Molecular pathology in bone and soft tissue tumors: a multifunctional key for diagnosis and prediction

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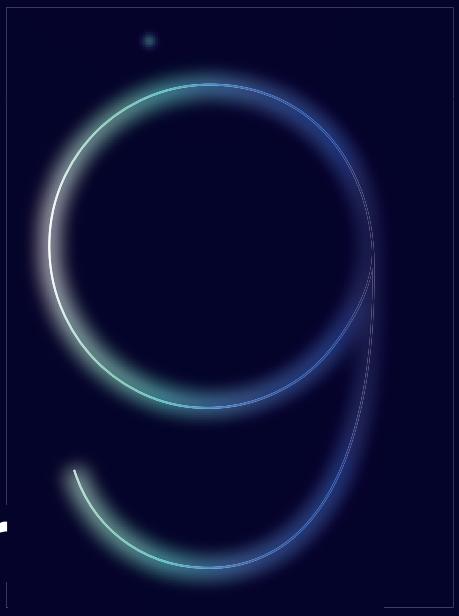

Chapter 9

Summary and concluding remarks

Summary

Molecular pathology in bone and soft tissue tumors

Bone and soft tissue tumors encompass a broad group of benign and malignant neoplasms and are considered difficult to diagnose for pathologists. These tumors are rare and distinction based on classic histomorphology can be challenging due to the overlapping morphology. Furthermore, traditional immunohistochemistry to identify the line of differentiation is less valuable in discriminating different bone tumors in comparison with soft tissue tumors. Fortunately, the field of bone and soft tissue sarcoma has rapidly evolved since the advance of new molecular techniques. The identification of novel genetic alterations has led to more insight into the genetic background of these tumors, which resulted in a more prominent role of molecular pathology in daily practice. The use of molecular alterations in bone and soft tissue tumors is not only confined to the improvement of diagnosis, but it also plays an important role in the prognosis and the identification of novel targets for molecular-based targeted therapy. In this thesis, the different key roles of molecular pathology in bone and soft tissue tumors are reflected in clinicopathological, immunohistochemical and molecular studies to aid diagnosis and prediction in a range of different bone and soft tissue tumors.

Chapter 2 summarized the current knowledge on genetic alterations in bone tumors, leading to a subclassification of bone tumors roughly into two groups. The first group consists of tumors with complex karyotypes, lacking any specific alterations. The second group shows simple karyotypes including tumors with translocations and tumors with specific gene mutations and/or amplifications. These specific recurrent genetic alterations can change transcription, cause altered signaling or alter gene function. This latter category is of explicit interest for diagnostic purposes since the specific molecular alteration can be employed as a diagnostic marker to improve diagnostic accuracy, either with molecular tests or surrogate immunohistochemistry [1]. With the acceleration of new molecular findings, which translates into novel diagnostic tools and therapeutic strategies, molecular pathology will play an increasingly central role in sarcoma patient care.

Chapter 3 focused on different assays for translocation detection. The applicability of a novel method for translocation detection, termed anchored multiplex PCR (AMP)-based targeted next-generation sequencing (NGS) [2], was investigated in different bone and soft tissue tumors. With the Archer® Fusionplex sarcoma panel, 26 genes relevant in bone and soft tissue sarcomas could be assessed in one assay using paraffin-embedded material. Results were compared to conventional fluorescence *in-situ* hybridization (FISH), RT-PCR and immunohistochemistry. A concordance of 90% between AMP-based targeted NGS and conventional methods was shown, and AMP-based targeted NGS was superior compared to RT-PCR and

FISH, demonstrating an NGS methodology with improved sensitivity compared to current methods for translocation detection. In our study, a failure rate of 14% for AMP-based targeted NGS was seen, probably due to decalcification and variability in tissue and fixation conditions, illustrating the daily challenges for pathologists. It remains difficult to achieve a decalcification method that can effectively and quickly decalcify while preserving genetic material. Currently, modification of standard decalcification procedures has been the subject of investigation and includes the use of microwave and ultrasonography combined with acid-based decalcification methods to reduce decalcification time [3, 4]. Others compared three next-generation sequencing approaches for fusion detection in sarcoma, including the AMP-based targeted NGS method using the Archer® Fusionplex sarcoma panel and two hybrid capture-based assays, which all demonstrated a good detection capability. The hybrid capture assays were more comprehensive and suitable for a research environment, since the Fusionplex sarcoma panel was limited to 26 genes, but the latter proved to be a fast and easy-to-analyze approach for routine diagnostic laboratories [5]. A limitation of AMP-based targeted NGS is that detection of fusions is limited to the selectively captured regions, which is especially relevant in bone and soft tissue tumors because of the accelerated identification of numerous novel fusions in the last years. Luckily, commercial companies are also focusing on the accelerated adoption of genetics through the development of more comprehensive assays, in which recently discovered sarcoma-associated fusion genes (e.g., FOS and FOSB) are currently included. Also, novel sequencing tools containing a larger number of genes involved in fusions are developed and customized panels can be designed for a more comprehensive panel to overcome this problem [5-7]. These targeted sequencing panels can screen for a wide range of genetic aberrations in a single test and have shown to be a reliable and cost-effective approach to improve sarcoma diagnostics compared to single-gene tests as well as more comprehensive methods, such as whole-genome sequencing (WGS) [5, 6, 8]. In the 100,000 Genomes project, close to 1000 sarcoma patients were recruited, but WGS data were only generated for 597 patients. The unsuitability of the samples was mainly caused by necrosis, secondary to neoadjuvant radiotherapy or chemotherapy, or fixation with formalin. Furthermore, not all alterations could be identified when validated against histology and standard of care diagnostic tests using the variant calling pipelines [9]. Therefore, targeted NGS serves as an excellent approach for the detection of aberrations in bone and soft tissue tumors in a clinical diagnostic setting.

Chapter 4 illustrated the key role of molecular pathology to improve diagnosis. We reported on a puzzling phenomenon rarely encountered by bone tumor pathologists, where conventional chondrosarcoma areas were admixed with clear cell chondrosarcoma areas. We performed extensive clinicopathological and molecular characterization of five cases. All five chondrosarcomas consisted predominantly of areas with conventional chondrosarcoma. Different grades were encountered,

including grade I (n=1), grade II (n=2) and grade III (n=2). Up to 20% of the tumor consisted of classical features of clear cell chondrosarcoma with a gradual transition between both components. Molecular analysis of conventional chondrosarcoma components revealed in two cases an *IDH1* c.395G>T, p.(Arg132Leu) mutation, and in one case an *IDH1* c.394C>T, p.(Arg132Cys) mutation, with identical *IDH* mutations in the clear cell chondrosarcoma counterpart (100%). Two cases were *IDH* wild-type. Approximately 50% of conventional chondrosarcomas harbor *IDH* mutations [10, 11], while these have never been found in classic clear cell chondrosarcoma [12]. Therefore, we conclude that the clear cell change is a phenotypic phenomenon occurring in conventional chondrosarcoma, rather than a collision between two types of chondrosarcomas, or clear cell chondrosarcoma with extensive conventional chondrosarcoma areas. This phenomenon has not been previously described in the literature, but knowledge of this phenomenon in conventional chondrosarcoma is crucial for bone pathologists, as this can be mistaken for dedifferentiated chondrosarcoma, clear cell chondrosarcoma or chondroblastic osteosarcoma, which require different treatments and have different prognosis [13].

In **chapter 5**, the translation of specific molecular findings into diagnostic tools to aid pathologists was illustrated in osteoid osteoma and osteoblastoma. These tumors were reported to harbor *FOS* (87%) and *FOSB* (3%) rearrangements [14]. We evaluated immunohistochemical expression of FOS in these tumors in comparison to other bone tumors, studied the influence of decalcification and correlated immunohistochemical findings with the underlying genetic alteration using FISH. Strong nuclear expression of FOS was observed in all osteoid osteomas (22/22), in 57% of osteoblastomas (12/21) and in 2% of control cases (3/197). FOS immunoreactivity disappeared after >3 days decalcification. *FOS* rearrangements were present in 94% of osteoid osteomas and osteoblastomas with a concordance of 86% between FISH and immunohistochemistry. This study illustrated that FOS immunohistochemistry can be used in decalcified biopsies to diagnose osteoid osteoma and osteoblastoma, as overexpression was seen in the majority while being rarely positive in their mimics. FOS immunohistochemistry should not be used after long decalcification and a low level of focal expression found in other lesions and tissues might cause diagnostic problems. For these cases FISH or AMP-based targeted NGS could be employed. Our results correspond with Amary *et al.*, who reported positivity rate of 83% in osteoblastomas and 73% in osteoid osteomas [15]. Of the osteosarcomas in their series, 14% showed focal to a more conspicuous expression of FOS, highlighting the importance of undertaking a thorough assessment of expression patterns of antibodies in the light of morphologic, clinical, and radiologic features.

Since cementoblastoma shows striking morphological resemblance to osteoblastoma, in **chapter 6** we set out to determine whether cementoblastoma

also harbors *FOS* rearrangements with overexpression of FOS. Sixteen cementoblastomas were analyzed for FOS expression by immunohistochemistry and for *FOS* rearrangements by FISH. We observed strong and diffuse staining of FOS in 71% of cementoblastomas and identified a *FOS* rearrangement in all three cases that were amenable to FISH. The morphologic similarities between cementoblastoma, osteoid osteoma and osteoblastoma combined with the shared molecular alterations suggests a relation between these lesions, as they probably represent parts of the spectrum of the same disease. The distinction between osteoid osteoma and osteoblastoma has been arbitrarily defined by size (cut-off 2cm) and despite the finding of *FOS* rearrangements underlying both lesions in the current World Health Organization (WHO) classification of soft tissue and bone tumors these are still considered as separate disease entities since clinical characteristics and behavior differ [14, 16]. In line with this conception, it would be logical to consider a clinically well-established term like cementoblastoma as a separate entity since these tumors also differ in the site and clinical characteristics.

The chapters **4**-**6** illustrated that molecular diagnostics can offer important benefits to patients since it improves diagnostic accuracy. However, the use of molecular diagnostics in clinical practice is still limited and access to these techniques remains unequal across countries and sometimes even within individual countries [17]. To consolidate genomic testing and to ensure that these tests are available for each sarcoma patient in the Netherlands, centralization of sarcoma patient care is inevitable, especially since multiple studies indicated that management of rare cancers in specialized hospitals by an experienced multidisciplinary team has a positive impact on survival outcomes [18]. While centralization of bone sarcoma patients is currently well organized, for soft tissue tumors further improvement should be obtained. To further improve management of sarcoma care, the chance of delayed diagnosis resulting in potentially more extensive surgery and decreased survival, should be minimalized. This can be achieved by raising more awareness among general practitioners and specialists and by the establishment of care pathways, which have shown to improve referral rates, reduce costs associated with local recurrence and result in better surgical results and overall patient outcomes [18, 19].

In **chapter 7** and **8**, the predictive role of molecular pathology stood central. In **chapter 7**, we focused on mismatch repair (MMR) deficiency as a predictive marker for potential immune checkpoint inhibiting (ICI) therapy in a broad spectrum of bone and soft tissue tumors. With immunohistochemistry for mismatch repair proteins MSH2, MSH6, MLH1 and PMS2 as a first screening method, eight out of 894 (1%) bone and soft tissue tumors were found to be mismatch repair-deficient. These included four leiomyosarcomas, two rhabdomyosarcomas, one malignant peripheral nerve sheath tumor and one radiation-associated sarcoma. Three patients were suspected of Lynch syndrome. Literature review revealed

30 MMR-deficient sarcomas of which 33% were undifferentiated/unclassifiable sarcomas. Most patients were genetically predisposed. Our findings were in line with Doyle *et al*., who reported an overall frequency of 2%. Although the frequency of MMR-deficient sarcomas is very low, identifying these tumors allows potentially novel treatment options for patients, especially since the advent of basket trails. Response to ICI therapy in MMR-deficient tumors is thought to be caused by a high tumor mutational burden (TMB). This leads to the production of more neo-antigens that might be recognized by the immune system and thereby eliciting an anti-tumor response [20-22]. Therefore, not only MMR-deficient tumors but also cancers associated with mutagens (i.e., UV exposure in melanoma and smoking in non-small-cell lung cancer), resulting in a high TMB demonstrate high response rates to ICI therapy [23]. Although the association between TMB and ICI response is robust, other factors are involved. For example, tumors associated with oncogenic viruses such as Merkel cell carcinoma respond better to ICI therapy than would be expected based on TMB alone. Besides genomic biomarkers, biomarkers involving the immune microenvironment have been a subject of investigation. PD-L1 was thought to be a promising predictive biomarker as its expression is expected to be required for response to ICI therapy. However, some studies found a positive correlation between PD-L1 and ICI response, while this result was not detected in other studies. Moreover, response to ICI therapy was observed in patients without expression of PD-L1. Also in sarcomas, where expression of PD-L1 is observed in approximately 50%, PD-1 blockade alone did not show promising efficacy clinically, suggesting that the PD-L1 status is not likely to be a sufficient comprehensive standalone biomarker [22, 24-27]. More recently, gene expression profiles were studied in soft tissue sarcoma, which led to an immune-based classification. Based on the composition of the tumor microenvironment five distinct phenotypes (i.e., immune-low (A and B), immune-high (D en E), and highly vascularized (C)) were identified. Interestingly, the class E group demonstrated improved survival and a high response rate to PD-1 blockade in a phase 2 clinical trial, suggesting that identification of this subgroup might be helpful to guide clinical decision-making and treatment [28].

In **chapter 8**, *NTRK* fusions in bone tumors as a predictive marker for TRK-inhibitors were explored. Immunohistochemical expression of pan-Trk was used to prescreen for *NTRK* fusions in a large series of bone tumors according to recent World Sarcoma Network (WSN) recommendations [29]. Osteogenic, chondrogenic tumors and Ewing sarcoma were included, thereby representing the three most common bone sarcomas. *NTRK* fusions were not identified among 354 examined bone tumors, which was in line with our expectations as only a few anecdotal cases are described in the literature. To date, a three-tiered screening method is proposed when screening for *NTRK* fusions in sarcomas and this is mainly based on the literature concerning soft tissue tumors [29]. It is recommended that screening in a clinical setting should be focused on histologic subtypes in which *NTRK* fusions are found

at high frequency and are diagnostic. For sarcoma patients with locally advanced and unresectable or metastatic disease, the WSN advises *NTRK* fusion testing using pan-Trk immunohistochemistry prescreening only for those sarcoma types known to harbor a complex genome (e.g., osteosarcoma). In sarcomas with recurrent gene fusions (e.g., Ewing sarcoma) or amplifications as driver alterations, *NTRK* fusion testing should be restricted to research [29], since *NTRK* fusions are typically mutually exclusive with other drivers [30]. We show that the likelihood of finding a *NTRK* fusion in bone tumors in clinical practice, even in tumors with complex genome lacking driver alterations, is extremely low. This may imply that, if more comprehensive large scale molecular studies confirm this, routine predictive *NTRK* testing in bone sarcoma patients with advanced disease may be reconsidered.

Chapter 7 & **8** highlighted a key role of molecular pathology in personalized medicine. The increasing importance of targeted therapy and checkpoint inhibitors in the treatment of several tumor entities and the necessity to screen for multiple predictive molecular alterations is causing new challenges [31]. Nowadays, targeted genomic sequencing, which focuses on a panel of genes or targets that have strong associations with the pathogenesis of disease and/or clinical relevance, is most commonly used in clinical practice. Although it provides numerous advantages such as greater sequencing depth with reduced costs and less data burden, it runs the risk of missing crucial variants outside the targeted regions. As the number of druggable gene aberrations and predictive biomarkers rapidly increases in oncology, a transition towards WGS, which has been mostly applied in study settings so far, is currently ongoing. In order to keep up with this demand, several hurdles need to be overcome to successfully implement WGS in routine practice [32]. Since frozen material is mandatory for WGS, this should be obtained in the routine diagnostic workflow. The time from sample to result should be dramatically reduced to obtain the result within a clinically relevant timeframe and further reduction of cost and easy-to-use software for data handling and analysis are required. Another challenge is that even if actionable targets are present, the lack of approved or investigational agents to match specific drivers hinders potential treatment options [33, 34]. Only when these processes reach maturity, WGS will be a potential future standard of care for genomic tumor profiling to improve personalized therapeutic management in advanced cancer patients.

Concluding remarks

The different key roles of molecular pathology in bone and soft tissue tumors have been addressed in several chapters of this thesis and has proven to provide a more accurate diagnosis and a better classification of tumor entities. Furthermore, molecular alterations could be translated into adjunctive markers that may aid diagnosis in routine diagnostics, such as FOS, and have allowed new opportunities

for novel therapeutic options. Despite the accelerated identification of numerous recurrent molecular alterations, it is likely that many potentially important molecular alterations in rare cancers are not yet discovered. Future studies using techniques such as WGS and RNA sequencing will definitely contribute to novel discoveries, especially since bioinformaticians have taken advantage of artificial intelligence to analyze these large datasets [35]. This will offer unprecedented opportunities to increase our understanding of molecular tumor biology, which could open new avenues and appealing targets for therapy. However, the access to molecular techniques, especially sequencing techniques is not always so self-evident, though the costs of next-generation sequencing have decreased at a dramatic rate, outpacing Moore's law. Translation of molecular findings into more easily accessible techniques such as immunohistochemistry, could help provide pathologists across the world diagnostic tools to improve bone and soft tissue tumor diagnostics.

References

1. Lam SW, van IJzendoorn DGP, Cleton-Jansen AM, Szuhai K, Bovee JVMG. Molecular Pathology of Bone Tumors. *J Mol Diagn* 2019;**21**;171-182.
2. Zheng Z, Liebers M, Zhelyazkova B *et al.* Anchored multiplex PCR for targeted next-generation sequencing. *Nat Med* 2014;**20**;1479-1484.
3. Raj AT, Patil S, Rao RS. A Comparison of Conventional and Microwave Decalcification and Processing of Tooth and Mandibular Bone Specimens. *J Clin Diagn Res* 2016;**10**;Zc121-zc126.
4. Chow DH, Zheng L, Tian L, Ho KS, Qin L, Guo X. Application of ultrasound accelerates the decalcification process of bone matrix without affecting histological and immunohistochemical analysis. *J Orthop Translat* 2019;**17**;112-120.
5. Racanelli D, Brenca M, Baldazzi D *et al.* Next-Generation Sequencing Approaches for the Identification of Pathognomonic Fusion Transcripts in Sarcomas: The Experience of the Italian ACC Sarcoma Working Group. *Front Oncol* 2020;**10**;489.
6. McConnell L, Houghton O, Stewart P *et al.* A novel next generation sequencing approach to improve sarcoma diagnosis. *Mod Pathol* 2020;**33**;1350-1359.
7. Gleason BC, Fletcher CD. Myoepithelial carcinoma of soft tissue in children: an aggressive neoplasm analyzed in a series of 29 cases. *Am J Surg Pathol* 2007;**31**;1813-1824.
8. Lam SW, Cleton-Jansen AM, Cleven AHG *et al.* Molecular Analysis of Gene Fusions in Bone and Soft Tissue Tumors by Anchored Multiplex PCR-Based Targeted Next-Generation Sequencing. *J Mol Diagn* 2018;**20**;653-663.
9. Prendergast SC, Strobl AC, Cross W *et al.* Sarcoma and the 100,000 Genomes Project: our experience and changes to practice. *J Pathol Clin Res* 2020;**6**;297-307.
10. Pansuriya TC, van Eijk R, d'Adamo P *et al.* Somatic mosaic IDH1 and IDH2 mutations are associated with enchondroma and spindle cell hemangioma in Ollier disease and Maffucci syndrome. *Nat Genet* 2011;**43**;1256-1261.
11. Amary MF, Damato S, Halai D *et al.* Ollier disease and Maffucci syndrome are caused by somatic mosaic mutations of IDH1 and IDH2. *Nat Genet* 2011;**43**;1262-1265.
12. Meijer D, de Jong D, Pansuriya TC *et al.* Genetic characterization of mesenchymal, clear cell, and dedifferentiated chondrosarcoma. *Genes Chromosomes Cancer* 2012;**51**;899-909.
13. Gelderblom H, Hogendoorn PC, Dijkstra SD *et al.* The clinical approach towards chondrosarcoma. *Oncologist* 2008;**13**;320-329.
14. Fittall MW, Mifsud W, Pillay N *et al.* Recurrent rearrangements of FOS and FOSB define osteoblastoma. *Nat Commun* 2018;**9**;2150.
15. Amary F, Markert E, Berisha F *et al.* FOS Expression in Osteoid Osteoma and Osteoblastoma: A Valuable Ancillary Diagnostic Tool. *Am J Surg Pathol* 2019;**43**;1661-1667.
16. Fletcher CDM, Bridge JA, Hogendoorn P, Mertens F. *WHO Classification of Tumours Editorial Board: Soft Tissue and bone tumours*. Fifth ed. Lyon (France): IARC, 2020.
17. Plun-Favreau J, Immonen-Charalambous K, Steuten L *et al.* Enabling Equal Access to Molecular Diagnostics: What Are the Implications for Policy and Health Technology Assessment? *Public Health Genomics* 2016;**19**;144-152.
18. Derbel O, Heudel PE, Cropet C *et al.* Survival impact of centralization and clinical guidelines for soft tissue sarcoma (A prospective and exhaustive population-based cohort). *PLoS One*

2017;**12**;e0158406.

19. Kasper B, Lecointe-Artzner E, Wait S *et al.* Working to improve the management of sarcoma patients across Europe: a policy checklist. *BMC Cancer* 2018;**18**;424.
20. Le DT, Durham JN, Smith KN *et al.* Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 2017;**357**;409-413.
21. Le DT, Uram JN, Wang H *et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* 2015;**372**;2509-2520.
22. Havel JJ, Chowell D, Chan TA. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat Rev Cancer* 2019;**19**;133-150.
23. Maleki Vareki S. High and low mutational burden tumors versus immunologically hot and cold tumors and response to immune checkpoint inhibitors. *J Immunother Cancer* 2018;**6**;157.
24. Tawbi HA, Burgess M, Bolejack V *et al.* Pembrolizumab in advanced soft-tissue sarcoma and bone sarcoma (SARC028): a multicentre, two-cohort, single-arm, open-label, phase 2 trial. *Lancet Oncol* 2017;**18**;1493-1501.
25. Burgess MA, Bolejack V, Tine BAV *et al.* Multicenter phase II study of pembrolizumab (P) in advanced soft tissue (STS) and bone sarcomas (BS): Final results of SARC028 and biomarker analyses. *Journal of Clinical Oncology* 2017;**35**;11008-11008.
26. Toulmonde M, Penel N, Adam J *et al.* Use of PD-1 Targeting, Macrophage Infiltration, and IDO Pathway Activation in Sarcomas: A Phase 2 Clinical Trial. *JAMA Oncol* 2018;**4**;93-97.
27. Zuo W, Zhao L. Recent advances and application of PD-1 blockade in sarcoma. *Onco Targets Ther* 2019;**12**;6887-6896.
28. Petitprez F, de Reyniès A, Keung EZ *et al.* B cells are associated with survival and immunotherapy response in sarcoma. *Nature* 2020;**577**;556-560.
29. Demetri GD, Antonescu CR, Bjerkehagen B *et al.* Diagnosis and management of tropomyosin receptor kinase (TRK) fusion sarcomas: expert recommendations from the World Sarcoma Network. *Ann Oncol* 2020;**31**;1506-1517.
30. Solomon JP, Benayed R, Hechtman JF, Ladanyi M. Identifying patients with NTRK fusion cancer. *Ann Oncol* 2019;**30**;viii16-viii22.
31. Dietel M. Molecular Pathology: A Requirement for Precision Medicine in Cancer. *Oncol Res Treat* 2016;**39**;804-810.
32. Samsom KG, Bosch LJW, Schipper LJ *et al.* Study protocol: Whole genome sequencing Implementation in standard Diagnostics for Every cancer patient (WIDE). *BMC Med Genomics* 2020;**13**;169.
33. Malone ER, Oliva M, Sabatini PJB, Stockley TL, Siu LL. Molecular profiling for precision cancer therapies. *Genome Med* 2020;**12**;8.
34. Zehir A, Benayed R, Shah RH *et al.* Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nat Med* 2017;**23**;703-713.
35. Ching T, Himmelstein DS, Beaulieu-Jones BK *et al.* Opportunities and obstacles for deep learning in biology and medicine. *J R Soc Interface* 2018;**15**.