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INDUCED PLURIPOTENT STEM CELLS A BRIEF REVIEW

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ABSTRACT

Induced pluripotent stem cell generation is regarded as one of the most important discoveries in cell biology of recent times. The demonstration of the reversion of somatic cells to cells which resemble embryonic stem cells (ESCs) by the viral transduction of 4 transcription factors has started an explosion of research into induced pluripotent stem cells (iPSC). iPSC have many intriguing possibilities, such as the generation of patient specific stem cells for cell replacement therapy, the study of diseases and the testing of novel drugs to treat such diseases. However, many hurdles need to be overcome before they are routinely used as true representations of ESC. Herein, iPSC studies to date are discussed alongside their potential advantages and disadvantages.

THE BEGINNINGS OF A REVOLUTION

The field of embryonic stem cell (ESC) research was shaken in 2006 by a paper from the lab of Shinya Yamanaka which demonstrated the generation of induced pluripotent stem cells (iPSCs). This publication, which entails the reprogramming of a mouse somatic cell genome to that resembling a pluripotent cell (1) has been heralded as one of the most important discoveries in cell biology and in the future may provide a limitless source of patient specific stem cells. In this seminal paper, the retroviral transduction of just four genes (Oct4, Sox2, Klf4 and c-Myc) partially reprogrammed mouse embryonic fibroblasts (MEFs) and adult tail tip fibroblasts (TTFs) to show characteristics reminiscent of mouse embryonic stem cells (mESCs). Initial studies utilised retrovirus-mediated gene transfer for their perceived ability to undergo silencing in the pluripotent state after genomic integration (2, 3). The iPSCs generated in this study were similar to mESC in terms of morphology, growth rate, gene expression and the epigenetic status, but were nevertheless obviously different to mESC. Further analysis showed that iPSCs were able to differentiate into cell types representative of all three primordial germ layers in teratoma assays and formed embryoid bodies, two key tests of pluripotency. However, these cells could not contribute to chimaeric animals when injected into mouse blastocysts, (perhaps a more robust test of pluripotency) and did not fully silence transgene expression.

Yamanaka's lab addressed some of the problems from this paper by using a slightly altered strategy (4). Previously, an antibiotic resistance gene was knocked into the Fbx15 locus, to allow for selection upon attainment of pluripotency (1). This study instead utilised the gene Nanog, which is more closely associated with the pluripotent nature of ESCs than Fbx15 (4). MEFs using this strategy gave rise to stable clones with similar gene expression and epigenetic status to ESCs, while they effectively silenced transgene expression. Importantly these iPSC showed contribution to chimaeric animals with germline transmission, another key test for the pluripotency of iPSC. However, it was also reported that up to 20% of chimaeric mice generated showed the formation of tumours attributed to the reactivation of the c-Myc transgene.

Soon, multiple studies began to show that iPSC technology was readily transferable and repeatable, so allowing an explosion of research interest in this field. Maherali et al and Wernig et al, both reported the generation of iPSC by retrovirally mediated transduction of Oct4, Sox2, Klf4 and c-Myc into MEFs and TTFs using Nanog or Oct4 selection, with reprogramming taking three weeks at an efficiency of 0.05-0.1% (5, 6), similar to Yamanaka's studies (1, 4). Both papers analysed the epigenetic status of their iPSC in some detail, and reported similarities between iPSC and mESC with regard to both histone modifications and DNA methylation. These iPSCs also showed contribution to chimaeric animals and germline transmission, with Wernig et al reporting the generation of embryo's derived entirely from iPSC following injection into tetraploid blastocysts (perhaps the best assay for pluripotency and developmental potential). However, while mid- and late-generation embryos were generated, no live births were reported, perhaps suggesting, that by the most rigorous tests, these cells were not equivalent to mESC.

HUMAN iPSC

The next major breakthrough came with the generation of human iPSC (7, 8). Yamanaka's group extended their earlier work and showed that adult dermal fibroblasts could be reprogrammed by retroviral transduction of OCT4, SOX2, KLF4 and C-MYC with good efficiency, but only after the cells where transduced with a mouse retrovirus receptor (7). Cells with hESC-like morphology were picked and expanded and showed all the hallmarks of pluripotency, including teratoma formation, appropriate gene expression and the ability to differentiate into multiple tissues. James Thomson's laboratory found that a slightly different set of factors, OCT4, SOX2,

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NANOG and LIN28, were sufficient to allow iPSC generation from foetal and adult fibroblasts (8) via lentiviral, rather than retroviral transduction, allowing the transduction of non-dividing cells (9), which was not previously permitted by the use of retroviruses (10). iPSC generated showed all the signs of a pluripotent cell type and their data further suggests that Lin28 was not required, but aided an increased frequency of reprogramming events. These two studies using similar but distinct, methods underscores the validity of iPSC technology and removes questions of reproducibility while also showing redundancy in which genes can affect the reprogramming process.

Closely following this came the generation of iPSC from another lab using a similar protocol (11). Addition of a ROCK-inhibitor, which can aid hESC growth when dissociated to single cells (12), enhanced the formation of iPSC from hESC-derived embryonic fibroblasts and importantly iPSC were also generated in the absence of C-MYC or KLF4, although at a lower efficiency. Although these initial strategies were useful for foetal cells, adult somatic human cells required the addition of hTERT and SV40 Large T antigen, two more potentially oncogenic factors, to allow for iPSC generation (11). Other studies have also reported that 6 factors are required for reprogramming of human fibroblasts (13, 14). Lentiviral expression of OCT4, SOX2, KLF4 and C-MYC in combination with NANOG and LIN28 were used to generate iPSCs from human neonatal foreskin fibroblasts and compared to four factor reprogramming (OCT4, SOX2, NANOG and LIN28) iPSC generation was quicker and more efficient (13). Another study found that SV40 Large T antigen, in addition to either of the four factor combinations of reprogramming factors, could increase efficiency by 23-70-fold in foetal fibroblasts and immortalised adult fibroblasts, using non-integrating lentivirus' (NILs) in order to minimise viral integration (14). This study also reported that SV40 Large T antigen could synergise with Oct4/Sox2/Nanog, Oct4/Sox2, or Oct4 alone to generate iPSCs, but only some clones were shown to be able to differentiate into cell types representative of all three primordial germ layers in teratoma formation assays. The use of Large T indicates that tumour suppressor pathways may need to be downregulated in order for the reprogramming process to take place at a respectable efficiency. A tumour suppressor response may be induced due to replicative stress on the cells, which induced a DNA-damage response (15, 16). This study also reported the generation of iPSC from fibroblasts homozygous for the sickle cell anaemia mutation, showing the capability of generating human models of disease in hESC-like cells. This is an important aspect of iPSC generation, with the generation of disease-specific iPSC perhaps as important as patient-specific iPSC and will be discussed further in this review.

iPSC generation from human neonatal foreskin fibroblasts was reported with four factors, although repeated retroviral transduction was required (17). Within this study, it was noted that partially reprogrammed clones still expressed the ectopic genes while the fully reprogrammed clones appeared to have silenced the exogenous genes more efficiently.

iPSCs Generation in More than Just Fibroblasts

It is important to establish that various cells types can be used for iPSC generation from a therapeutic aspect and also to assess the potential for a defined terminally differentiated cell type to be an amenable target of iPSC generation. It is still not fully understood if all cells can be targets for iPSC generation, although studies have shown that not just fibroblasts can be reprogrammed. Mouse β-pancreatic cells (18), and adult mouse liver and stomach cells (19) were shown to be relevant targets for iPSC generation. All cell types were deemed pluripotent and iPSC contributed to cells of all three germ layers in chimaeric embryos, although only one animal survived to birth. Interestingly iPSC from liver and stomach were less tumourigenic than MEF-derived iPSC, although perinatal death was higher and, interestingly, when c-Myc was removed only a 20-40% reduction in efficiency was observed, compared to a 90% drop in MEF reprogramming.

Another study reported the reprogramming of a terminally differentiated cell type but required the interruption of the cellular state before iPSC could be generated (20). Terminally differentiated mature B-cells from an iPSC-derived chimaeric mouse could only be reprogrammed after cellular "sensitisation" by expression of the myeloid transcription factor C/EBPα. C/EBPα can disrupt the function of Pax5, a transcription factor understood to play a major role in mature B-cell development and function, and hence suggests that not all terminally differentiated cells could be reprogrammed using the same factors.

iPSC technology was utilised in a novel reprogramming study in which transdifferentiation of an adult cell type to another adult cell type, without the requirement to revert these cells to an ESC-like state using transcription factors specific to that cell lineage was demonstrated (21). Specifically, they converted pancreatic exocrine cells to cells that closely resemble pancreatic β-cells by retroviral transduction of 3 genes; Ngn3, Pdx1 and Mafa, which are all involved in β-cell development. As β-cells are the insulin secreting cells destroyed in diabetes, this study is of obvious therapeutic interest.

An elevated efficiency and speed of reprogramming of human keratinocytes in comparison to fibroblasts was also observed (22). The advantage of using keratinocytes was further demonstrated by the generation of iPSC from cells isolated from single plucked human hairs, thus bypassing the requirement for skin biopsies when requiring fibroblasts.

REVEALING THE REPROGRAMMING PROCESS - MOLECULAR MECHANISMS AND SECONDARY iPSC GENERATION

The process of reprogramming somatic cell genomes towards a pluripotent-like state seems now to be a reachable goal, but little is actually known about how this is achieved at the molecular level. Understanding the roles of each of the reprogramming factors is of importance to understand the process and the relatively low efficiency of reprogramming and extended lengths of time required suggests that reprogramming is a gradual stochastic event rather than a set of specific events, which also requires to be understood.

Oct4, Nanog and Sox2 had been previously associated with the pluripotent state (23), with the use of such factors in iPSC generation thought to "kick-start" pluripotency-associated transcriptional circuits in non-pluripotent target cells. c-Myc induces the expression of genes involved in proliferation and self-renewal (24) and can also mediate global histone acetylation(25) perhaps mediating chromatin decondensation to allow the functions of DNA-binding transcription factors 26). Klf4 may function to downregulate p53 transcription (26), which has been shown to regulate Nanog expression (27) and also inhibits ES differentiation (28). Lin28 is involved in RNA processing (29), and modulates the expression of Let7 micro-

RNA's (miRNAs) in stem cells to maintain pluripotency in mESC (30). The importance of miRNA in the maintenance of pluripotency is just becoming uncovered (31, 32).

Studies intent on understanding the molecular mechanisms behind iPSC generation have begun to make use of "secondary systems" in which iPSC are generated by lentiviral transduction of doxycycline (dox) -inducible transgenes and are then injected into blastocysts to form chimaeric mice from which fibroblasts, or other cell types, carrying the dox-inducible transgenes can be isolated (33-39). Dox-induction of these cells allows for reprogramming of a homogenous cell type, allows the induction process to be controlled to more fully understand the process and importantly, this also allows for self-selection of reprogrammed cells after dox-removal, as only cells with activated endogenous pluripotency genes will grow effectively. SSEA1 and alkaline phosphatase (AP) activity have been shown to be early markers of the reprogramming process, while endogenous Oct4 and Nanog expression and telomerase activity were only found in fully reprogrammed cells (33). Longer transgene expression was linked to enhanced reprogramming, but continued transgene expression ablated the differentiation capacity of iPSC. In a similar study, secondary iPSC generation was reported to be 20-50 fold more efficient that the "primary" system and multiple cell types could be used, suggesting that iPSC can be generated from cells with different developmental origins and epigenetic states, using this system (34). Again, longer transgene expression led to enhanced iPSC generation and, interestingly a DNA methyltransferase inhibitor, **5-Aza-deoxycytidine (5-Aza)** was shown to boost the reprogramming process while a histone deacetylase inhibitor, Trichostatin A (TSA), did not. This suggests that DNA methylation is more important to the reprogramming process, although the effects of modulating histone methylation were not studied. The time scale of reprogramming of the secondary cells (9-13 days) was noted to be similar to that of directly reprogrammed cells (10-14 days) suggesting that direct viral integration is not an essential part of the reprogramming process, and further suggests that reprogramming is driven by stochastic epigenetic events requiring a minimum time of transgene expression.

Integrative genomic analysis of reprogramming of mouse fibroblast and B-lymphocytes using this secondary system was reported next (35). An immediate response of transgene induction was the decrease of mesenchymal related genes and an increase in proliferation associated, stress-induced and anti-proliferative genes. This study also reported that the vast majority of transduced cells remain in an intermediate reprogramming state, suggesting that stochastic events are required for cells to become completely reprogrammed. Such partially reprogrammed clones were studied in order to understand the barriers to the reprogramming process and showed incomplete activation of pluripotency related genes, incomplete deactivation of genes related to the original cell phenotype and incomplete epigenetic reprogramming. Interestingly, it was also reported that inhibition of DNMT1 led to the full reprogramming of these stable partially reprogrammed cells, indicating that reprogramming of the epigenotype of the cell is vitally important in iPSC generation.

A more detailed analysis of reprogramming at different stages was undertaken in mouse cells with transcription factor binding, gene expression and histone methylation examined at different stages of reprogramming (37). c-Myc was observed to be very important to early reprogramming, especially for the silencing of fibroblasts-specific genes, contrary to its usually perceived function as a transcriptional activator. Further, it is proposed that the lack of Oct4, Sox2 and Klf4 co-binding in partially reprogrammed cells contributes to the lack of full reprogramming. This lack of binding was suggested to be due to the lack of expression of Nanog, which allows the targeting of reprogramming factors.

Human cells were also studied by generation of secondary system, by generating iPSC from fibroblasts and/or keratinocytes using dox-inducible lentviruses and then differentiating them to fibroblasts, for further dox-mediated reprogramming studies (38, 39). Secondary human fibroblasts were reprogrammed with an efficiency of 1-3% upon dox-induction. Induction times of 6-8 days were required at the minimum to produce iPSC, but prolonged dox-induction (20-25 days) gave highly homogenous pluripotent iPSCs with good efficiency.

Overall, these papers suggests that timing of transgene expression/silencing is critical to the reprogramming process, but also that reprogramming is, in part, a defined set of molecular events, although stochastic events are likely also to be required for fully reprogrammed iPSCs to be generated.

CHANGING THE REPROGRAMMING MIXTURE

Changing the Factors

It has already been demonstrated that there is some redundancy in factor requirement for reprogramming with the reports of two differing sets of four reprogramming factors working to a similar degree (7, 8). This suggests that other factors may also be able to influence the reprogramming process, and indeed, the removal of some factors, such as the oncogene c-Myc, would be beneficial. Analysis of gene expression in mESC has demonstrated that N-Myc, which has a less oncogenic nature (40), was expressed at a higher level than c-Myc and could substitute for c-Myc in iPSC generation (41). Other studies have also shown that c-Myc is non-essential for the human (42) and mouse (42, 43) reprogramming process with the tumourigenic potential of mouse iPSC generated being much lower, with all chimaeras generated surviving past 100 days. Similarly, it has been found that Sox3, 15 and 18 could substitute for Sox2, Klf1, 2 and 5 could substitute for Klf4 and both N-Myc and L-Myc could substitute for c-Myc in iPSC generation from MEFs (42). Use of the "substitutes" Sox1 and 3, Klf2 and N- and L-Myc led to iPSC generation from MEFs that gave rise to teratomas upon injection into nude mice.

Further additional reprogramming factors beyond the six "classic" factors have been reported. Addition of viruses carrying UTF1 and siRNA against p53 highly enhance the generation of fully pluripotent iPSC and could substitute for C-MYC (44). UTF1 is linked with chromatin mediated transcriptional repression (45) and so may function to aid differentiation, but is also **reported to highly expressed in ESCs and downregulated upon differentiation (46). Enhanced generation of iPSC after p53 siRNA expression again suggests that perhaps the downregulation of tumour-suppressor pathways are required as previously discussed with the use of SV40 Large T antigen**. iPSCs were also reported to be derived from MEFs with the addition of the nuclear receptor Esrrb, in the place of c-Myc and Klf4 (47) and were shown to be fully pluripotent, formed chimaeric animals and contributed to the germline. **Esrrb has been previously reported to be important in pluripotency**

and self-renewal of ES cells (48, 49) and it's capability to substitute for Klf4 and c-Myc was reported to be partly due to Essrb's ability to upregulate many key ESC-associated genes and a high degree of similarity in their transcriptional regulatory circuits.

A novel paper in the field of iPSC generation next showed the possibilities of reprogramming by transduction of miRNA (50). **miRNA are single-stranded RNA molecules of 21-23 nucleotides in length, which are partially complementary to one or more messenger RNA (mRNA) molecules and are involved in regulation of gene expression. Multiple miRNAs are expressed as cluster of genes in a single cistronic sequence which is later cleaved to give the mature miRNA**. A miR302-cluster transgene was retrovirally transduced into several tumour cell lines and cells with ES-like morphology, marker expression and rate of cell division were readily produced. All iPSC clones formed teratomas and also formed EBs and differentiation led to the production of neuronal cells. It is noted that MECP2 and the p66 component of MECP1 are targets of miR302 and analysis of the Oct4 promoter did show demethylation.

Changing the Cell to Change the Factors

Other cell types have been studied for their potential use in iPSC generation to minimise transgene requirements. Many cell typed in mouse and human, may already express reprogramming factors at a high enough level to remove them from the factors used in viral transduction. Multiple recent studies have shown that mouse neural cell types can be reprogrammed by lower numbers of transgenes, due to their endogenous expression of Sox2 and c-Myc (51-58). Mouse neural progenitor cells (mNPCs) were reprogrammed to iPSC with only two factors (Oct4 and Klf4) at a low efficiency (51) and use of an inhibitor of the K9-specific histone methyltransferase inhibitor (BIX-01294) improved the efficiency to that of the 4 factors, functionally replacing c-Myc and Sox2 (59). Mouse adult neural stem cells (mNSCs) (52) were used to generate iPSC without Sox2 and without c-Myc or Klf4, but with much delayed kinetics. Further studies showed that the use of two factors (Oct4/Klf4 or Oct4/c-Myc) allowed generation of iPSC which were shown to be pluripotent in nature with no reported tumourigenesis in chimaeric and F1 mice. Changes in growth conditions to minimise differentiation stimuli (use of serum free medium with MEK/ERK and GSK3 inhibitors supplemented with LIF) allowed for the generation of iPSC with only Oct4 and Klf4 in mNSCs in another study (55). iPSC were also generated from mouse meningiocytes (56), a multipotent cell type which expresses high levels of Sox2 and are easy to extract (in animals). iPSC clones generated were highly homogenous and gave a high yield of chimaeric animals. Recent studies have been able to generate iPSC from mouse NSCs, by expressing Oct4 alone (57). These cells were shown to be pluripotent, formed teratomas and exhibited transmission through the germline showing that, in principle, Klf4, c-Myc and Sox2 transgene expression are not required for reprogramming.

Small Molecules and Soluble Factors: Towards Transgene Free iPSC?

The addition of small molecules into the reprogramming mix has allowed improvement of iPSC generation suggesting the possibility of transgene free reprogramming, simply by the addition of appropriate small molecules or soluble factors to the cell culture medium.

Alongside previous uses of the small molecule BIX-01294, a K9-specific histone methyltransferase inhibitor (51), retroviral-mediated generation of iPSC was shown to be greatly enhanced using MEFs by the addition of **5-Aza**, dexamethasone (synthetic glucocorticoid), and various histone deacetylase inhibitors (valproic acid (VPA), TSA and suberoylanilide hydroxamic acid (SAHA)) (60). MEFs have also been reprogrammed by Oct4 and Klf4 alone, with the aid of two small molecules which inhibited K9-specific histone methyltransferase activity (BIX-01294) and an L-channel calcium agonist **(BayK8644)** (58). Similarly, primary human neonatal fibroblasts have been reprogrammed with only OCT4 and SOX2 transduction followed by one month in ESC culture conditions, following an initial two weeks of treatment with the histone deacetylase inhibitor, VPA (61).

Naturally occurring signaling molecules, rather than artificial drugs, that can modulate the expression of ESC-associated transcription factors could be utilised as soluble factors that enhance reprogramming. For this reason, the WNT pathway was studied (62) and it was discovered that modulation of the WNT-pathway can affect iPSC generation (63). In an attempt to compensate for excluding c-Myc, reprogrammed cells were cultured in WNT3a-conditioned medium and by approx 3 weeks, homogenous ESC-like colonies of cells were observed which were deemed to be fully pluripotent.

IPSC- FROM BENCH TO BEDSIDE?

Models of Human Disease

Multiple studies have addressed the potential use of iPSC to treat disease utilising studies in mouse models and using human fibroblasts carrying mutations relevant to specific diseases. Mouse models of human sickle cell anaemia (SCA) (64), Parkinson's Disease (PD) (65) and Haemophilia A (66) have all been used to investigate possible therapeutic uses of iPSC technology. iPSC technology was combined with gene therapy to correct the mutant human SCA allele, with subsequent haematopoietic progenitor differentiation and transplantation effectively leading to the recovery of the mouse (64). In another study, iPSC generated from MEFs were differentiated to mNPCs, which were then transplanted into foetal mouse brains where they showed functional integration and differentiation into various neural cells (65). The same cells were then transplanted into adult rat brain after administration of 6-hydroxy dopamine (6-OH-DA) into the striatum, which specifically kills dopamine neurons, providing a useful model of Parkinson's disease. **Encouragingly, improved behaviour was observed 4 weeks after transplantation**. TTFs were used to generate iPSC without c-Myc and were differentiated towards an endothelial progenitor cell type and where then injected into the livers of mice carrying the mutation associated with Haemophilia A (65). Following a deliberate injury to the tail, mice with the mutation that did not receive the iPSC-derived progenitors died wheras those that did survived as well as control mice. However, it was noted that 16 of the 36 chimeras generated in one of these studies died within 8 months, with many showing tumours making it clear that the use of integrating viruses and oncogenes as reprogramming factors will hinder any therapeutic use of iPSC technology.

iPSC have also been generated from other model animals (67-69) such as the rhesus macaque (67) and rat (68, 69). These studies are useful as the rhesus macaque is the most relevant primate model for human disease, and also shows that reprogramming is conserved across species.

Disease and Patient Specific iPSC

iPSC technology has been immensely useful in the generation of "disease-specific" iPSC in humans (70-73). This offers the opportunity to recapitulate and study normal and "affected" tissue formation, enabling further studies into respective diseases and drug development or treatments.

Possible treatment of type 1-diabetes was studied by combined use of iPSC technology with directed differentiation of human iPSCs to reprogram human foreskin fibroblasts to iPSC with subsequent generation of islet-like clusters which were shown to partially recapitulate their normal in vivo function (70). Dermal fibroblasts from an 82 year old patient with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease, were also used for iPSC generation (71). iPSC were differentiated into motor neurons and glial cells which still carried the mutation present in ALS, allowing for more in-depth studies of the molecular mechanisms of this disease. These studies underline the ability to generate human models of disease with iPSC technology and also that the technology can also be applied to "old" fibroblasts, proof of concept that iPSC technology can be utilised with any age of cell. Activation of telomerase and elongation of telomeres is likely to be an important part of establishing stable iPS cells, especially if using donor cells from aged patients. Telomerase activity and telomere extension has been observed in iPSC generated from MEFs and also from "old" dermal skin from aged mice, which had telomeres of similar length to iPSC generated from "young" donors (74). iPSC have also been generated from a patient with spinal muscular atrophy (72) in which disease-specific changes in cell survival and function were reported. Differentiation towards the cell type affected in SMA (motor neurons) was allowed at early time points, but was affected upon further culture of the cells where motor neuron production was slowed or motor neurons were degenerated. A further study also reported the generation of iPSC from a wide range of genetic diseases, with either Mendelian or complex inheritance (73).

Safe iPSC?

Initial iPSC studies utilised retroviruses for gene transfer into target cells, for their ability to be silenced (2, 3), but alongside their inability to infect non-dividing cells (10), it was noted that silencing was not maintained in iPSC (11, 71). Constitutive lentiviral use, in which transgene silencing is poor (8, 41, 75) was superseded by inducible lentiviral methods which hoped to attain full silencing of transgene expression upon attainment of the pluripotent state. However, the common problem with these vector types is the possibility of integrative mutations, or reactivation of transgenes, which has been shown to lead to tumourigenesis (4). Studies on viral integration seem to demonstrate that there are possible "hot-spots" for viral integration, but the majority seem to be random (19, 76-78).

Solutions to these problems have been the centre of intense recent research and multiple techniques have been found to enhance iPSC technology. The use of a polycistronic transcript, in which all transgenes are present in a single linear form, could theoretically reduce the number of integrations required for iPSC generation down to just one and has been utilised to generate iPSC from both MEFS and TTFs with good efficiency (0.5-1%) (79, 80). Most of the iPSC showed only one integration site and full transgene silencing. A further study in this area was able to show that human neonatal human foreskin fibroblasts could be reprogrammed similarly (81). The iPSC were generated with relatively low efficiency, although they did express common pluripotency markers, formed teratomas with cells representative of all three primordial germ layers and some differentiation potential was reported.

The use of adenoviral transgene transduction, which does not lead to transgene integration, was studied next (82). Hepatocytes, which are easily infected by adenovirus, were successfully reprogrammed with no integration, and iPSC generated showed endogenous expression of pluripotency-associated genes. The adeno-iPSC also formed teratomas in nude mice, while some chimerism and germ line transmission was observed. However, this was only observed in one cell type, the efficiency of reprogramming was extremely low (0.001-0.0001%) and 23% of the iPS lines were tetraploid. A similar study generated iPSC utilising a plasmid based approach (83), which did not require the use of a virus, although again this was only in one cell type and at very low efficiency.

Recent studies have solved problems associated with the techniques outlined above by the use of plasmid-based transduction of transgenes using the piggyBac (PB) transposition system and Cre-lox technology (84, 85) which allows for seamless integration then excision of transgenes (86). In the first study, stable iPSC are generated from MEFs and HEFs using dox-inducible transgenes using the piggyBac system (85) and seamless removal of the transgenes from iPSCs was observed following transient transposase expression. Further, pluripotency of the MEF-derived iPSCs was confirmed via a tetraploid embryo complementation assay. The second study utilised a single plasmid with a 2A-peptide-linked reprogramming cassette, flanked by loxP sites, to generate iPSCs in MEFs, with subsequent removal of the transgene by Cre expression (84). The single plasmid cassette was then combined with piggyBac technology to induce stable formation of iPSC from HEFs, although removal of the plasmid was not reported. These methods seem to be simple (use of plasmids and accessible transfection products), can be used in any cell type (not restricted by tendency to be infected by virus) and may allow for accurate transgene removal subsequent to iPSC generation. This technique, while still initially requiring transgene integration, does remove the possibility of transgene re-expression and associated tumourigenic consequences. This enhanced technique was then utilised in a therapeutic context, with the generation of disease-specific transgene-free human iPSCs (87). Fibroblasts from sporadic-PD sufferers were reprogrammed into iPSC, and subsequently, the transgenes were excised through expression of Cre-recombinase, moving the major therapeutic goals of iPSC technology significantly closer. Further, the paper also suggests that after the excision of the viral transgenes, the expression profile of these iPSC was more similar to that of hESCs than previously generated iPSC lines. This suggests a small amount of "leaky" expression from transgenes is still enough to perturb the gene expression profile of such iPSCs.

Selection Criteria for iPSC

If iPSC technology matures rapidly, it will be possible to generate large numbers of iPSC lines, but what criteria do we

use to select for therapeutically useful iPSC? Pluripotent ESC can be distinguished by morphological criteria, by surface-antigen detection, by loss of transgene expression and by analysis of gene expression, protein expression and studies of DNA methylation and histone modifications. Lineage specific differentiation, teratoma formation, germline transmission and tetraploid complementation assays are also routinely used to identify pluripotent cells. However, to do all these assays for each iPSC clones would be a great undertaking, or can not be undertaken, such as chimera formation and tetraploid complementation in the case of human iPSC.

This conundrum has been the topic of some debate and minimum criteria to test the pluripotent nature of iPSC have been suggested (88-90). These include, morphological attributes (including self-renewal), correct expression of pluripotency and lineage-associated genes, transgene independence and proof of functional differentiation. For human iPSCs, the main functional test is suggested to be the differentiation if iPSC into cell types representative of all three primordial germ layers in teratoma formation.

SO IS THIS THE END OF ES RESEARCH?

The short answer, of course, is no.

Research into ESCs has allowed us to understand some of the key genetic and epigenetic pathways which govern ESC pluripotency and self-renewal (91, 92), and without this data iPSC technology may not have been developed. Indeed, iPSC technology was partly inspired by the fact that somatic human cells could become reprogrammed by fusion with hESC so suggesting that factors present in hESC allows the reprogramming of a somatic cell genome (93). Further, there are still many questions which can be answered by more studies in ESC, as we still do not fully understand the pluripotent state of ESC, although gene expression (49, 91, 94, 95), protein interaction (96), epigenetic (92, 97-99) and miRNA analysis (31, 32) are surging forward. Only by such analysis and comparisons will we understand if iPSCs are true representations of ESCs. We also still do not have gold standard differentiation assays for many cell types and as functional analysis is mooted as being a possible test for the "worthiness" of iPSCs for therapy (88-90) it seems reasonable to suggest that further extended studies in ESCs are essential. Also, mentioned throughout the literature is the possible tumourigenic nature of iPSC. This is a major problem, as both the transgenes utilised and integrative viruses have been associated with tumourigenesis (100-107). Perhaps further studies in ESC biology, SCNT and associated techniques will allow us to discover new and improved ways to induce pluripotency in somatic cells without the risk of tumourigenesis,

Indeed, many top scientists, some highly involved in the iPSC field, have called for iPSCs to be studied alongside, rather than instead of, ESC biology and associated techniques, such as somatic cell nuclear transfer (SCNT) (108-112). Although, iPSC technology does not have the associated ethical problems that hESC derivation/biology and SCNT technologies (113, 114), there are many reasons why these associated technologies should progress side-by-side. Current disadvantages in iPSC technology may mean that proper therapeutic use may be decades away, in which time hESC could produce many important breakthroughs. hESCs will also be required as controls for analysis of iPSCs, further giving reason for continued research on them. Also, importantly, iPSCs will not tell us anything about early human development, which could be uncovered by the use of techniques, such as SCNT. Important processes, which are likely to be epigenetic in nature (115), that control early development may be totally by-passed, meaning many discoveries which may actually make iPSC a better, quicker and more therapeutically relevant technology may lie undiscovered.

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FIBRINOLYTIC PARAMETERS FOR HAEMODIALYSIS IN PATIENTS WITH ARTERIOVENOUS FISTULAE DYSFUNCTION

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FIBRINOLITIČKI PARAMETRI KOD BOLESNIKA SA DISFUNKCIJOM ARTERIOVENSKE FISTULE ZA HEMODIJALIZU

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ABSTRACT

High levels of serum fibrinogen and fibrin D-dimer are independent risk factors for thrombosis of vascular access. The purpose of this study was to determine the concentrations of fibrinogen and fibrin D-dimer in haemodialysis patients with arteriovenous fistulae and to investigate their potentials as markers of vascular access dysfunction. A prospective, non-randomised clinical study was carried out at the Kragujevac Clinical Center from 2004 to 2007. The study involved 228 patients, 133 (58.3%) male and 95 (41.7%) female. They were divided into a group (n = 133) of patients with initial functioning of arteriovenous fistulae and a group (n = 95) of patients with initial dysfunction of arteriovenous fistulae. Data were collected regarding the demographic structure, aetiology of renal disease, blood pressure, body mass index, biochemical parameters and concentrations of fibrinogen and fibrin D-dimer of the patients. Dysfunction of arteriovenous fistulae was significantly higher in older people, (66.5 ± 11.47 vs. 61.13 ± 13.8 yrs; p = 0.048). High diastolic blood pressure is a very important factor in the functioning of arteriovenous fistulae (83 ± 15.7 vs. 80 ± 13.9 mmHg; p = 0.03) Values of fibrinogen (6 ± 2.56 vs. 5.72 ± 2.16; p = 0.042) and D-dimer (332.03 ± 149.48 vs. 219.56 ± 193.05; p = 0.012) at the end of study showed statistically significant differences between the disease and control groups. By Cox regression analysis, the concentration of D-dimer (Beta = 0.002; SE = 0.001; p = 0.006) was shown to be an independent variable at the end of the study. The age of the patient, high concentrations of fibrinogen and D-dimer and low diastolic blood pressure were significantly associated with limited functioning of arteriovenous fistulae for haemodialysis.

Key words:
Haemodialysis, Arteriovenous fistula, Fibrinogen, D-dimer

SAŽETAK

Fibrinogen i D-dimer su parametri fibrinolize i značajni faktori tromboze. Cilj ovog proučavanja bio je da se odredi uticaj fibrinogena i D-dimera na disfunkciju arteriovenskih fistula za hemodijalizu. Istraživanje je organizovano kao prospektivna, nerandomizovana, klinička studija u periodu od 2004 do 2007. godine u Kliničkom Centru "Kragujevac" u kojem je uključeno 228 bolesnika, 133 (58,3%) mu{karaca i 95 (41,7%) žena. Oni su podeljeni u grupu bolesnika koji su imali inicijalno funkcionalne arteriovenske fistule (n = 133) i grupu ispitanika sa nefunkcionalnim fistulama (n = 95). U studiji su analizirane demografske odlike bolesnika, etiologija bubrežne bolesti, arterijski krvni pritisak i indeks telesne mase, rutinski biohemijski parametri, kao i koncentracija fibrinogena i D-dimera. Disfunkcije arteriovenskih fistula su značajno če{će kod starijih bolesnika, (66,5 ± 11,47 vs. 61,13 ± 13,8 yrs; p = 0,048). Visok dijastolni pritisak (83 ± 15,7 vs. 80 ± 13,9 mmHg; p = 0,03) je važan faktor u proceni funkcionisanja arteriovenske fistule. Vrednosti fibrinogena (6 ± 2,56 vs. 5,72 ± 2,16; p = 0,042) i D-dimera (332,03±149,48 vs. 219,56±193,05; p=0,012) na kraju studije, u korelaciji ispitivanih grupa, imaju statistički značajnu razliku. Cox regresiona analiza koncentracije D-dimera na kraju studije nezavistan je faktor rizika za funkcionisanje arteriovenskih fistula (Beta = 0,002; SE = 0,001; p = 0,006). Starije osobe, visoka koncentracija fibrinogena i D-dimera na kraju studije, kao i nizak dijastolni pritisak, značajno limitiraju funkcionisanje fistule za hemodijalizu.

Ključne reči:
Hemodijaliza, Arteriovenska fistula, Fibrinogen, D-dimer

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INTRODUCTION

The endothelium forms a natural barrier that prevents leakage from blood vessels and also functions as a contact between the blood and subendothelial structures in the formation of a thrombus. The normal haemostatic reaction is initiated by damage to the blood vessel wall and the exposure of sub-endothelial structures to blood flow, resulting in the formation of a thrombus (1).

A high level of serum fibrinogen is an independent risk factor for thrombosis of blood access in patients on haemodialysis (HD). Complications of vascular access caused by thromboses are the main reason for hospitalisation in 17-25% of patients on HD (2).

An elevated level of D-dimer is usually considered a marker of increased clotting activity. This assumption is one of the key elements in the controversy regarding the cause and consequences of hypercoagulability (1). Plasma levels of fibrin fragment D-dimer are elevated during acute venous thrombosis because D-dimer is a marker of fibrin formation and reactive fibrinolysis. In clinical settings, a low D-dimer concentration may be a useful diagnostic tool for the exclusion of acute thrombosis (3). The purpose of this study was determine the concentrations of fibrinogen and fibrin D-dimer in patients with arteriovenous fistulae (AVF) for HD and to investigate the factors that influence the function and survival of these patients.

MATERIAL AND METHODS

This investigation was designed as prospective, non-randomised clinical study. The study was performed at the Department of Nephrology and Dialysis, Urology and Nephrology Clinic, Kragujevac Clinical Center from 2004 to 2007. It involved 228 patients, 133 (58.3%) male and 95 (41.7%) female. AVF failure was defined as initial dysfunction. The criterion for enrolment of the patients was the formation of AVF thromboses within the first month following the creation of anastomosis, the most common AVF complication. The examined patients consisted of two groups:

I. A group of patients with initial functioning of AVF and
II. A group of patients with dysfunction of AVF for HD

Blood samples for laboratory analysis were taken from each individual in the middle of the week before HD. Blood was collected from the arterial end of the vascular access using a 16G AVF needle (1.65x25 mm) in HD patients before HD and from the antecubital fossa using a 21G needle. Haematological and biochemical analyses presumed to have an influence on the survival and function of vascular access were performed, and the concentrations of fibrin D-dimer at the beginning and end of the study were measured for all patients. The numbers of erythrocytes and thrombocytes, haemoglobin levels, and haematocrit count were determined with a COULTER device via flow cytometry. Concentrations of urea, creatinine, fibrinogen, cholesterol, triglycerides, total protein, albumin, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were determined by spectophotometry (ILab-600), while levels of D-dimer were measured by immunoassay (ACL(IL)). The clinical characteristics of groups were determined by the aetiology of end stage renal disease. Blood pressure was measured in dialysis patients at the beginning and at the end of the study. Body mass index (BMI) was determined in all patients as an anthropometrical parameter using the following formula: BMI=body weight kg/body height m^2.

Statistical analyses

Continuous variables are presented as means ± SD. Univariate comparisons between the groups were made using the two-tailed, unpaired Student's t test for continuous variables. Non-parametric data were compared by the $\chi 2$ test. Cox regression analysis was used to analyse relationships between predictor variables. A two-tailed P-value <0.05 was considered statistically significant.

RESULTS

Dysfunction of AVF were detected in 76 (33.3%) patients The group of patients with initial function of AVF comprised 152 (66.7%) patients, 133 (58.3%) male and 95 (41.7%) female. There was no statistically significant difference in sex structure of examined patients; p=0.07. Patients with initial dysfunction of AVF were significantly older than the patients with initial function of AVF (66.5 ± 11.47 yrs dysfunctional vs. 61.13 ± 13.8 yrs functional; p = 0.048). In patients with initial function of AVF, glomerular disease was predominant while those patients with dysfunction of fistula primarily suffered from tubulointerstitial diseases although the difference was not statistically significant. A high diastolic blood pressure (83 ± 15.7 mmHg functional vs. 80 ± 13.9 mmHg dysfunctional; p = 0.03) was very important factor in functioning of AVF but there was no significant difference in BMI in the patients we examined (Table 1).

High levels of plasma fibrinogen and D-dimer were associated with patients with dysfunctional AVF at the end of the study. Plasma fibrinogen levels were 6.25 ± 2.56 in patients with dysfunction versus 5.72 ± 2.16 in control patients; p = 0.042. D-dimer levels likewise were 332.03 ± 149.48 compared to 219.56 ± 193.05 in the controls; p = 0.012. No statistically significant differences in other biochemical parameters tested were found between the two groups (Table 2).

There was a statistically significant association between fistula survival and D-dimer concentration at the end the study, when analysed by Cox regression analysis (Beta 0.002, SE 0.001; p = 0.006) (Table 3).

DISCUSION

In HD patients, the tendency to suffer enhanced bleeding is primarily based on functional platelet abnormalities and defective adhesion to the vessel wall. On the other hand, a variety of coagulation abnormalities contribute to an increased thrombotic tendency (2). The results of this study suggest that a native AVF represents a primary vascular access in all patients for whom it is possible to create a long-term functioning AVF. The number of older dialysis patients (>65 years), has increased significantly more than that of the younger patients (4). The most common problem in older patients receiving HD is the creation of a vascular access. Initial dysfunction of AVF in our study occurred more frequently in the older patients, which is in agreement with earlier findings.

No statistically significant difference was found in the gender structure between our patients. There is no consensus in the literature there about influence of gender structure on fis-

Table 1. Demographic, anthropometric and clinical data for the two groups of patients.

		Patients with initial function of AVF (N = 152)	Patients with initial dysfunction of AVF (N = 76)	*P*-value
Age (yrs)		61.13 ± 13.18	66.5 ± 11.47	0.048*
Gender (M/F)		95/57	38/38	0.07
Systolic blood pressure (mmHg)		150 ± 23.1	145 ± 29.8	0.08
Diastolic blood pressure (mmHg)		83 ± 15.7	80 ± 13.9	0.03*
BMI† (m^2/kg)		21 ± 0.02	21 ± 0.018	0.069
Renal disease‡ (%)	Glomerular	20.4	17.1	0.55
	Tubulo-interstitial	19.7	26.3	0.26
	Diabetic nephropathy	17.7	10.5	0.15

*statistically significant results;†Body mass index;‡Only the most important diseases leading to terminal renal insufficiency

Table 2. Biochemical analyses of the examined patients.

Test	Patients with initial function of AVF (N = 152)	Patients with initial Functioning of AVF (N = 76)	*P*-value
Erythrocytes x 10^9/L	2.96 ± 25.2	3.15 ± 0.78	0.25
Haemoglobin g/L	88.5 ± 18.4	93 ± 16.2	0.06
Thrombocytes x 10^9	205 ± 82.5	195 ± 90.8	0.38
Urea mmol/L	25.1 ± 9.9	24.8 ± 9.14	0.23
Creatinine μmol/L	639 ±228.9	597 ± 284.5	0.43
Fibrinogen§ g/L	4.85 ± 2.1	5.53 ± 2.2	0.31
Fibrinogen´ g/L	5.72 ± 2.16	6 ± 2.56	0.042*
Cholesterol mmol/L	4.27 ± 1.54	4.23 ± 1.47	0.29
Triglycerides mmol/L	0.93 ± 0.8	0.91 ± 0.23	0.17
Total proteins g/L	64 ± 8.7	64 ± 9.25	0.48
Albumins g/L	35 ± 5.4	37 ± 6.4	0.30
HDL mmol/L	0.93 ± 0.25	0.91 ± 0.23	0.17
LDL mmol/L	2.5 ± 1.35	2.8 ± 0.98	0.38
D-dimer¶ ng/ml	317.28 ± 177.64	249.46 ± 111.29	0.106
D-dimer£ ng/ml	332.03 ± 149.48	219.56	0.012*

*statistically significant results;§fibrinogen at the beginning;¥fibrinogen at the end of the study;¶D-dimer=D-dimer at the beginning;£D-dimer=D-dimer at the end of the study

Table 3. Cox regression analysis of the fibrin D-dimer as a marker of thrombogenesis in the examined patients.

Parameters	Cox regression model		
	Beta	SE	*P*-value
D-dimer¶ (ng/ml)	0.000	0.002	0.384
D-dimer£ (ng/ml)	0.002	0.001	0.006*

*statistically significant results;¶D-dimer=D-dimer at the beginning; £D-dimer=D-dimer at the end of the study

tula function (5-9). In the our study 58.3% patients were male. BMI as value of malnutrition was not risk factor for the AVF dysfunction (10-15) in our study.

The major components of blood flow through the potential fistula are: cardiac output, mean arterial blood pressure, functional diameter of the feeding artery and the venous pressure of the draining vein. It is common for the cardiac output of renal dialysis patients to be lower than normal and for these patients to be unable to augment cardiac function. Therefore, their blood pressure is ultimately decreased in response to fistula placement. Patients with poor cardiac function may either go into high output cardiac failure or develop hypotension, which can ultimately clot the fistula itself (16, 17). As a consequence, the most important factor along with good vascular selection is stable blood pressure (150/83 mmHg), because hypotension can lead to AVF failure.

Glomerular diseases and polycystic renal disease are more frequent among patients without AVF failure (18). Tubulointerstitial diseases were prevalent in our patients with dysfunction of AVF, while chronic glomerular diseases were predominant in the patients without AVF failure. Moreover, a large number of glomerular disease diagnoses were established without any biopsy verification. However, differences between the groups in our study were not statistically significant.

Fibrinogen and other adhesive proteins bind to this receptor, causing platelets to aggregate. High levels of plasma fibrinogen may trigger thrombus formation in AVF (16). Plasma levels of D-dimer, a fragment of fibrin, are elevated during acute venous thrombosis because D-dimer is a marker of fibrin formation and reactive fibrinolysis (3, 11). Decreased fibrinogen concentrations and increased D-dimer concentrations at the end of a one-year study have repercussions in AVF failure due to their roles in the activation of coagulation (18, 19). In our study, there was a statistically significant positive relationship between the group of patients with initial function of AVF and patients with dysfunction of AVF with respect to the measured values of fibrinogen and fibrin D-dimer at the end of the study. This indicated that patients with thrombosis of AVF had a significantly disordered haemostatic function, in agreement with published data (2, 3, 11, 20). However, no significant differences were detected between the patient groups in indicators of the uremic syndrome and uremic complications, and other biochemical analyses.

However, Cox regression analysis indicated a significant correlation between increased fibrin D-dimer concentration at the end of our one-year study and patients with AVF complications. Moreover, D-dimer concentration in our patients was higher even when there was no clinical manifestation of intravascular thrombosis (12). This is of potentially great importance as the routine detection of D-dimer levels and its

changes in plasma may be used to predict thrombus. This detection could play a role in the prevention of thrombosis, a leading complication of AVF.

In conclusion, dysfunction of AVF was found more frequently in older patients. Careful evaluation of frequent measurements of blood pressure, and fibrinogen and D-dimer concentrations is useful for predicting thrombosis of vascular access.

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CORRELATION OF P53 EXPRESSION LEVEL AND COLORECTAL CARCINOMA LOCATION

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KORELACIJA EKSPRESIJE P53 I LOKALIZACIJE KOLOREKTALNIH KARCINOMA

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ABSTRACT

The purpose of this study was to determine the significance of the correlation of clinical-morphological parameters and the expression levels of p53 in colorectal carcinomas. This was a prospective, clinical-experimental study. We believed that there would be a correlation between the expression levels of proto-oncogenes and the pathological stage as well as the degree of histological differentiation of colon cancer. The study researched and evaluated the correlation between p53 expression and the location of colon and rectal carcinomas.

Key words: *carcinoma, colon, p53, location*

SAŽETAK

Predmet istraživanja tokom ove studije bio je utvrđivanje značaja korelacije kliničko - morfoloških parametara i nivoa ekspresije p53 kod kolorektalnih karcinoma. Istraživanje je urađeno kao prospektivna, kliničko-eksperimentalna studija. Hipoteza studije je da postoji povezanost nivoa ekspresije protoonkogena sa patološkim stadijumom i stepenom histološke diferencijacije tumora debelog creva. Ispitivana je i procenjivana korelacija ekspresije p53 sa lokalizacijom karcinoma kolona i rektuma.

Ključne reči: karcinom, kolon, p53, lokalizacija

INTRODUCTION

Colon cancer is one of the most frequent malignant tumours and the second leading cause of carcinoma-related deaths in developed countries 1. Advancements in the area of molecular medicine have led to new findings regarding the mechanisms of pathogenesis of this type of carcinoma. These findings have led to new research approaches for potential medications and diagnostic procedures for the prevention and treatment of this type of carcinoma 1-4.

Gene p53 is the most frequently mutated gene found in tumours. It is located on chromosome 17, and it encodes the p53 transcription factor. p53 regulates the cell cycle by activating gene transcription, and some p53 gene targets act to arrest the cell cycle in G1, and when necessary, it also initiates programmed cell death (apoptosis); thus, p53 belongs to the group of tumour-suppressing genes 5. The basic role of a tumour-suppressing gene is to arrest the cell cycle in order to repair errors in the DNA structure 6. Mutations or the inactivation of tumour-suppressing genes result in uncontrollable cell division and a failure to apoptose. They belong to a group of recessive oncogenes, because they are expressed after both alleles are deactivated.

The wild-type form of p53 takes part in the DNA repair process, controls the cell cycle, cell proliferation and cell differentiation, and under certain conditions, triggers apoptosis by inducing expression of the Bcl-2 gene family. The Bcl-2 family includes both anti-apoptotic (Bcl-2, Bcl-XL) and pro-apoptotic (Bad, Bcl-2-associated X protein (Bax)) proteins. Mutated p53 either looses its tumour-suppressing ability or has it inactivated through the interaction of p53 with other cellular proteins or viral oncoproteins (e.g., HPV E7) 7.

Wild-type p53 is expressed in normal tissues at undetectable levels, but the mutated form is expressed in over 50% of all tumours. The method of p53 expression and the level of p53 expression are both significant for monitoring tumour development and prognosis only if it is coexpressed with other tumour markers. However, this is not the case in terminal phases of illness when the success rate of any therapy, as well as the outcome, is almost completely known. Numerous studies have concluded that it is not enough to monitor the expression of only p53 and that other tumour markers must also be monitored, whose behaviours may point to better therapies for malignant diseases 8.

The objective of this study was to examine the p53 gene expression levels in tumour samples taken from different areas of the resected colon segments from patients with colorectal carcinoma in order to determine if a correlation exists between clinical and morphological parameters and p53 expression levels. The possible prognostic significance of this correlation was also determined.

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MATERIALS AND METHODS

This study was a prospective, clinical-experimental study. Postoperative material was obtained from the resected colorectal tumours of 63 patients (male and female) at the Surgical Clinic of the Medical Faculty in Kragujevac. Immunohistochemical analyses were made at the Department of Pathology and Forensic Medicine of KC "Kragujevac" in Kragujevac.

Immunohistochemical-staining procedures included antigen unmasking, blocking endogenous peroxidases, incubating sections with primary anti-serum and staining with the specific antibody, followed by LSAB +- HRP, according to the standard protocol 9. DAKO monoclonal mouse anti-p53 (clone DO-7) antibody diluted 1:200 was used to detect p53. The selected threshold value for determining positive or negative expression of p53 was >30% (i.e., a positive readout on >30% of tumour cells was classified as positive for p53 expression). Therefore, the scoring system was based on determining the percentage of immunoreactive tumour cell nuclei as well as determining the intensity of immunoreactive staining (Table 1).

Table 1. Adding the points for the percentage of immunoreactivity and for the intensity gives the complete possible maximal score for the evaluating expression levels.

% of immunoreactive nuclei	Intensity of immunoreactive staining
0=< 5% of nucleic staining	0 = no staining of nucleus
1= 5% to 30% of stained nuclei	1 = weak intensity of nucleus staining
2= 30% to 50% of stained nuclei	2 = mild intensity of nucleus staining
3= 50% to 70% of stained nuclei	3 = very intensive staining
4= 70% to 90% of stained nuclei	
5> 90% of stained nuclei	

Statistical methods

The Mann-Whitney test was used to compare the variable averages of two populations; for two or more populations, the Kruskal-Wallis test was used. The chi-square test and Fischer's exact test were used to determine the correlation between two descriptive variables.

RESULTS

Most of the primary colorectal carcinomas (CRCs) were located in the sigmoid colon 23/69 (36.5%). In addition, 17/63 (27%) were located in the rectum, 14/63 (22.2%) in the first section of the large intestine (cecum), and the smallest number (9/63; 14.3%) in the proximal and transverse areas of the colon.

Nuclear expression of p53 was present in 33/63 (52.4%) tumours, and the remaining 30/63 (47.6%) had no p53 expression.

Statistical analysis of CRC location and p53 expression

The Kruskal-Wallis test demonstrated that the percentage of cells with nuclear p53 expression varied significantly between areas of the colon (p = 0.000). Nuclear p53 expression in the sigmoid colon was significantly different than that in other areas, but the other areas did not differ significantly from each other (Figure 1). In contrast, there were no significant differences between the areas of the colon for the intensity of tumour cell p53 staining (p = 0.359).

Of the total 63 analysed CRC samples, 17 tumours on the sigmoid flexure (pelvic colon) were positive for p53 expression, while 6 were negative. In the rectum, 7 tumours were positive, while 10 tumours were not. Similarly, 10 tumours on the cecum were p53 negative, while only 4 were positive. Only 2/7

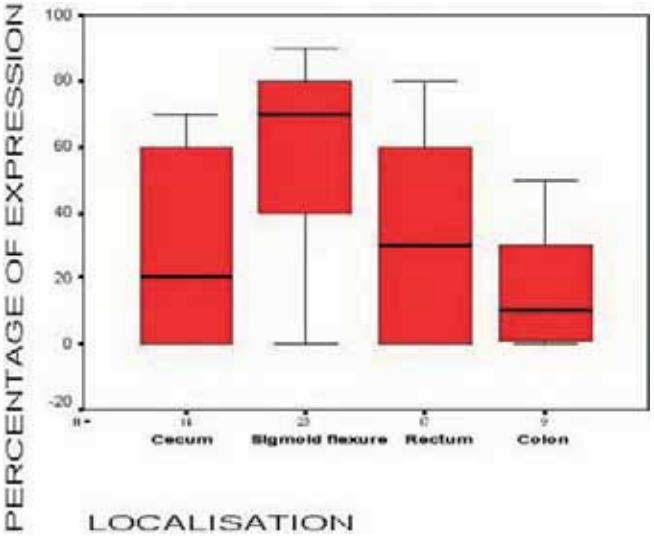

Figure 1. Comparison of colorectal carcinoma location with the percentage of p53 nuclear expression.

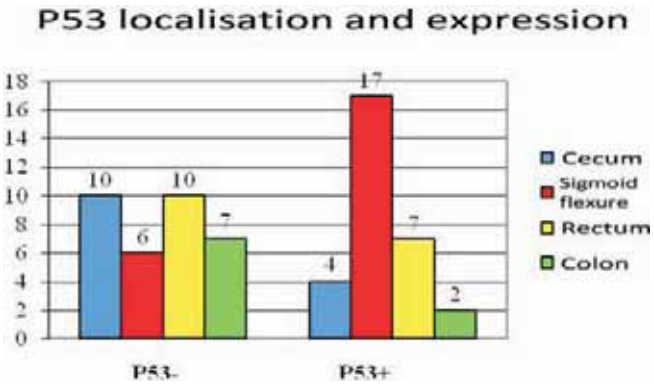

Figure 2. Comparison of the number of colorectal carcinoma samples with p53 expression and their location.

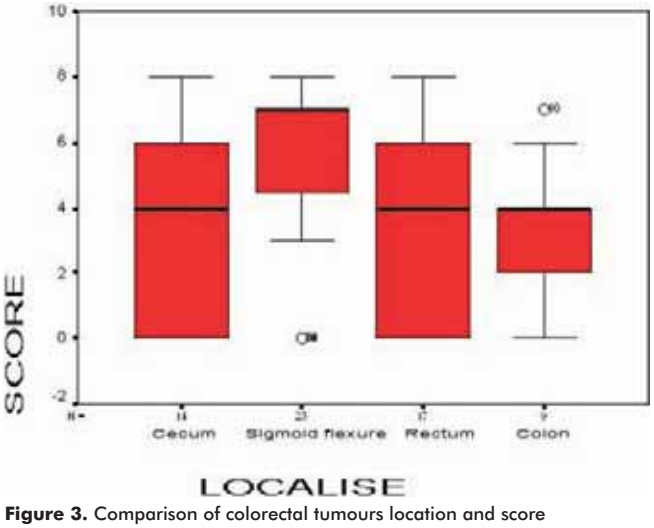

Figure 3. Comparison of colorectal tumours location and score

colon tumours were positive for p53 expression. The tumour location and p53 expression were dependent (p = 0.012) (Figure 2).

There were no significant differences in score between the analysed CRC locations (p = 0.053), although it is indicative (Figure 3).

DISCUSSION

Results of the analysis of 63 CRCs obtained in this study demonstrate that most of the primary CRCs were located in the sigmoid colon, 23/63 (36.5%). In addition, 17/63 (27%) were found in the rectum, 14/63 (22.2%) were situated in the cecum, and only 9/63 were in the proximal and transverse areas of the colon. The results obtained in this study are consistent with the results of several published studies 10-14. According to the other studies, most CRCs are located in the sigmoid colon and rectum (75%), followed by the cecum and other colon segments (16%).

We also demonstrated that the nuclear expression of p53 was present in 33 cases (52.4%), and p53 expression was used as a positivity mark in more than 30% of tumour cells. However, the remaining 30 samples (47.6%) had no p53 expression. Our results are consistent with data in the available literature regarding p53 marker expression in CRCs in which p53 expression in all carcinomas was found to be 45%-70%, while the expression of p53 in CRCs was 42%-67% 15,16.

Many studies have researched the numerous mechanisms of tumour progression in different anatomical regions. p53 mutations are more frequent in tumours of distal parts of the colon and rectum, with underlying invasion of blood and lymphatic vessels in these tumours 17,18. This may explain the highlighted aggressiveness and vascular invasion of proximal colon carcinomas with p53 overexpression. The connection and relationship between p53 mutations and other clinical-pathological indicators has not been confirmed. Some authors have demonstrated that the specific p53 mutations and elevated p53 expression are connected with the aggressiveness of distal-region tumours 19,20. According to the available data, the allele deletion of 17p correlates with a higher risk of emergence of outlying metastases, especially with tumours on the colon's left side 21.

Examination of the difference between the biological behaviour of p53 in tumours and the rectum does not support

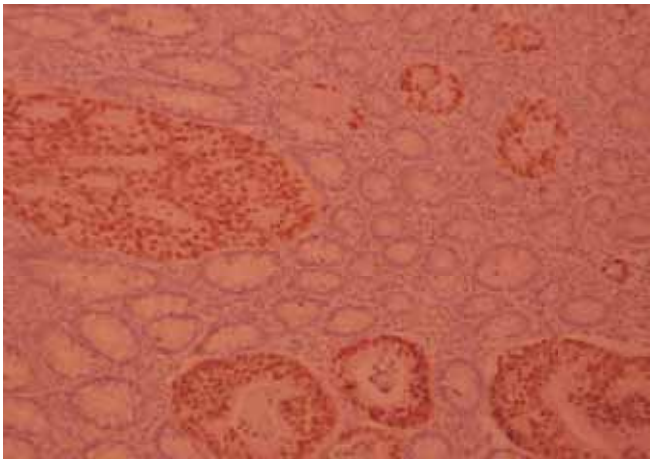

Figure 4. Immunohistochemical staining of p53 (x200).

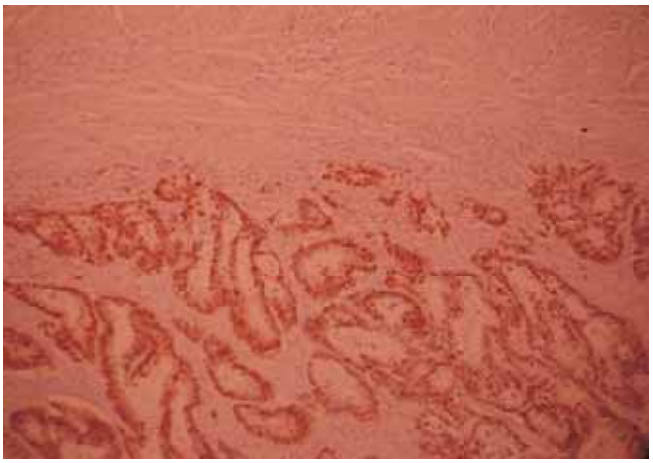

Figure 5. Immunohistochemical staining of p53 (x100).

p53 as an independent prognostic factor for survival in rectal tumours 22 (Figure 4). Immunohistochemically detected p53 has a limited predictive value, especially in proximal colon tumours. However, an increased p53 expression level in CRC was found in distal parties 23.

A high frequency of p53 mutations in left-side tumours (71%) and the rectum (91%) suggest that the molecular mechanisms of tumourigenesis in synchronous left- and right-side tumours are probably different 24. Still, the fact that a Tp53 mutation's prognostic value regarding a tumour's biological behaviour is dependent on the tumour's location remains 25.

According to the literature, CRC genesis begins with a series of mutations through the adenoma-carcinoma sequence: first APC (tumour suppressor), which results in dysplasia, then K-Ras (oncogene), resulting in anaplasia, and lastly p53, which gives a malignant character to the already mutated tissue 26. Cancerogenesis and CRC development include mutations in multiple genes (APC, C-myc, K-Ras, β-catenin, SMAD4). However, the cumulative effects have a bigger role in cancerogenesis than the particular sequence of changes in those genes 26,27. Molecular markers can help determine the risk of developing an invasive carcinoma from premalignant lesions (adenoma). Therefore, gene therapy should target a number of genes involved in cancerogenesis. According to the same data, these tumours have some genetic instability and a loss of their normal karyotype 26-28. Other authors also suggest its value in the progression of the genetic errors 29.

Today, the p53 mutation is the most frequently found mutation in breast, oesophagus and non-small cell lung carcinoma 30,31. p53 is the base of Lane's functional model of the žmolecular policeman'. Wild-type p53 is the transcriptional regulator in the G1 phase of the cell cycle 32. When DNA damage occurs, p53 is activated, and through p21, it inhibits cyclin-dependant kinases and the subsequent phosphorylation of proteins needed to enter S phase. The G1 pause allows DNA error repair or the induction of apoptosis and the prevention of mutated cell proliferation 33. p53 mutations result in an accumulation of p53 proteins, leading to greater proliferation, loss of apoptosis, chromosome instability and disruption of differentiation 34. On the other hand, p53-induced cell death can prevent Bcl-2 expression of proto-oncogenes and inhibit apoptosis 35. All previously used literary information states that the majority of CRCs begin through a successive process of sequential genetic and phenotypic changes, i.e., through hyperplasia, adenoma, carcinoma and metastasis.

Some studies have presented CRC cases in which p53 expression was 76%, and in those, the increased expression was linked with a shorter survival period 36-40. Many studies have dealt with the indicators of prognosis in patients with malignant diseases regarding p53 protein accumulation, genetic mutation of the gene encoding it and the loss of heterozygosity of the allele on chromosome 17. These clinical and basic studies suggest that damage to or mutation in p53 leads to excessive genetic amplification of p53 and a loss of cell-cycle and apoptosis control. The level of these changes has represented the degree of unfavourable prognosis for patients, as tumours with p53 hyper-expression have proven to be resistant to radiation and most chemotherapeutics 41. However, some authors have presented a contradictory relationship between p53 expression and survival rate, arguing that the expression has limited value when it comes to estimating the clinical outcome 42,43. Contrary to those, other authors believe it is a clinically significant marker 44. Some studies have shown that p53 expression is an indication of shorter survival 45,46. Vice versa, some regard p53 expression as a better prognostic factor for colon tumours in the Australian population 47. Recent studies point out that only simultaneous expression of K-Ras and p53 can be an indicator of unfavourable prognosis 47.

In thirty-five other studies (twenty-four including immunohistochemical staining), p53 expression was found to be a predictor of an unfavourable outcome (Figure 5). Paradoxically, twenty-four other studies have shown the opposite results 48. Some studies have shown that the expression level does not have a connection with biological behaviour, tumour stage, histological type, location, tumour size, presence of lymph node and venal invasion, perineural invasion, metastases in lymph nodes or outlying metastases (hepatic and peritoneal), all of which disputes the significance of p53 49.

Based on information that the growth of p53-transfected malignant cells is arrested and that mutant p53 gives way to a vaccine therapy, other studies have proposed the possibility of gene therapy 50.

Wild-type p53 is expressed in normal tissues at undetectable levels. However, mutated p53 is expressed in over 50% of all tumours, when it is possible to detect it (commonly through immunohistochemistry). As a multi-marker, whose level and method of expression is monitored in many tumours, it is used to monitor its connection with the development of malignant diseases 5,7.

Today, colon and rectal tumourigenesis is a known process, from adenoma to carcinoma through a series of mutations (as described above). In an attempt to examine the correlation between the three key molecules in cell transformation (APC, K-ras, and p53) in colorectal neoplasm, some studies have monitored the level of expression of these three tumour markers. The results have shown that p53 is expressed in more than 50% of tumours, APC in approximately 50% of tumours and K-ras in less than 30%. Co-expression of p53 and APC was present in 27% of all tumours, and co-expression of all three only in a low percentage (less than 7%). However, the co-expression of all three corresponded with the most aggressive form of disease and thus, an unfavourable prognosis. Based on the gathered results, the authors concluded that K-ras and p53 use two independent genetic mechanisms of cancerogenesis, which when activated together, lead to the development of aggressive forms of the disease 5,7.

Other studies monitored the expression levels of normal and mutated p53 in breast cancer in situ. Identical p53 mutations were found in approximately equal percentages in in situ carcinoma and in invasive forms of the disease, suggesting that there is a clonal connection between the in situ carcinoma and invasive disease. Thus, in situ carcinoma formation is the critical moment in the disease development pathway; on the other side, it disproves the modern understanding of the p53 cancerogenesis mechanism, which implies that the accumulation of mutations leads to an invasive form of the disease 51. In these studies, p53 expression was noted in 36% of all tumours, and in 7%, only mutated p53 expression was noted; in 10%, wild-type p53 hyper-expression was observed, while in 19%, mutated p53 was expressed and wild-type p53 was hyper-expressed 1.

In other mutant p53 studies, the effects of recessive and transdominant mutants were examined; these differed in only one amino acid, and this was the consequence of an error on codon 72. However, the structural difference in only one amino acid differentiated the paths of inhibition of wild-type p53 and mutant p53. The recessive mutation directly inhibited wild-type p53 and the transdominant indirectly inhibited it through p73. This is significant, because recessive mutants are formed more frequently in the process of tumourigenesis (73%) 7.

The occurrence of any of the two mutants in heterozygotic form with wild-type p53, through the inhibition of the latter, allows the coexistence of both wild-type and mutated p53 in the tumour, where both exert their effects. This explains the results of studies that have shown hyper-expression of both wild-type p53 and mutated p53 in the cases with the highest tumour p53 expression. In fact, the smallest percentage (same study) of tumours expressed only mutated p53, but those tumours were the most aggressive. Thus, balance exists in wild-type/mutant p53 coexistence: mutant p53 develops its tumourigenic effects, and wild-type controls them, preventing an already severe disease from becoming even more dangerous 8.

The results of the mentioned studies are somewhat contradictive, since it is now known that tumourigenesis occurs only if both p53 alleles mutate or are missing, meaning that while p53 is heterozygote, one normal p53 is preserved. The previously mentioned studies have shown that p53 is the watchdog of cell functions and can drive the cell in two directions: apoptosis or uncontrollable cell division. However, some studies noted wild-type p53 hyper-expression in tumours when mutant p53 was not expressed. In such cases, it is presumed that some other genetic pathway is activated and that it crosses over with wild-type p53's expression pathway, as is the case with mutated Mdm-2, which now would not be able to inhibit p53 expression 52,53.

The results of these studies lead to the conclusion that it is not enough to monitor only p53 expression for prognosis, but that the expression of other tumour markers whose behaviours may point to some better therapeutic approaches for malignancies must also be monitored54-57.

A number of recent studies have monitored the correlation of p53, Ki67, HER2 and hormonal receptor expression with clinical-pathological parameters in ductile breast carcinoma. The size of the tumours has been correlated with HER2+, ER+ and Ki67 expression, and necrosis has been correlated with p53 expression. ER-PrR-HER2+p53+ status was the sign of an unfavourable prognosis 8.

Other studies have examined the predictive factors of metastases genesis in the breast cancers of patients undergoing a mastectomy during postoperative radiotherapy. These studies monitored HER2, p53 and Bcl-2 expression and demonstrated that HER2 and p53 were independent predictors of breast carcinoma metastasis 52,57,58. Bcl-2 has also been identified in 38% of tumours, and it correlates with a small tumour size, low histological grade and the lack of metastases. p53 was expressed in 19% of tumours and correlated only with ER+ status. HER2 was expressed in 53% of tumours and correlated with a high number of metastases and a high histological grade 8,52,53. These results suggest that HER2 can be referred to as an independent indicator of disease prognosis, but p53 expression can only be used as a prognostic factor when coexpressed with HER2 52,53,57,58.

Studies of tumour marker expression on tumour cells isolated from the bone marrow of breast cancer patients (early stage) have shown that early metastasis is rare in patients with ER+PrP+ primary tumours, while it is more frequent in HER2+ primary tumours. However, studies have not examined the correlation between early metastasis occurrence and p53-/p53+ primary tumours 53.

Studies examining the expression of the potential prognostic factors pRB2/p130, p107, p27kip1, p53, Mdm-2 and Ki67 in prostate carcinoma have shown that the expression of p53, Mdm-2, Ki67 and low-level expression of p27kip1 correlates with an unfavourable prognosis. It has also been shown that RB mutations (tumour-suppressor) are early events in tumourigenesis and that p53 expression is not a good prognostic factor in the early stage of disease 54.

Changes in p16, p53, SMAD4 and Ki67 expression in intraductal papillary-mucinous pancreatic tumours are also present in pancreatic adenoma. However, mutations in these molecules were more frequent in pancreatic cancer, which supports the theory that the invasiveness of tumours rises alongside the accumulation of mutations, meaning that the prognosis is more unfavourable 55. In Barrett's adenocarcinoma, there is an inverse relationship between the expression of mutated p53 and p21 59.

The results of the mentioned studies suggest that p53 expression can only be used for disease prognosis when it is coexpressed with other tumour markers. A direct correlation between p53 expression and prognosis is noted in the later stage of the disease, when any therapy is questionable, meaning there is no significance in measuring the level of its expression 60.

CONCLUSION

The greatest number of colorectal carcinomas is located in the sigmoid flexure. There is a correlation between p53 expression and location, meaning that the location and p53 are dependant. The percentage of p53 nuclear expression in the sigmoid flexure is significantly different from the expression percentages in other locations, while the differences between other locations are not statistically important, but are indicative. On the basis of the previously stated facts, we conclude that p53 does not have prognostic potential, but its value regarding the understanding of oncogenesis is undisputable.

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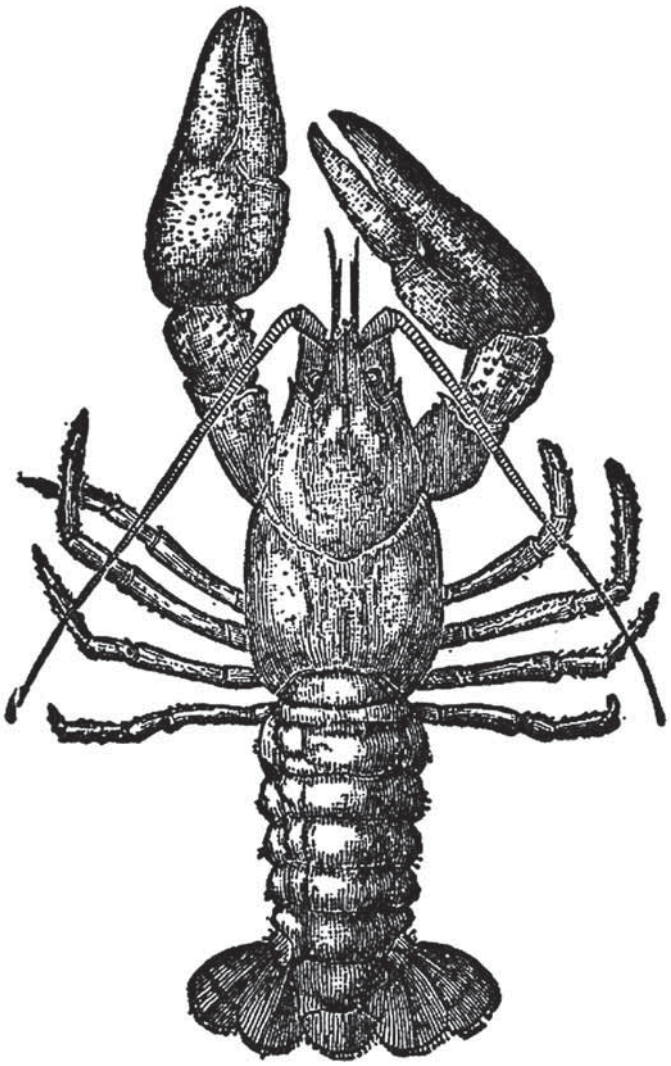

COSTS OF CAESAREAN SECTION AND VAGINAL DELIVERY IN AN UPPER-MIDDLE-INCOME COUNTRY: A CASE SERIES

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TROŠKOVI CARSKOG REZA I VAGINALNOG POROĐAJA U ZEMLJI SA SREDNJE-VISOKIM PRIHODIMA: SERIJA SLUČAJEVA

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ABSTRACT

Background. *Elective Caesarean section is an economically favourable delivery method in some countries, but an unfavourable option in other high-income economies.*

Objective. *The aim of this study was to compare the immediate costs of Caesarean section and vaginal delivery, identifying their main determinants in the health system milieu of a higher-middle-income Balkan country.*

Methods. *The study was designed as in-depth case series study of Caesarean sections and vaginal deliveries in a tertiary care Serbian hospital within a six month period. The perspective of the Serbian Republic Institute for Health Insurance was followed, and direct costs caused by mother and newborn during the hospitalisation were registered. In total 943 pregnant patients at term entered the study. Elective Caesarean section was the method of delivery for 113 patients, while 830 patients had vaginal deliveries.*

Results. *The average total direct costs per delivery case during hospitalisation of both mother and child were significantly higher ($t = 8.187$; $p < 0.001$) in the group with Caesarean section delivery (96,992.84 ± 67,842.82 RSD), as compared with those who had a vaginal delivery (41,869.60 ± 42,843.16 RSD). The main determinants of increased costs were delivery method, length of hospitalisation, Apgar score, weight at birth and pregnancy-induced hypertension.*

Conclusions. *Within the economic settings of Balkan countries, Caesarean section bears higher immediate costs than vaginal delivery due to prolonged maternal hospitalisation, worse immediate delivery outcomes and a higher rate of complications in pregnancy.*

Key words: *Caesarean section, delivery, costs*

SAŽETAK

Uvod. *Planirani carski rez je metod porođaja čija pogodnost primene, sa ekonomskog aspekta varira u zemljama sa visokim prihodima.*

Cilj. *Upoređenje neposrednih direktnih troškova carskog reza i vaginalnog porođaja, uz identifikaciju odrednica koje u najvećoj meri utiču na njih u zdravstvenom sistemu zemlje Balkana sa srednje-visokim prihodima.*

Metode. *Studija je dizajnirana kao serija slučajeva carskih rezova i vaginalnih porođaja sa detaljnom analizom svakog od njih, u tercijarnoj zdravstvenoj ustanovi Republike Srbije u periodu od šest meseci. Korišćena je perspektiva Republičkog zavoda za zdravstveno osiguranje Srbije, a praćeni su i analizirani direktni troškovi i majke i deteta tokom perioda hospitalizacije. Istraživanje je obuhvatalo ukupno 943 trudnice u terminu za porođaj, od čega je planirani carski rez bio metod porođaja kod njih 113, dok je 830 trudnica porođeno vaginalnim putem.*

Rezultati. *Prosečni ukupni direktni troškovi i majke i deteta su, po porođaju, tokom hospitalizacije bili značajno viši ($t = 8,187$; $p < 0,001$) kod grupe učesnica u studiji koje su se porodile carskim rezom (96,992.84 ± 67,842.82 RSD) u odnosu na grupu sa vaginalnim porođajem (41,869.60 ± 42,843.16 RSD). Determinate koji su značajno uticale na porast troškova bile su metod porođaja, dužina hospitalizacije, Apgar skor, težina novorođenčeta na porođaju i hipertenzija indukovana trudnoćom.*

Zaključak. *U ekonomskim uslovima koji vladaju u zemljama Balkana, carski rez ostvaruje više neposredne troškove u odnosu na vaginalni metod porođaja usled produžene hospitalizacije majki, lošijih neposrednih postporođajnih ishoda vezanih za novorođenče kao i, uopšte učestalijih komplikacija u trudnoćama koje su završene ovim putem.*

Ključne reči: *Carski rez, porođaj, troškovi*

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INTRODUCTION

The prevalence of Caesarean section deliveries has risen across the world, with the total number of Caesarean sections continuing to rise (1). This may have a profound influence on national and regional health budgets, provided that significant differences exist in the costs of these two delivery methods. Economic studies conducted in high-income economies had shown conflicting results when comparing costs of Caesarean section and vaginal delivery. While in Canada, Caesarean delivery during labour for the first delivery was associated with increased cumulative costs (2), in the USA the average total costs of vaginal deliveries (maternal plus total baby costs) is higher than the average total costs of Caesarean section (1). However, the results of such comparisons are highly dependent on the scope of the costs that are taken into account (e.g., whether the cost of newborns were included or not, and whether only immediate costs were considered or both immediate and delayed costs were included) (3), on the type of Caesarean delivery itself (4) (with or without labour) and on foetal presentation during vaginal delivery (5) (breech of cephalic).

The economic aspects of Caesarean section delivery in upper-middle-income economies have not been studied. The majority of the Balkan countries (Serbia, Montenegro, Bulgaria and Romania) have made rapid socio-economic transitions during the last decade from planned economies of the socialist type to liberal, market-driven economies, resulting in their shift from the lower- to the higher-middle-income countries group (2007 gross national income per capita from $3,706 to $11,455) (6). These countries also have similar annual health budget per capita, which are as follows: Bulgaria $272, Serbia $212, Montenegro $212 and Romania $250 (7). While drug and medical device costs in Balkan countries are similar to costs in developed European countries, the cost of health care services is much lower (8). This creates a different economic environment in health care, and could produce different cost relations between the same pair of medical procedures in high-income European countries and higher-middle-income Balkan countries.

The aim of our study was to compare the immediate costs of Caesarean section and vaginal delivery, identifying their main determinants in the health system milieu of the higher-middle-income Serbian economy.

MATERIALS AND METHODS

Our study was designed as a detailed, in-depth case series study (9) of Caesarean sections and vaginal deliveries in a tertiary care Serbian hospital.

The perspective and the time frame

In Serbia, the Republic Institute for Health Insurance (RIHI) is the institution that makes contracts with health facilities and controls the prices of services, drugs and materials. Our study was carried out within this framework. Only services, drugs and materials endorsed by RIHI were taken into account when calculating costs. The observations of costs occurred during the periods for which the mother and newborn were hospitalised.

The patients

The study was conducted among patients of the Gynaecology-Obstetrics Clinic, Clinical Centre "Kragujevac", in Kragujevac, Serbia. All pregnant patients of the Clinic who came for a delivery during the period January the 1st, 2008 – June the 30th, 2008 were enroled in the study. In total, 943 pregnant patients at term entered the study; none was lost to follow-up. The investigators did not interfere with the clinical decision of choosing Caesarean section or vaginal delivery by the obstetricians. Elective Caesarean section was the method of delivery in 113 patients, while 830 patients had a vaginal delivery. The characteristics of the patients are shown in Table 1.

The costs

Only direct costs made during the stay of mother and child in the hospital were taken into account in this study. The costs of hospital days, services, materials and drugs used were collected from invoices sent to the Republic Institute for Health Insurance by the Clinical Centre "Kragujevac" for each mother and newborn studied. The hospital day cost is a flat-rate cost, which includes only bed and food (1,255.31 RSD/day). The following services and materials were invoiced separately: physical examination (284.01 RSD), Caesarean section delivery (7,726.25 RSD), vaginal delivery (898.19 RSD), intravenous injection (93.49 RSD), intramuscular or subcutaneous (48.51 RSD) injection, blood sample collection (48.51 RSD), ECG (195.26 RSD), blood count (241.40 RSD), blood chemistry (593.72), wound dressing (93.49 RSD), etc. (services), and infusion sets, needles (surgical and syringe), syringes, surgical gloves, catheters, urine bags, dressings, etc. (materials). The drugs used were also invoiced separately. The costs are expressed in Serbian official currency, the dinar (RSD); the average exchange rate in June 2008 was 1 Euro = 77 RSD.

Statistics

The costs are presented as mean values, with standard deviations (SD). Statistically significant differences between the treatment groups were tested by Student's t-value for differences between small, independent groups, or by chi-square contingency tables with Yates' continuity correction (for comparing frequencies). The difference was considered significant if the probability of null hypothesis was less than 0.05 ($p < 0.05$). The main determinants of costs were identified by means of multiple linear regression (10); all calculations were made using SPSS-10 statistical software for Windows.

RESULTS

The average total direct costs per delivery case during hospitalisation of both mother and child were significantly higher ($t = 8.187$; $p < 0.001$) in the group who had a Caesarean section delivery (96,992.84 ± 67,842.82 RSD), as compared with those who had a vaginal delivery (41,869.60 ± 42,843.16 RSD).

The average total direct costs per delivery case during hospitalisation of the mother alone were also significantly higher ($t = 20.112$; $p < 0.001$) in the group who had a Caesarean section delivery (55,785.04 ± 15,436.11 RSD), as compared to those who had a vaginal delivery (24,910.34 ± 11,124.80 RSD).

The average total direct costs per delivery case during hospitalization of the child alone were significantly higher ($t = 3.671$; $p < 0.05$) in the group who had a Caesarean section delivery (41,679.45 ± 67,215.23 RSD), as compared with those who had a vaginal delivery (17,146.37 ± 40,845.37 RSD).

Characteristic	Caesarean section delivery (n=113)	Vaginal delivery (n=830)	Test value and significance of difference
Age (years)	30.4 ± 5.5	27.6 ± 5.4	t = 5.018; p < 0.001*
Length of mother stay in days	7.4 ± 1.2	4.5 ± 1.8	t = 22.771; p < 0.001*
Length of child stay in days	12.3 ± 14.7	5.5 ± 8.5	t = 4.788; p < 0.001*
Apgar score (0 – 10)	8.2 ± 1.8	8.8 ± 1.4	t = 3.483; p < 0.001*
Weight at birth (grams)	3096.4 ± 831.0	3370.4 ± 631.6	t = 3.376; p < 0.001*
Tocolysis during pregnancy, n (%)	14 (12.2%)	109 (13.1%)	chi-square = 0.011; p > 0.05
Pregnancy-induced hypertension, n (%)	19 (16.8%)	49 (5.9%)	chi-square = 16.102; p < 0.001*
Cerclage, for incompetent cervix, n (%)	4 (3.5%)	24 (2.9%)	chi-square = 0.006; p > 0.05
Urinary tract infection, n (%)	3 (2.6%)	53 (6.4%)	chi-square = 1.855; p > 0.05
Colpitis in pregnancy, n (%)	1 (0.9%)	95 (11.4%)	chi-square = 11.004; p < 0.001*
In vitro fertilization, n (%)	3 (2.7%)	2 (0.2%)	chi-square = 6.888; p < 0.01*
Diabetes mellitus, gestational, n (%)	4 (3.5%)	22 (2.7%)	chi-square = 0.055; p > 0.05
Anti-phospholipid syndrome, n (%)	2 (1.8%)	6 (0.7%)	chi-square = 0.350; p > 0.05
Premature rupture of the membranes, n (%)	2 (1.8%)	18 (2.2%)	chi-square = 0.005; p > 0.05
Vacuum extraction, n (%)	0 (0%)	4 (0.5%)	chi-square = 0.001; p > 0.05
Placenta praevia, n (%)	2 (1.8%)	0 (0%)	chi-square = 7.546; p < 0.01*
Premature detachment of placenta, n (%)	2 (1.8%)	1 (0.1%)	chi-square = 4.124; p < 0.05*
Pregnancy followed-up by a gynecologist, n (%)	112 (99%)	813 (97.9%)	chi-square = 0.232; p > 0.05

* Significant difference

Table 1. The characteristics of Caesarean section and vaginal delivery cases

When total direct costs per delivery case during hospitalisation of both the mother and child were taken as dependent variable, and delivery method (Caesarean section or vaginal), length of mother stay in days, length of child stay in days, Apgar score, weight at birth (grams), tocolysis during pregnancy, pregnancy-induced hypertension, cerclage for incompetent cervix, urinary tract infection, colpitis in pregnancy, in vitro fertilization, gestational diabetes mellitus, anti-phospholipid syndrome, premature rupture of the membranes, vacuum extraction, placenta praevia, premature detachment of placenta, and pregnancy follow-up by a gynaecologist, as independent variables, the results of multiple regression showed that only delivery method, length of newborn stay (in days), Apgar score, weight at birth and pregnancy-induced hypertension had a significant influence on the total costs (Table 2).

When total direct costs per delivery case during hospitalisation of the mother alone were taken as the dependent variable, and the delivery method (Caesarean section or vaginal), length of mother stay in days, length of child stay in days, Apgar score, weight at birth (grams), tocolysis during pregnancy, pregnancy-induced hypertension, cerclage, for incompetent cervix, urinary tract infection, colpitis in pregnancy, in vitro fertilization, gestational diabetes mellitus, anti-phospholipid syndrome, premature rupture of the membranes, vacuum extraction, placenta praevia, premature detachment of placenta and pregnancy follow-up by a gynaecologist, as independent variables, the results of multiple regression showed that only delivery method, length of mother stay in days, Apgar score, weight at birth, vacuum extraction, pregnancy-induced hypertension, urinary tract infection and diabetes mellitus, gestational, had a significant influence on the total costs (Table 3).

When total direct costs per delivery case during hospitalization of the newborn alone were taken as the dependent variable, and delivery method (Caesarean section or vaginal), length of mother stay in days, length of child stay in days, Apgar score, weight at birth (grams), tocolysis during pregnancy, pregnancy-induced hypertension, cerclage, for incompetent cervix, urinary tract infection, colpitis in pregnancy, in vitro fertilization, gestational diabetes mellitus, anti-phospholipid syndrome, premature rupture of the membranes, vacuum extraction, placenta praevia, premature detachment of placenta and pregnancy follow-up by a gynaecologist, as independent variables, the results of multiple regression showed that only length of mother stay in days, length of newborn stay in days, Apgar score, weight at birth and pregnancy-induced hypertension, had a significant influence on the total costs (Table 4).

DISCUSSION

In order to be cost-effective when compared to vaginal delivery, Caesarean section delivery should be associated with a low rate of surgical complications, shorter length of stay in a hospital for both mother and newborn, and a more rapid adaptation of a newborn to extrauterine life, as measured by the Apgar score (11,12). In our study, the obstetricians chose vaginal delivery more frequently (7.3:1), with excellent results. When compared with Caesarean section, vaginal delivery was associated with significantly shorter hospitalisation of both mother and newborn, and with better average Apgar scores. In addition, the average cost of Caesarean section delivery was twice as high as that of vaginal delivery, making Caesarean section an economically less favourable delivery option in Balkan countries settings.

The multiple regression analysis showed that Caesarean section primarily increases costs caused by the mother during hospitalisation, while the costs caused by child are not directly dependent on the delivery method. The costs caused by a mother are also increased due to longer hospitalisation, worse immediate delivery outcomes (lower Apgar score and lower

Variable	Regression coefficient (B)	Standard Error	t	p - value
Delivery method	-15,796.4	3,928.7	-4.021	0.000
Length of newborn stay in days	7,468.5	192.3	38.844	0.000
Apgar score	-1,261.7	412.9	-3.056	0.002
Weight at birth	-7.6	1.9	-4.048	0.000
Pregnancy-induced hypertension	11,525.4	4,145.6	2.780	0.006

Table 2. Multiple regression of total direct costs per delivery case during hospitalisation of both mother and child

Variable	Regression coefficient (B)	Standard Error	t	p - value
Delivery method	-18,902.7	1,149.7	-16.441	0.000
Length of mother stay in days	4,058.9	185.5	21.889	0.000
Apgar score	-287.6	120.8	-2.380	0.018
Weight at birth	-1.4	0.5	-2.472	0.014
Pregnancy-induced hypertension	2738.8	1213.2	2.258	0.024
Vacuum extraction	24,328.9	4,686.1	5.192	0.000
Urinary tract infection	-2,623.4	1,313.5	-1.997	0.046
Diabetes mellitus, gestational	5,316.4	1,887.5	2.817	0.005

Table 3. Multiple regression of total direct costs per delivery case during hospitalisation of mother only

Variable	Regression coefficient (B)	Standard Error	t	p - value
Length of mother stay in days	-3,354.8	577.1	-5.813	0.000
Length of newborn stay in days	7,476.1	175.1	42.700	0.000
Apgar score	-974.1	376.0	-2.591	0.010
Weight at birth	-6.2	1.7	-3.651	0.000
Pregnancy-induced hypertension	8,786.6	3,774.9	2.328	0.020

Table 4. Multiple regression of total direct costs per delivery case during hospitalisation of newborn only

birth weight), and complications of the pregnancy (higher rate of pregnancy-induced hypertension and gestational diabetes mellitus). It seems like obstetricians chose Caesarean section as a delivery method in patients with complicated pregnancy, which led to prolonged post-delivery hospitalisation and higher costs. However, Caesarean section itself results in longer hospitalisations and postpartum maternal medical care utilization than vaginal delivery, regardless of the complications in pregnancy (13,14).

The costs caused by newborns were linked primarily to the immediate delivery outcomes (lower Apgar score and lower birth weight increased costs), longer hospitalisation and complications of pregnancy (hypertension), which could have led to worse outcomes. Higher costs caused by the newborns after Caesarean section probably reflect bias deliberately made by the obstetricians, choosing Caesarean section for complicated pregnancies, and may not be a reflection of the effects of this delivery method itself. However, our study has serious limitations due to short follow-up period, which resulted in our registering only immediate costs. The delayed costs of children with birth injuries remains unknown (15), and their potential to change the overall cost ratio of the two delivery methods is unclear.

In conclusion, within the economical settings of Balkan countries, Caesarean section bears higher immediate costs than vaginal delivery due to prolonged maternal hospitalisation, worse delivery outcomes and a higher rate of complications in pregnancy.

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MARKERS OF INFLAMMATION IN TROPONIN T-NEGATIVE UNSTABLE ANGINA PECTORIS

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MARKERI INFLAMACIJE U TROPONIN T NEGATIVNOJ NESTABILNOJ ANGINI PEKTORIS

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ABSTRACT

Introduction. *Chronic inflammatory processes are crucial in the pathogenesis of atherosclerosis and are considered risk factors for the development of cardiovascular disease. Troponin T-negative unstable angina pectoris, a clinical syndrome in acute coronary syndrome, accompanied by increased values of systemic inflammatory markers is a possible sign of further deterioration in acute myocardial infarction and sudden cardiac death.*

Aim. *We examined the inflammatory status of patients with troponin T-negative unstable angina pectoris (CRP, Homocysteine, fibrinogen, leukocyte and sedimentation) in comparison to patients with no cardiovascular disease.*

Method. *We examined the levels of CRP-a, homocysteine, leukocytes, fibrinogen, and sedimentation in 39 patients over a period of 4 months; 20 patients were included in the experimental group, with values of troponin T ≤0.03 ng/ml; 19 patients did not have cardiovascular disease (control group). The criteria used for cardiovascular patients were troponin T values on the second day after admission of less than 0.03 ng/ml, pain in the chest that did not resolve with therapy and lasted longer than 20 minutes, dynamic changes in T waves without ST elevation and worsening of pain in patients who previously had angina pectoris.*

Results. *In this study, we showed that patients with troponin T-negative unstable angina pectoris had statistically significantly high values of CRP (P = 0.000), Homocysteine (p = 0.000), leucocytes (P = 0.000), fibrinogen (p = 0.001) and sedimentation (P = 0.033) relative to the control group.*

Conclusion. *Our research shows that markers of inflammation are significantly increased in troponin T-negative unstable angina pectoris. This may indicate that the assessment of inflammation markers in troponin T-negative unstable angina pectoris as a separate clinical entity in ACS could be important in determining the best treatment for unstable angina.*

SAŽETAK

Uvod. *Hronični inflamatorni proces je ključan u patogenezi ateroskleroze i smatra se jednim od faktora rizika za pojavu rizika kardiovaskularnih bolesti. T troponin negativna nestabilna angina pektoris kao poseban klinički sindrom u sklopu akutnog koronarnog sindroma, praćena povišenim vrednostima sistemskih inflamatornih markera je mogući predznak daljeg pogoršanja u pravcu akutnog infarkta miokarda i iznenadne srčane smrti.*

Cilj. *U našem radu ispitivan je inflamatorni status pacijenata sa T troponin nestabilnom negativnom anginom pektoris (CRP, Homocistein, Fibrinogen, Leukociti i Sedimentacija) i upoređeni sa istim vrednostima kod pacijenata koji nisu kardiovaskularni bolesnici.*

Metod. *Ispitivan je nivo CRP-a, Homocisteina, Leukocita, Fibrinogena i Sedimentacije kod 39 pacijenata u razdoblju od 4 meseca od kojih su 20 pacijenata bili ispitivana grupa sa vrednostima T troponina ≤ 0,03 ng/ml; 19 pacijenata koji nisu kardiovasularni bolesnici.*

Kriterijumi za kardiovaskularne bolesnike su bili vrednosti T troponina, odredjivan drugi dan nakon prijema, manje od 0,03 ng/ml, izražen bol u grudima koji ne prolazi na ranije ustanovljenu terapiju i traje duže od 20min, dinamičke promene na T talasu bez ST elevacije i pogoršanje bola kod ranijuih anginoznih bolesnika.

Rezultati. *Istraživanjem smo pokazali da bolesnici sa T troponin negativnom, nestabilnom anginom pektoris, imaju visoko statistički značajne vrednosti CRP ($p<0.01$), Homocistein ($p<0.01$), leukociti ($p<0.01$), fibrinogen ($p<0.01$), sedimentacija ($p<0.05$) u odnosu na kontrolnu grupu.*

Zaključak. *Naše istraživanje pokazuje da su markeri inflamacije značajno povećani u T troponin negativnoj nestabilnoj angini pektoris. To može da ukaže da praćenjem markera inflamacije u T troponin negativnoj nestabilnoj angini pektoris kao posebnog kliničkog entiteta u sklopu AKS će imati značaja u odredjivanju pravovremenog puta u lečenju nestabine angine.*

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INTRODUCTION

Biochemical cardiac markers play an important role in evaluation of the degree of the severity of pathological processes and treatment of patients with ACS (1). Several systemic inflammatory markers may show different degrees of inflammation and coronary atherosclerosis, especially in ACSS (2, 3). Inflammation, both local and general, plays an important role in the development of ACS. Inflammatory processes determine plaque stability and instability (4, 5). One important question is whether markers of inflammation can be helpful in assessing the degree of risk and in the identification of patients for the purpose of determining the appropriate therapeutic procedures. The markers of inflammation investigated in this study were CRP, homocysteine, leucocytes, sedimentation and fibrinogen. CRP is a powerful independent predictor of cardiovascular disease, although it is not clear whether CRP is an excellent indicator or a trigger for ACS (6, 7, and 8).

Recent research shows that high values of CRP correlate with the amount of NO (Nitric Oxide) from the endothelium in patients with coronary disease (9). The data suggest that CRP itself attracts potential monocytes and facilitates the entry and oxidation of small LDL particles in macrophages, forming foamy cells and causing complement activation (10). CRP has a direct proatherothrombotic effect on the levels of vascular smooth muscle cells (11) The results show that macrophages and cells similar to smooth muscle cells produce seven times more CRP than the liver (12)

Homocysteine is also a powerful and independent predictor of ACS, sudden cardiac death and stroke. Homocysteine is toxic for the endothelium, especially free homocysteine, which acts prothrombotically, increasing collagen production and reducing the availability of NO, which explains the connection between high homocysteine concentrations in the plasma and more severe atherosclerosis (13, 14, and 15). Free homocysteine is a more accurate predictor of new cardiovascular events in ACS than total homocysteine (16). An increase in the number of leukocytes, which may be an adequate indicator of risk in ACS, begins with the growth in the first two to four days after the establishment of angina pain.

Furthermore, red blood cell sedimentation increases in ACS from the second to the fifth day, and lasts for several weeks after that, followed by an increase in fibrinogen with the same dynamics, but without a significant correlation with the severity of ACS.

Based on the above noted points, the aim of our research was to find possible correlations between inflammatory markers in troponin T-negative unstable angina pectoris.

SUBJECTS AND METHODS

Institution and study duration. The research was conducted in the coronary unit and the internal medicine department of the health centre in Gornji Milanovac from September 2008 to December 2008. This was a prospective and controlled study.

Subjects and research settings. The study included 39 patients of whom 14 (35.9%) were women and 25 (64.1%) were men, with the youngest being 36 years old and the oldest 72 years old. The average age in both groups was 60. Twenty patients (51.3%) had unstable angina pectoris and negative troponin-T; 19 patients (48.7) did not have cardiovascular disease and were considered the control group. The criteria used for the inclusion of patients in the experimental group were troponin-T <0.03 ng/ml, pain in the chest lasting more than 20 minutes that was unresponsive to previously established therapy or sublingual therapy, dynamic changes in T waves without ST elevation, and aggravation of pain in previously established angina pectoris.

Each patient from the coronary protocol was matched with one patient without cardiovascular disease of the same age, forming the control group.

Methods. The objective of the research was to determine the values of the markers of inflammation as measured on the second day after receiving the patients in the department. The concentration of CRP in serum was determined by immunohaemia nefelometria method. Normal concentration of CRP is ≤5 mg/l. The concentration of total homocysteine tHcy in serum was determined using the FPIA (Fluorescence polarisation immunoassay) method. The normal concentration in serum is ≤15 μmol/l. To determine the leukocyte count, a sample of full blood was treated with K3-EDTA anticoagulants. The method is automatic haematology counter using the principle of volumetric independence. To measure sedimentation, the full sample of blood was treated with the anticoagulant sodium citrate at 3.8% and the sedimentation rate was measured using the Westergren method, comparing the results of two measurements in the first hour. Fibrinogen was determined by the Claus method.

Statistical analysis. The results were analysed in the SPSS Windows program. "Student's T test" was used as the parametric test and Mann-Whitney as non parametric test. For the normal distribution the Kolmogorov-Smirnov test and Shapiro-Wilk tests were used. For the comparison we considered probability p <0.005 as significant.

RESULTS

The results presented in Table 1 and Fig. 1 show the values obtained for each of the individual inflammation markers.

Table 1 represents the average age of the patients in the experimental group and control groups, and shows that they are almost the same: 60.05 in the experimental group and 60.85 in the control group, which is not statistically significant (p>0.05). The next row shows a statistically significant difference (p = 0.000) in the CRP of the experimental group, where the average value was 23 while in the control group it was 8. There is also a statistically significant difference in the values of homocysteine (p<0.01), with an average value of 22.42 in the experimental group compared to 8.20 in the control group. Another statistically significant difference (p<0.01) was found in the values of leucocytes, showing an average value of 8.75 in the experimental group in comparison to an average value of 4.60 in the control group. Statistical significance is also present in fibrinogen (p<0.01) with average values of 4.28 in the experimental group in comparison to the control group (2.93). Sedimentation rate also expressed statistically significant values (p<0.05), with an average value of 22 in the experimental group in comparison to the control group with an average value of 8.

The average values for each marker with comparison between the examined and control groups from Table 1. are represented in Fig. 1. From the chart diagram 1.2 it can be seen that the most important differences were the average values of

	Age	CRP	Homocysteine	Fib	Leuk	Se
Patients	60.05 ± 11.47	23.00 (19.00 – 26.00)	22.42 ± 4.18	4.28 ± 1.82	8.75 (6.15 – 11.37)	22.00 (4.00 – 58.00)
Controls	60.85 ± 10.03	8.00 (6.00 – 10.75)	8.20 ± 2.57	2.93 ± 0.70	4.60 (3.70 – 6.70)	8.00 (5.25 – 11.75)
p	p>0.05	p<0.01	p<0.01	P<0.01	p<0.01	p<0.05

Table 1.

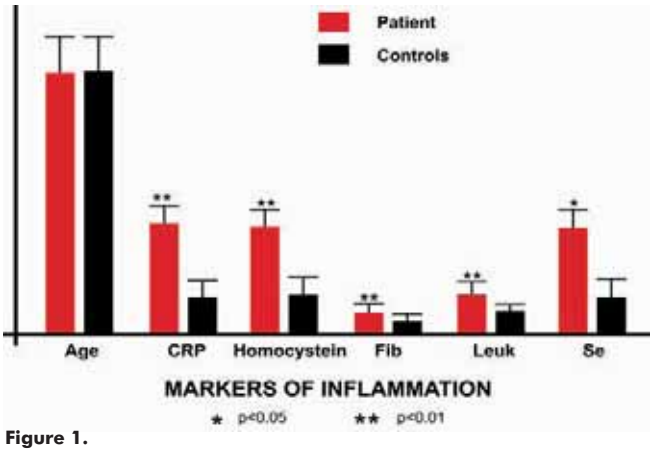

Figure 1.

CRP between the control and the experimental group, followed by the homocysteine and sedimentation values.

DISCUSSION

Our study examined the levels of inflammation markers for CRP, homocysteine, sedimentation, fibrinogen and leucocytes in patients with troponin T-negative unstable angina pectoris in comparison to a control group. The study showed that patients with troponin T unstable angina had statistically significant higher values of these markers in plasma than the group of patients who had no cardiovascular disease. Our results show that the levels of the inflammation markers in the patients with unstable troponin T-negative angina correspond to the results previously obtained in this field. One of the studies conducted by Niccoli (6) shows that the value of CRP correlates with the vulnerability and degree of inflammation of atherosclerotic plaque more than with the range and seriousness of coronary artery disease. Tanaka and associates (5) indicate that CRP values have a positive correlation with the number and degree of plaque ruptures in ACS.

Experimental data support the view that CRP in ACS is localised to the blood vessel wall and its origin is cardiac, with direct proinflammatory effect on the endothelium and mononuclear cells (17, 18). Patients with troponin T-negative unstable angina pectoris and high CRP values have an increased risk of suffering acute myocardial infarction, cardiac death and need for early revascularisation, in relationship to patients without increased CRP values (9).

Free homocysteine is also a powerful and independent predictor of acute coronary events, sudden cardiac death and stroke. Values of total homocysteine of 5-15 μmol/l and values of free homocysteine of 2.8 μmol/l are considered normal (19). Free homocysteine, rather than total homocysteine, is a more specific and more independent predictor of cardiopulmonary event in ACS (16). High concentrations of homocysteine are directly associated with advanced atherosclerosis, toxic effects on the endothelium, NO reduction and prothrombotic effects (15).

The results of our study support the above mentioned data in troponin T-negative unstable angina pectoris. We realised that levels of inflammation markers do not depend on gender or age. Our research shows that the values for fibrinogen, leukocyte and sedimentation rate are significantly higher in patients with troponin T-negative unstable angina than in the control group. Patients who have higher CRP values also have increased values of fibrinogen. Furthermore, our results indicate that increased values of CRP, homocysteine and fibrinogen are associated with a worse clinical outcome in patients with ACS, which differs from other reports (20).

In summary, the results of our study support the importance of inflammation in coronary heart disease. Based on our results, the most significant markers in coronary heart disease are CRP and homocysteine, particularly with respect to the final clinical outcome.

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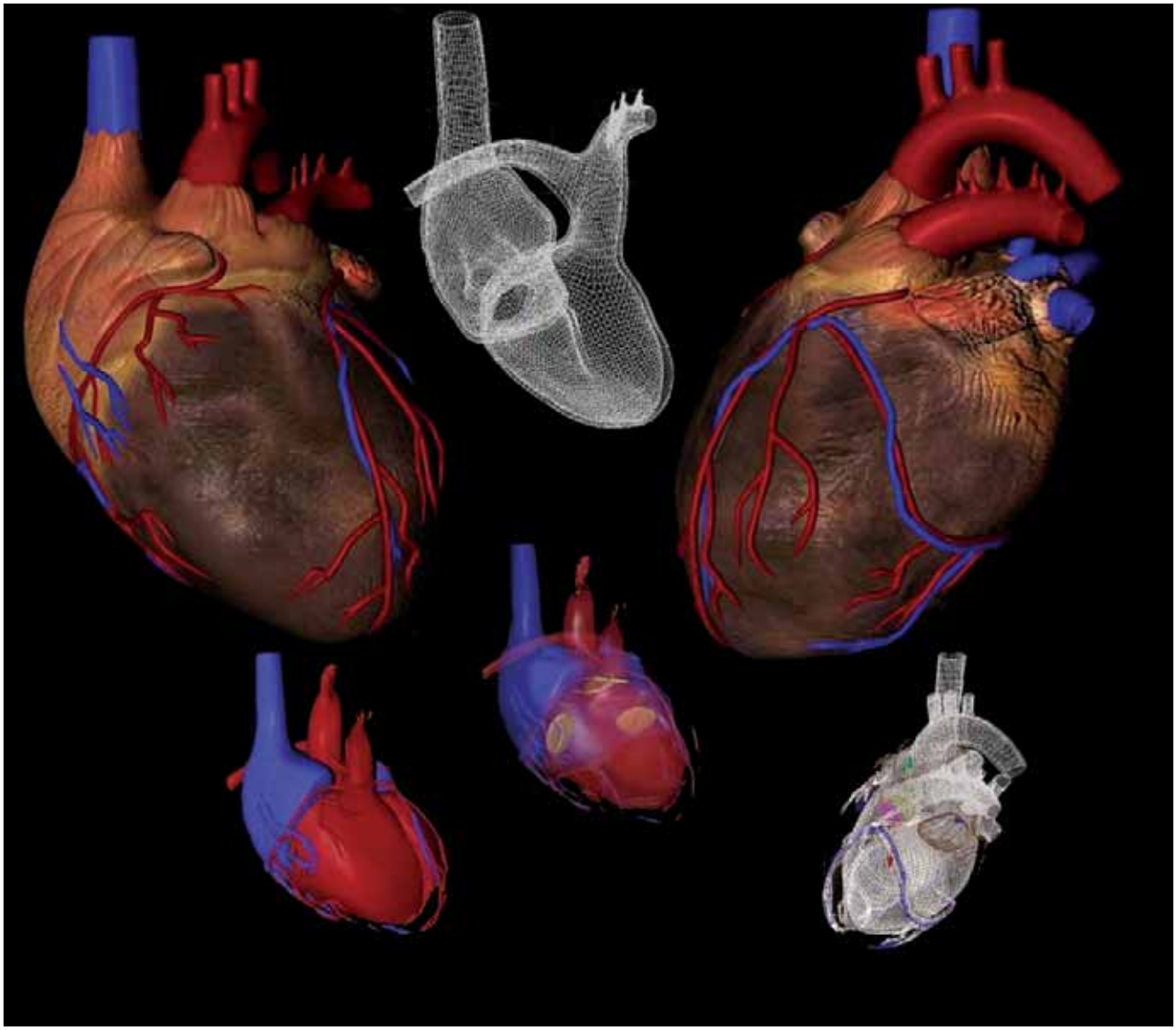

CERVICAL SYMPATHETIC CHAIN SCHWANNOMA

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ŠVANOM SIMPATIČKOG CERVIKALNOG LANCA

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ABSTRACT

Introduction. *Schwannomas of the head and neck develop from the sheaths of cranial, peripheral or autonomic nerves. Between 25% and 45% of schwannomas occur in this region. Schwannomas of the sympathetic chain are very rare, and the literature describes just over 40 cases, localised mostly in the neck or retroperitoneal areas.*

Case report. *We present the case of a 38 year-old woman with a large schwannoma of the cervical sympathetic chain. At its widest, the tumour reached a maximum diameter of 130 mm. Since the mediastinal component was so large, the tumour was operatively excised via a right posterolateral thoracotomy. Microscopic analysis confirmed the diagnosis of benign schwannoma. A computed tomography scan six months after resection showed no evidence of local recurrence.*

Conclusion. *Schwannomas of the cervical sympathetic chain are extremely rare tumours. Proper diagnosis requires microscopic tumour tissue analysis. Although these tumours are benign, they can cause a variety of symptoms while growing, necessitating urgent operative treatment.Key words. Open angle glaucoma, argon laser trabeculoplasty , intraocular pressure.*

Keywords: *schwannoma, sympathetic chain, head and neck neoplasms, surgery, pathology*

SAŽETAK

Uvod. *Švanomi glave i vrata nastaju iz omotača kranijalnih, perifernih ili autonomnih nerava. Na ovoj lokalizaciji se javljaju u 25 % do 45% slučajeva. Švanomi simpatičkog lanca su veoma retki, i u literaturi je opisano nešto više od 40 slučajeva, sa najčešćom lokalizacijom u vratnoj ili retroperitonealnoj regiji.*

Prikaz slučaja. *Predstavljamo slučaj, 38 godina stare žene sa džinovskim švanomom vratnog simpatičkog lanca, maksimalnog prečnika 130mm. Zbog veličine medijastinalne komponente, tumour je operativno uklonjen kroz desnu posterolateralnu torakotomiju. Mikroskopskim ispitivanjem postavljena je dijagnoza benignog švanoma. Šest meseci nakon operacije pacijent je bez radioloških znakova recidiva bolesti.*

Zaključak. *Švanomi vratnog simpatičkog lanca su izuzetno retki tumouri čija se dijagnoza potvrđuje mikroskopskom analizom tumourskog tkiva. Najčešće su benignog karaktera ali svojim rastom mogu usloviti pojavu različitih simptoma, zbog čega je indikovano hitno operativno lečenje.*

Ključne reči: *švanomi, simpatički lanac, neoplazme glave i vrata, hirurgija, patologija.*

INTRODUCTION

Schwannoma (also known as "neurilemmoma") is a rare tumour arising from the nerve sheaths of cranial, peripheral and autonomic nerves. Between 25% and 45% of these tumours are located in the cranial and cervical regions, and schwannomas most commonly arise from cranial nerves and their branches.1 Clinically, they present as solitary, painless, slow-growing tumours, sometimes followed by neuralgia and paresthesia in the corresponding region. The treatment of choice is complete surgical excision to prevent neurological deficits and recurrence. Since these tumours rarely undergo malignant transformation, the primary goals of surgery should be preserving and repairing nerve function.

Schwannomas in this location often imitate other conditions such as infections or metastatic tumours. Although their incidence is quite low, surgeons must consider the potential neurogenic origin of these tumours. When a schwannoma is incompletely resected, permanent nerve damage may occur. Schwannomas are diagnosed using computed tomography (CT) and magnetic resonance imaging (MRI) imaging in addition to angiography, and histological examination of resected tissue is required to confirm the diagnosis.

ABBREVATIONS
α-SMA - smooth muscle actin; CT - computerised tomography; GFAP - glial fibrillary acidic protein; EMA - epithelial membrane antigen; MRI - magnetic resonance imaging; US - ultrasound.

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CASE REPORT

A 38 year-old woman was admitted to our hospital for swelling in the right half of her neck. She described a one-year history of occasional aching in the right half of her chest and difficulty swallowing. Physical examination revealed a painless, movable mass located in subcutaneous tissue in the right supraclavicular region. No neurological deficit was detected. Laboratory tests and spirometry parameters were within normal limits. Ultrasound (US) scan of the neck revealed a hypoechogenic mass in the right supraclavicular area, inferior to the main blood vessels. Radiographic, posterior-anterior films of the chest revealed a clearly defined shadow that increased the pressure on the trachea, causing leftward tracheal deviation. CT scans revealed an expansive mass, sized 98 x 78 x 70 mm, located in the upper mediastinum, with the intensity of the non-homogenous soft tissues (the attenuation of 40HU). No pathological changes were observed in the surrounding lung parenchyma. The tumour compressed trachea and brachiocephalic vein, but there was no clear evidence of infiltration (Figure 1).

Based on these imaging procedures, the patient was given a clinical diagnosis of a tumour in the upper mediastinum. A multidisciplinary medical team recommended a right posterolateral approach for surgical excision because of the bulky mediastinal portion of the tumour, despite expansion of the tumour into the neck.

After thoracotomy, a rounded mass was visualised, with a maximum diameter of 130 mm, below the upper thoracic aperture. The root originated over the upper edge of the first rib. The mass was identified as a cervical sympathetic chain tumour. To safely resect the tumour, we first reduced the tissue and then removed it together with the tumour capsule.

The patient experienced Horner's syndrome after the operation, but the postoperative period was otherwise uncomplicated. The patient was released from the hospital after two weeks. A CT scan performed six months after the resection showed no signs of a local recurrence.

Three tissue samples of irregular shape (maximum diameter of 23, 52 and 61 mm), with total weight of 210 grams, were sent for pathological examination (Figure 2). Macroscopically, they had smooth and shiny surfaces, and the cross-sections were white-yellowish in colour with a glassy and firm consistency. Microscopic analysis revealed an encapsulated tumour with a clear border and spindle-shaped cells grouped in short and long fascicles oriented in different directions (Figure 3a). The tumour was uniformly cellular, though there were a few hypercellular zones with palisade nuclei forming Verocayžs bodies and areas of myxoid degeneration (Antoni A and Antoni B component). Pathologists found no evidence of cytological atypia, mitosis or necrosis. The tumour had scant macrophage infiltration, focal aggregates of lymphocytes, and zones of cystic degeneration together with hyaline in the blood vessel walls. Immunohistochemically, tumour cells diffusely expressed vimentin (Figure 3b), S-100 protein (Figure 3c) and glial fibrillary acidic protein (GFAP) (Figure 3d), but lacked epithelial membrane antigen (EMA), desmin, CD 31, CD 34, smooth muscle actin (α-SMA) and CD 68 (Figure 3e). We found focal areas with macrophages by CD68 staining, as well as regions of blood vessels staining positively for CD31, CD34 and α-SMA in tumour tissue. Application of Ki-67 antibodies showed a very low proliferation index, less than 1%

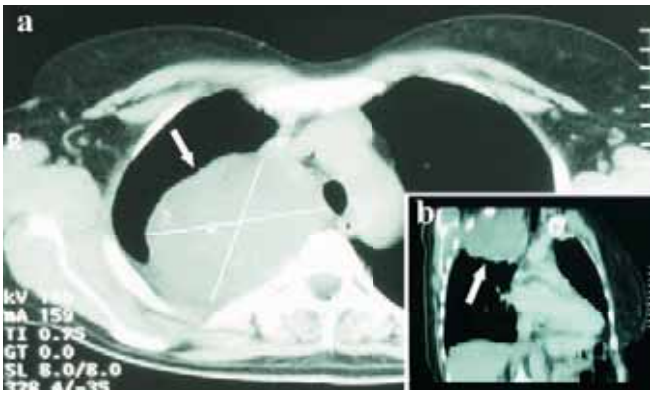

Figure 1. Heterogeneous soft tissue shadow located in the upper mediastinum: a) CT of the chest; b) CT reconstruction

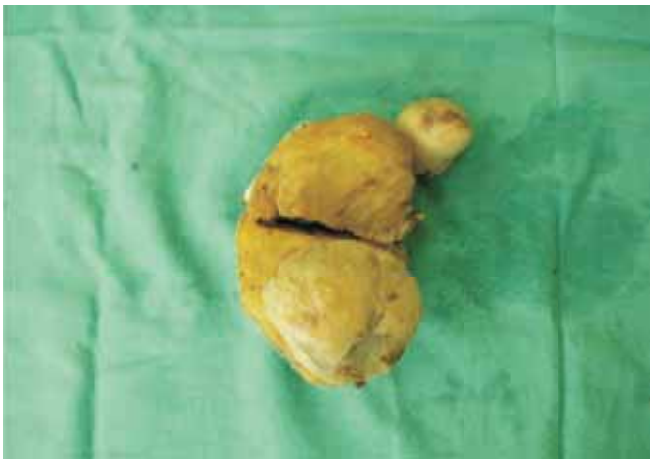

Figure 2. Macroscopic appearance of the resected tumour: three tissue samples of irregular shape, with maximum diameter of 130 mm and total weight of 210 g.

(Figure 3f). Taken together, the microscopic and immunohistochemistry results led to a final diagnosis of benign schwannoma.

DISCUSSION

Schwannomas were first described in 1910 by Verocay.2 They have alternatively been called neurilemmomas, solitary tumours of nerve sheets, and perineural fibroblastic tumours but the WHO has recommended the term schwannoma.3, 4 In general, schwannomas are solitary tumours, whereas the presence of multiple schwannomas is a component of von Recklinghausen disease. In the head and neck, schwannomas can originate from any peripheral, spinal or cranial nerves except the optic or olfactory nerves.5

Schwannomas of the sympathetic chain are very rare. Approximately 40 cases have been described in the literature, and these are most often localised to the cervical and lumbar retroperitoneal region.6-11, 12-16 These tumours usually affect patients from 20 to 50 years old7, and men are affected three times more often than women.16 Schwannomas can grow to 20 cm in diameter.17 These large tumours are often associated with focal haemorrhage, calcifications or cystic degeneration of the lesion.18

Most schwannomas of the cervical region are asymptomatic. Depending on their exact location, cervical schwan-

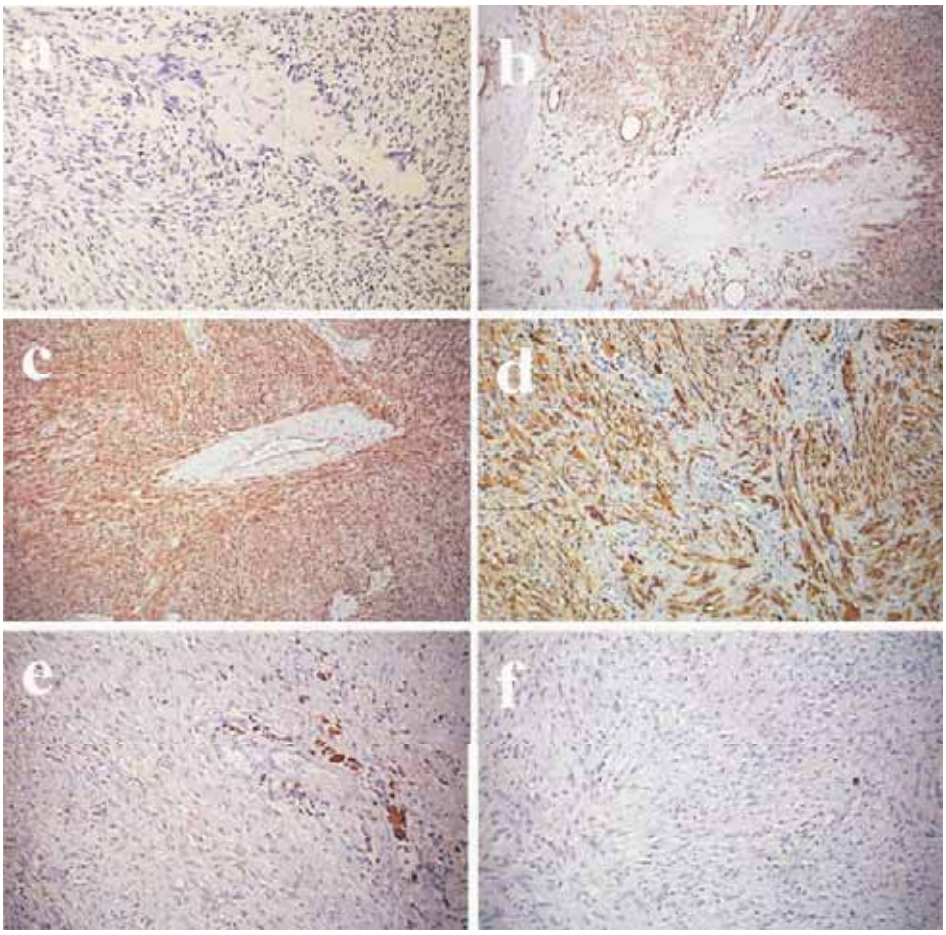

Figure 3. Histological features of a schwannoma: a) the tumour is composed of spindle cells grouped in shorter and longer fascicles (HE staining technique, x200). The tumour cells express: b) vimentin (x100), c) S-100 protein (x200), and d) GFAP (x200). e) The tumour cells are weakly, focally positive for CD 68 (x200), and f) the Ki-67 proliferation index is very low, less than 1% (x200).

nomas can cause tonsillitis, hoarseness, dysphagia and pain. Although schwannomas infrequently compress or infiltrate surrounding organs, in these rare cases, the sympathetic chain is usually affected, resulting in pain and paresthesias. Several cases have been associated with Horner's syndrome.6, 8, 10, 11, 13, 14 Sympathetic hyperactivity can also occur, and one report describes a cervical sympathetic chain schwannoma that caused ipsilateral lacrimation, palmar hyperhidrosis, conjunctival injection, and nasal congestion.9

The challenge in the treatment of these tumours is to differentiate a benign schwannoma of the sympathetic chain from other pathological processes with similar presentations. Depending on the location of the tumour, the differential diagnosis could include lesions of the carotid artery, paragangliomas, sarcomas and malignant schwannomas. Careful clinical examination is important, but histopathological and immunohistochemical analyses are required to confirm the diagnosis and exclude malignancies.

Preoperative imaging procedures including MRI, CT and angiography are particularly helpful. On an MRI scans, schwannomas show a high intensity signal on T2-weighted imaging and a low intensity signal on T1-weighted scans. In comparison to paragangliomas, schwannomas are not vascularised. Non-contrast CT shows a mass less dense than surrounding muscle tissue. CT scans with contrast show heterogeneous distribution of the contrast agent.

US can help to distinguish a vagal nerve schwannoma from a schwannoma of the sympathetic chain. Carotid angiogram can also be important in the diagnosis of cervical tumours. Carotid body tumours are characterised by hypervascularity while schwannomas lack this distinguishing feature.12, 15

Cervical anatomy can give insight into the appropriate differential diagnosis for a cervical tumour. Paragangliomas have a cranial origin and spread laterally. Schwannomas mostly occur in the parapharyngeal region, affecting cranial nerves IX, X, XI and XII. If they include the vagal nerve, the schwannomas divide the common carotid artery and internal jugular vein, while schwannomas the of the sympathetic chain do not divide these vessels.7

Microscopic and immunohistochemical analysis are key to establishing a definite diagnosis. According to the WHO classification of central nervous system tumours, schwannomas are defined as the tumours originating from nerve sheaths.3, 4 They are further classified into cellular, plexiform and melanotic types.3, 4 Immunohistochemically, they are characterised by expression of S-100 protein and GFAP.19

In our case, the presence of Antoni A and Antoni B zones, microscopic patterns pathognomonic for schwannomas, confirmed our diagnosis.20 We observed nuclear palisades in several tumour specimens, which formed Verocay's bodies. Nuclear palisades in Antoni A regions are more commonly associated with spinal, rather than at intracranial, schwannomas. In addition, schwannomas of cranial nerve VIII characteristically lack nuclear palisades.20 Schwannomas are typified by cystic and hyaline degeneration of blood vessel walls, and they have macrophages and lymphocytic aggregates.19 Ki-67 is expressed only in some tumour cells, which aligns with reports that mitosis is rarely observed in schwannomas.21

The appropriate schedule for surgical resection and postoperative follow-up remains controversial.22 In our case, there were several indications for immediate intervention. Our patient was young, and she had a large sympathetic chain schwannoma that was likely to grow quickly and/or convey novel symptoms during the disease course. Furthermore, our differential diagnosis included other tumour types that require urgent surgical intervention. Since some schwannomas are malignant and benign schwannomas can undergo malignant transformation, young patients may have a higher risk of having cancerous schwannomas.23 Surgical intervention was indicated since only microscopic examination of biopsy specimens could yield a precise diagnosis.

In our patient, the surgeon had to resect the tumour through a right posterolateral thoracotomy. Direct visualisation revealed that the tumour originated from the sympathetic chain. After the schwannoma and cervical sympathetic chain were removed, the patient experienced Horner's syndrome postoperatively. Previous studies reported similar neurological deficit in patients with this kind of tumour.10, 11, 13, 16, 23, 24 Classical Horner's syndrome is characterised by lesions of the oculosympathetic pathway at any point between the hypothalamus and eye. It presents as pupillary miosis, ptosis, enophtalmos and facial anhydrosis. Ptosis is a consequence of paralysis of Mueller's muscle, and it can be corrected by strengthening the levator aponeurosis or resecting the adjacent conjunctiva and muscles.7, 25 Aside from the obvious aesthetic consequences, Horner's syndrome does not cause functional deficits.

Schwannomas of the cervical sympathetic chain are extremely rare tumours. They are difficult to diagnose preoperatively, and clinical diagnosis must be confirmed by microscopic analysis of resected tumour tissue. Complete surgical excision is the treatment of choice and recurrence is rare. Since most schwannomas are benign, long term follow-up is usually not required.

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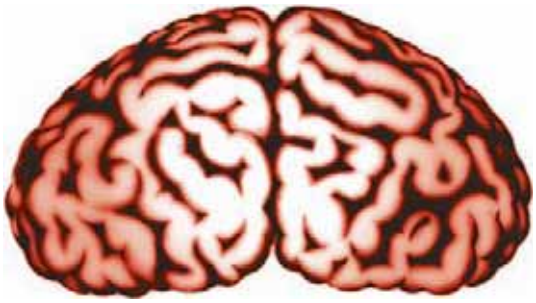

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