene [27,28]. The most common type of *CXCR4* gene mutation in the WM patients is the S338X (C1013G) nonsense mutation causing impairment in the receptor internalization and increase in the activation of intracellular kinase pathways resulting in increased migration, adhesion, growth and survival of the aberrant WM cells (Tab. 1) [27,29]. Incidence of this variant analysed from BM was 28% in WM patients and 20% in IgM MGUS [29], nevertheless incidence from PB samples remains unknown.

Tab. 1. Known mutations in CXCR4.
List of 40 frame shift or nonsense mutations in the *CXCR4* gene located in C-terminal domain.

Nucleotide change	Amino acid change	Type of mutation
931_933insT	T311fs	frameshift
945_946insC	H315fs	frameshift
951_953delACCTC	T318fs	frameshift
952_953insA	T318fs	frameshift
953_954delC	T318fs	frameshift
954_956insC	S319fs	frameshift
958_960delTG	V320fs	frameshift
963_964insC	R322fs	frameshift
969_971insG	S324fs	frameshift
977_978insC	L326fs	frameshift
978_980insT	K327fs	frameshift
979_985delAGATCCT	K327fs	frameshift
982_983delAT	I328fs	frameshift
984_986insT	L329fs	frameshift
993_995insA	G332fs	frameshift
997A<T	K333X	nonsense
1000C<T	R334X	nonsense
1005_1007insT	G336fs	frameshift
1006G<T	G336X	nonsense
1012_1015delTCAT	S338fs	frameshift
1012_1013insT	S338fs	frameshift
1012_1013delT	S338fs	frameshift
1013C<A	S338X	nonsense
1013C<G	S338X	nonsense
1013_1015delATCT	S338fs	frameshift
1013_1015delATCTGTTTCCACTGAGT	S338fs	frameshift
1015_1017delCT	S339fs	frameshift
1017_1018delT	S339fs	frameshift
1020_1021delT	S341fs	frameshift
1022_1023insT	S341fs	frameshift
1024_1026delCT	S342fs	frameshift
1030_1041CTGAGTCTTCGT	S344fs	frameshift
1031_1033delCT	S344X	nonsense
1033_1035delAG	E345fs	frameshift

Ibrutinib, the inhibitor of Bruton tyrosine kinase (BTK), is one of the small molecules approved by FDA for the treatment of WM patients. Despite high response rates and durable remissions of patients [19], disease progression can occur during ibrutinib therapy. Whereas mutated *MYD88* promotes ibrutinib efficacy [30], *CXCR4* mutations are associated with slower response kinetics and lower response rates among WM patient on ibrutinib. In other words, *CXCR4* mutations impact ibrutinib clinical activity in WM [31].

While detection of $MYD88^{L265P}$ and $CXCR4^{S338X}$ mutation in BM sample is common for WM diagnosis, the aim of our study was to compare sensitivity of detection of these mutations from different cell fraction found in PB (whole PB – PB, PB mononuclear cells – PBMC, $CD19^+$ PB cells – $CD19^+$ PB) and BM ($CD19^+$ BM cells – $CD19^+$ BM). The possibility of collection and analyzing PB instead of BM

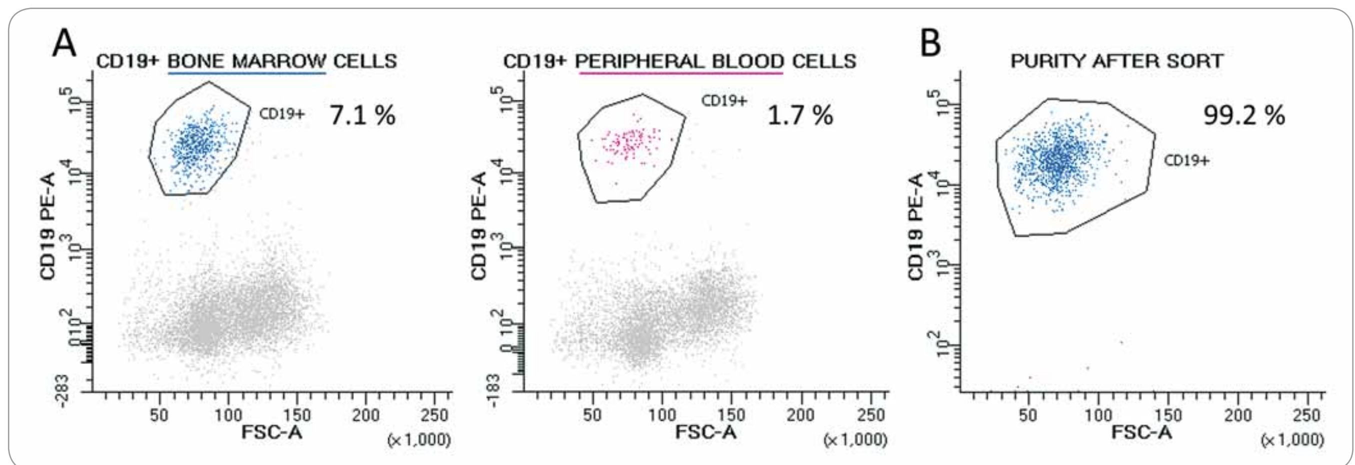

Fig. 5. Separation of the cells of interest entering molecular analyses of mutation status.

A. WM cells are characterised by expression of CD19 on their surface, therefore this marker was used for enrichment of analysed cell population with aberrant WM cells from BM (blue dots) and from peripheral blood (purple dots).

B. Purity after sorting was controlled and achieved at least 98% in all cases (blue dots). For cell staining monoclonal antibody CD19-PE (Exbio) was used.

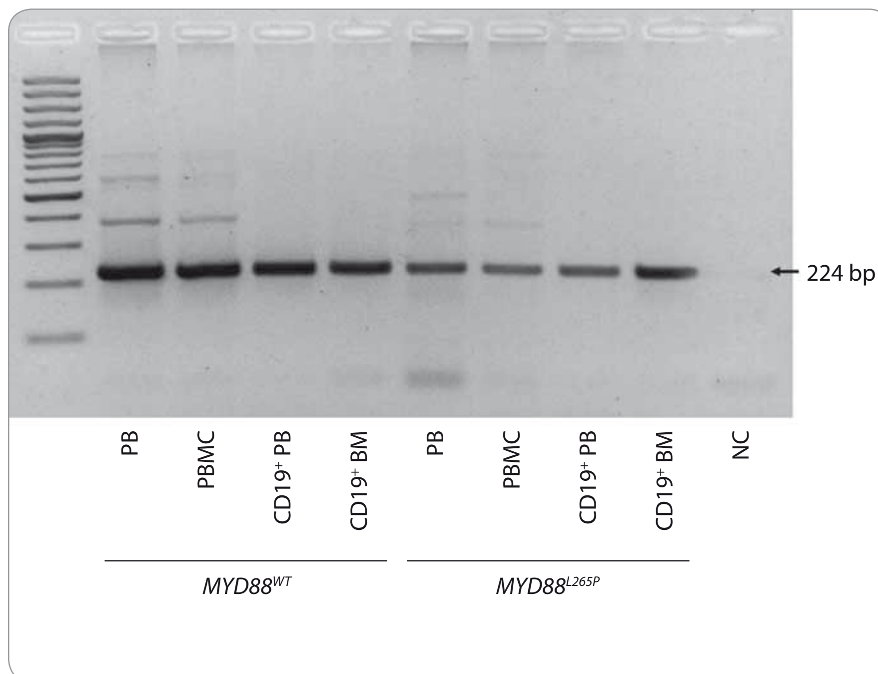

Fig. 6. Detection of *MYD88*^{L265P} in different input material.

Detection of *MYD88*^{L265P} from PB, PB mononuclear cells (PBMC), CD19^{+} peripheral blood cells (CD19^{+} PB) and CD19^{+} bone marrow cells (CD19^{+} BM) was done using allele specific PCR. All input materials were sufficient for detection of mutant allele (line 6–9).

would be significantly easier and most importantly less invasive for patients.

Material and methods

Material and samples

PB and BM samples were collected at the Department of Haematooncology of University Hospital Ostrava (Czech Republic), University Hospital Bratislava (Slovak Republic) and City Hospitals' Group in Chorzow (Poland). This study was approved by institutional ethical review boards and all individuals provided written informed consent. Mononuclear cells were separated from PB and BM by Ficoll-Plaque PLUS (GE Healthcare) gradient centrifugation. Infiltration of CD19^{+} cells was analysed by flow-cytometry analysis using anti-CD19-PE (Exbio) antibody. The CD19^{+} cells were sorted from PBMC and BM mononuclear cells (BMMC) by fluorescence activated cell sorting (FACS Aria III, Becton Dickinson). DNA from whole blood and PBMC were isolated by Magnesia® 16 magnetic bead extraction system (Anatolia Geneworks). DNA from the CD19^{+} cells of PB and BM was isolated by QIAamp DNA Mini kit (Qiagen).

ASO-PCR (MYD88)

Detection of somatic mutation L265P in the *MYD88* gene was performed by the allele-specific PCR. Two forward primers were used to differentiate the mutant and the wild type alleles. Sequences of the MUT forward primer was 5'-GTGCCCATCAGAAGCGCCC-3' and the WT forward primer was 5-GTGCCCATCAGAAGCGCCT-3'. The common reverse primer was 5'-AGGAGGCAGGGCAGAAGTA-3' [32]. PCR was performed in the final volume of 20 ul with 100 ng of each primer and 50 ng of DNA using GoTaq® G2 Hot Start Polymerase (Promega). Conditions of PCR were following – 1 cycle: 94 °C for 1 min, 35 cycles: 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min and the final extension step: 72 °C for 10 min. Final products of 224 bp were detected on 2% agarose gel.

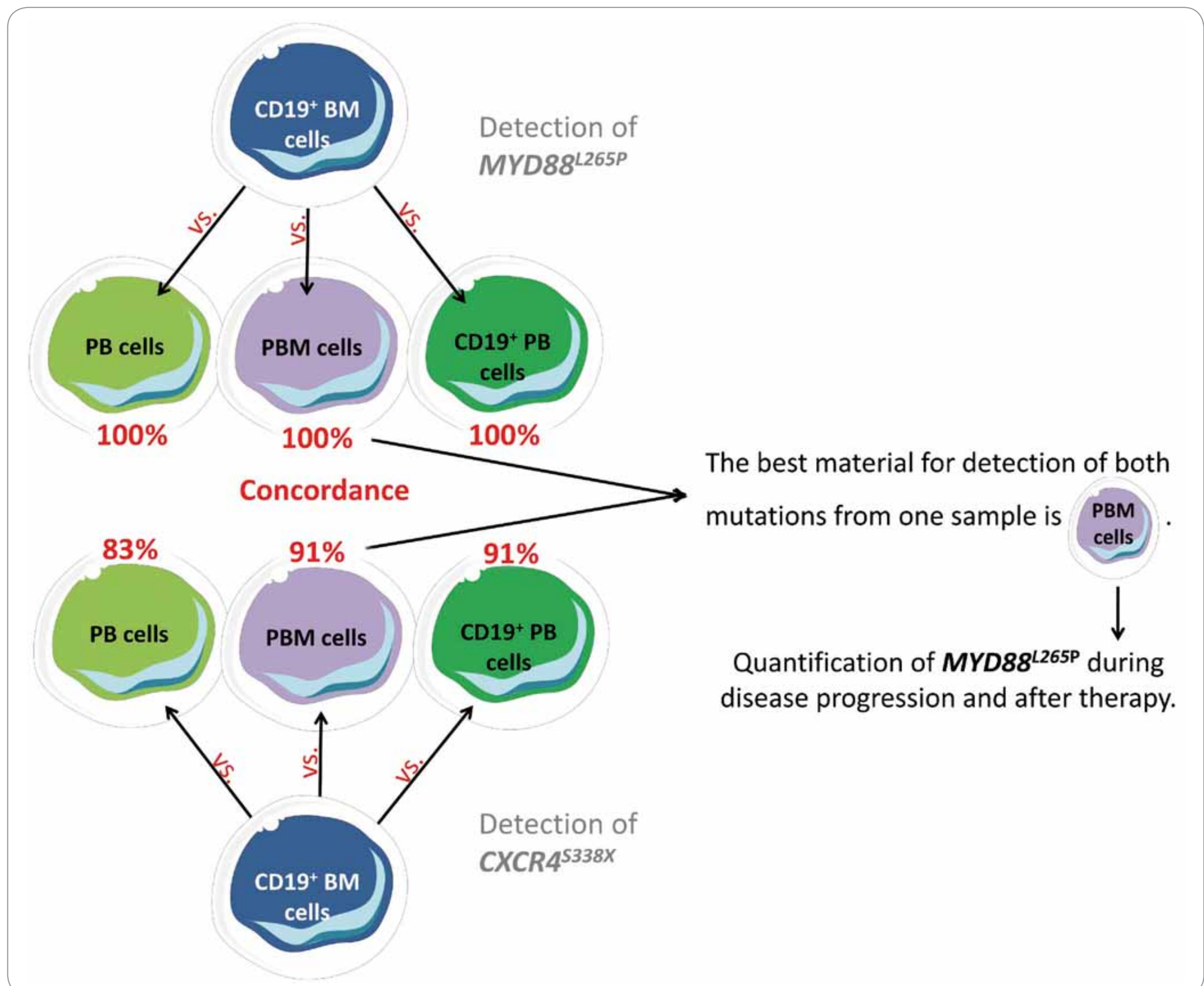

Fig. 7. Scheme showing comparison of ASO-PCR sensitivity detecting *MYD88*^{L265P} and *CXCR4*^{S333X} from different input material.

Use of different input material for detection of *MYD88*^{L265P} shows 100% concordance (red numbers in %) and 83–91% concordance for detection of *CXCR4*^{S333X} (red numbers in %). Although analysis of peripheral blood mononuclear cells and CD19+ PB cells shows same results, PB mononuclear cells (PBM cells) were chosen as best material for detection of both mutations from same material as well as for detection of *MYD88*^{L265P} during therapy and disease progression, because of simpler sample processing. PB cells (light green), PB mononuclear cells (violet), CD19+ PB cells (dark green) and CD19+ BM cells (blue).

Real time ASO-PCR (CXCR4)

Detection of somatic mutation S338X in the *CXCR4* gene was performed by the real-time allele-specific PCR. The common forward primer was 5′-TTTCTTCCACTGTTGTCTGAACC-3′. Two reverse primers were used to differentiate the mutant and WT allele. Sequence of the MUT reverse primer was 5′-GACTCAGACTCAGTGGAAACAGATG-3′ and the WT reverse primer 5′ GACTCAGACTCAGTGGAAACAGAAC-3′ [29]. PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems). The probe was tagged by 6FAM-MGB1 and its sequence was 5′-TATGCTTTCCTTGGAGCCA-3′. StepOne Real Time PCR system (Applied Biosystems) was used for detection of mutations. Conditions of reaction were following – 1 cycle: 95 °C for 10 min, 50 cycles: 95 °C for 15 s and 60 °C for 1 min.

Quantification of *MYD88*^{L265P}

Continuous quantification of *MYD88*^{L265P} during disease development and treatment was performed by qPCR from PB samples. Set of three primers was used, common forward primer with sequence 5′-CCTTGTACTTGATGGGGATCA-3′, mutated reverse primer 5′-CCTTGTACTTGATGGGGATGG-3′ for detection of *MYD88*^{L265P} and WT reverse primer 5′-CCTTGTACTTGATGGGGATGG-3′ as a control of amplification [15]. For each experiment concentration line was prepared using mutant DNA diluted to final concentrations – 100, 50, 25, 12.5, 6.25, 3.12,

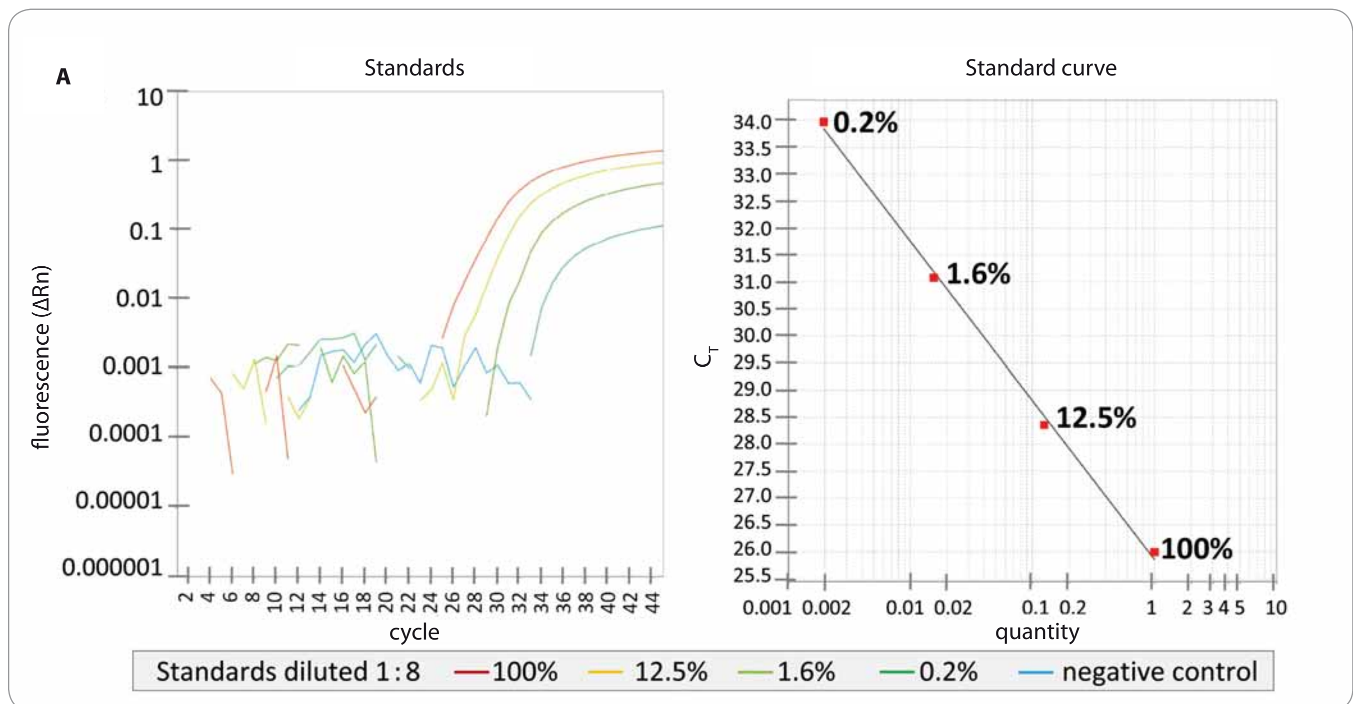

Fig. 8. Quantification of *MYD88*^{L265P} from PB mononuclear cells during therapy and disease progression.

A. Standards were prepared from *MYD88*^{L265P} BM cells with dilution coefficient 1 : 8 (100%, 12.5%, 1.6%, 0.2% of *MYD88*^{L265P} positive sample).

1.56 and 0%. Final volume of each reaction was 20 μl, containing 300 nM primers, 200 nM probe and 20 ng of DNA. Experiments were performed using TaqMan Gene Expression Master Mix and StepOne Real Time PCR system (Applied Biosystems) with following conditions – 1 cycle: 95 °C 10 min, 50 cycles: 95 °C for 15 s and 60 °C for 1min.

Results

Comparison of different input material for detection of *MYD88*^{L265P} and *CXCR4*^{S338X} mutations

The aim of this study was to compare the sensitivity of PCR detection of *MYD88*^{L265P} and *CXCR4*^{S338X} in different cell fraction of PB and BM. And to choose the best material for routine diagnostics in the view of reproducibility and patients comfort.

Aberrant WM cells are characterised by expression of specific surface markers. Specifically, immunophenotype of WM cells is associated with high level of CD19, CD20, CD22, CD25, CD27 and IgM and compare to the other B-cell non-Hodgkin lymphomas they lack expression of CD5, CD10, CD11c and CD103 [13,33,34]. The fluorescence activated cell sorting (FACS) is a common technique to separate cells of interest even if the infiltration is less than 5%. The use of FACS for separation of clonal WM plasma cells might be problematic as their immunophenotype is similar to the healthy plasma cells CD19$^+$/CD56$^-$/CD138$^+$ [10]. For the purpose of this study, the CD19$^+$ cells were separated to enrich for the WM cells in analysed samples (Fig. 5). The alternative way for cell separation based on the immunophenotype is the magnetic activated cell sorting (MACS) that is recommended when infiltration of malignant cells is > 5%. Nevertheless the purity of magnetic separation is significantly lower than fluorescence based separation [35], which is the main reason for using FACS in our study (purity of sort was > 98% in all cases; Fig. 5).

BM and PB samples of WM (27) and IgM MGUS (10) patients were tested for presence of *MYD88*^{L265P} and *CXCR4*^{S338X} mutations. Allele specific PCR for detection of studied mutation was done from four different cell fractions (PB, PBMC, CD19$^+$ PB, CD19$^+$ BM) in each patient. Although the sensitivity is different (bands on agarose gels have different intensity), the concordance is relatively high (Fig. 6). When the results of allele specific PCR detecting *MYD88*^{L265P} in CD19$^+$ BM are compared with those in PB, PBMC or CD19$^+$ PB the concordance is 100% in all cases. In other words, when the mutation was present in BM it was always detected also in other cell fractions (PB, PBMC, CD19$^+$ PB) and in case of absence of mutation all cell fractions were negative. For *CXCR4*^{S338X} the concordance is slightly lower, CD19$^+$ BM vs. CD19$^+$ PB or PBMC show 91% concordance, in case of CD19$^+$ BM vs. PB concordance was 83% (Fig. 7).

Based on our results we choose PBMC as the best cell fraction to determinate mutation in both studied genes. PBMC shows 100% concordance with BM samples when detecting *MYD88*^{L265P} and 91% concordance in case of *CXCR4*^{S338X}. Collection of blood is much more comfortable for patients and analysis of both mutations (*CXCR4*^{S338X}, *MYD88*^{L265P}) from the same material is money and time-saving for purpose of routine examination. Summarising schema of our result suggesting a laboratory guide for future analyses is presented in Fig. 7.

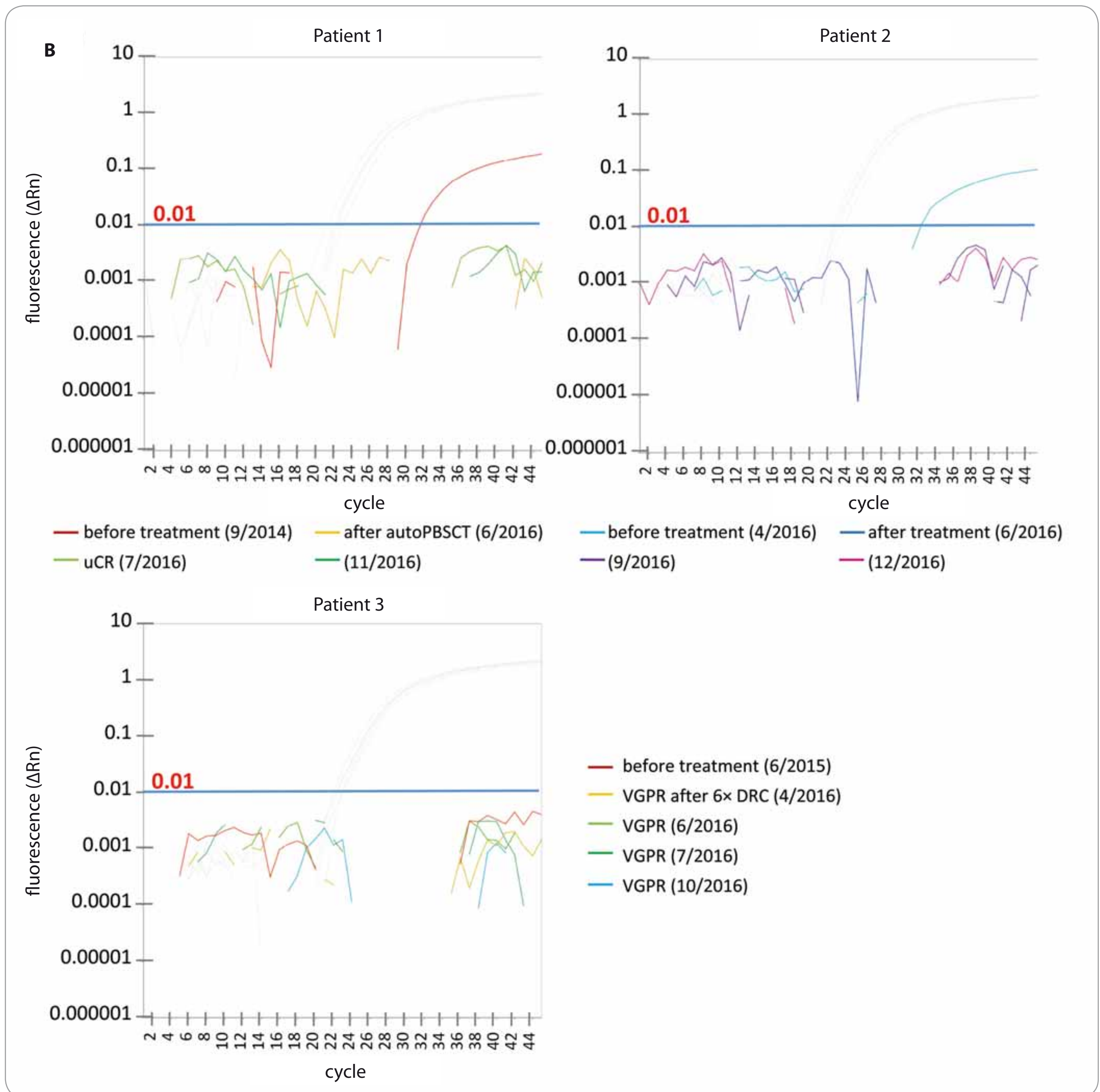

Fig. 8 – continuing. Quantification of *MYD88^{L265P}* from PB mononuclear cells during therapy and disease progression.

B. Presence of *MYD88^{L265P}* was detected before treatment initiation and subsequently in 1 to 4 month intervals from PB mononuclear cells. *MYD88^{L265P}* was present in patient 1 and patient 2 before treatment initiation, patient 3 was *MYD88^{WT}*. Level of *MYD88^{L265P}* cells in all samples of all three analysed patients during therapy was decreased under detection level of used method (qPCR).

C. Sensitivity of detection of *MYD88^{L265P}* using qPCR was 0.1%.

autoPBSCT – autologous peripheral blood stem cell transplantation, uCR – uncertain complete response, VGPR – very good partial response, DRC – dexamethasone, rituximab and cyclophosphamid

Abundance of *MYD88^{L265P}* during therapy correlates with the clinical status

We analysed presence of somatic mutations *MYD88^{L265P}* and *CXCR4^{S338X}* in 37 WM and IgM MGUS patients. Comparison of the detection sensitivity from different input materials shows PMBC as the best choice for the analysis of both mutations. This cohort of PBMC sample was used for quantification of *MYD88^{L265P}* and set of samples from three patients collected at the time of diagnosis and during therapy was used. Patients #1 and #2 were *MYD88^{L265P}* positive at the time

of diagnosis (#1 Ct = 31; #2 Ct = 32), whereas patient #3 was negative. Data shows undetectable (Ct > 45) amount of *MYD88*^{L265P} in all three patients after initiation of the therapy (Fig. 8B). This trend is in compliance with the treatment response (Fig. 8). Patient #1 achieved uncertain complete response (uCR) after autologous PB stem cells transplantation (autoPBSCT) and patient #3 achieved very good partial response (VGPR) after six cycles of DRC.

Conclusion

MYD88^{WT} is connected to differences in clinical and biological characteristics. Patients with the *MYD88*^{WT} gene were characterised by lower level of IgM, higher lactate dehydrogenase level, less somatic hypermutations in the Immunoglobulin Heavy Chain Variable gene (IGHV). But these differences do not have any impact on time to first therapy, response to treatment or progression-free or overall survival [36]. Absence of *MYD88* mutation in WM patient is associated with a female predominance, splenomegaly, gain of chromosome 3, and CD27 expression [37]. Presence of MYD88 mutations in IgM-MGUS patients relates to significantly higher levels of IgM and more frequent Bence-Jones proteinuria at the time of diagnosis. Moreover, it positively correlates with a risk of progression from the IgM monoclonal gammopathy of undetermined significance (IgM MGUS) to WM [32]. Based on our results, we were able to introduce detection of both *MYD88*^{L265P} and *CXCR4*^{S338X} mutations from PB mononuclear cells as a routine diagnostic method in our lab.

Monitoring of minimal residual disease in monoclonal gammopaties is becoming an attractive topic with high future potential. We collected PB samples from three patients during treatment and analysed *MYD88*^{L265P} using qPCR. Although sensitivity of detection for qPCR is not sufficient to detect less than 0.1% of aberrant cells, the results correlate with treatment response (Fig. 8). As flow cytometry analysis is a conventional strategy to detect aberrant cell clones, monitoring of the *MYD88* mutation status in WM patients during therapy seems to be effective and easy way for detection of MRD. Potential improved method for future analysis based on PCR is a digital droplet quantitative PCR (ddPCR) with a sensitivity of 5×10^5 that is suitable for identification of *MYD88*^{L265P} in the MRD samples, where the aberrant cells are rare [38].

Acknowledgments

We are grateful to Fedor Kryukov and Elena Kryukova for their participation in project designing and discussing of experimental work. We would also like to thank to Biobank Ostrava (FN Ostrava) for providing BM and PB samples. Figures were created using http://www.servier.com/Powerpoint-image-bank.

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1 SPC přípravku Imnovid 24. 6. 2016
2 Dimopoulos et al., Haematologica. 2015 Oct;100 (10):1327-33

IMNOVID 1 mg, 2 mg, 3 mg, 4 mg – základní informace o léčivém přípravku

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Registrační značka MK ČR 5158. ISSN 0862-495X. ISSN pro on-line přístup 1802-5307.

On-line verze je přístupná na adrese www.linkos.cz nebo www.klinickaonkologie.cz.

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Toto číslo vychází 15. 9. 2017.

První a jediná imunostimulační protilátka proti SLAMF7 v kombinaci s Rd* k léčbě pacientů s mnohočetným myelomem a nejméně jednou předchozí terapií[1]

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*Rd = lenalidomid (Revlimid) + dexamethason
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Reference: 1. SPC přípravku Empliciti, červenec 2017. **2.** Hsi ED et al. Clin Cancer Res. 2008;14(9):2775-84. doi: 10.1158/1078-0432.CCR-07-4246. **3.** Collins SM et al. Cancer Immunol Immunother. 2013;62(12):1841-9. doi: 10.1007/s00262-013-1493-8. **4.** Lonial S et al. N Engl J Med. 2015;373(7):621-31. doi: 10.1056/NEJMoa1505654. **5.** Dimopoulos M et al. ELOQUENT-2 Update: A Phase 3, Randomized, Open-Label Study of Elotuzumab in Combination with Lenalidomide/Dexamethasone in Patients with Relapsed/Refractory Multiple Myeloma – 3-Year Safety and Efficacy Follow-up. Oral presentation. ASH 2015. Abstr. 27.

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689CZ17PR06713-01 Datum schválení: 24.8.2017

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PŘEVRATNÉ OBJEVY NOVÝCH LÉKŮ VZNIKAJÍ V LABORATOŘÍCH,

ALE RODÍ SE UŽ V NAŠICH SRDCÍCH.

Přes 100 let pohání zaměstnance MSD společná touha: vyvíjet léky a vakcíny, které zlepšují miliony lidských životů. S podobnou péčí se věnujeme novým veterinárním přípravkům. Uvědomujeme si však, že je před námi ještě dlouhá cesta, proto klademe důraz na výzkum a vývoj. Stejné úsilí věnujeme zlepšování dostupnosti lékařské péče a spolupráci s těmi, kteří sdílejí naše nadšení pro zdravější svět. Víme, že to zvládneme. Dáváme do toho vše.

Chcete vědět více o našem poslání? Navštivte www.msd.cz

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09-2018-CORP-1228831-0000