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Marlena Szalata

Methylation and oxidative changes in DNA samples of cattle living in environmental conditions contaminated with heavy metals

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List of abbreviations

2dG	2-deoxyguanosine 2-deoksyguanozyna
5hmC	5-hydroxymethylcytosine 5-hydroksymetylocytozyna
5mC	5-methylcytosine 5-metylocytozyna
8OHdG	8-hydroxy-2'-deoxyguanosine 8-hydroksy-2'-deoksyguanozyna
8-OHdG	8-hydroxy-2'-deoxyguanosine 8-hydroksy-2'-deoksyguanozyna
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine, tautomeric form of 8OHdG 8-okso-7,8-dihydro-2'-deoksyguanozyna, forma tautomeryczna 8OHdG
8-oxoG	8-hydroxyguanine 8-hydroksyguanina
AP	apurinic- or apyrimidinic site miejsce apurynowe lub apirymidynowe
BER	base excision DNA repair naprawa DNA przez wycięcie zasady
bp	base pair para zasad
BS-PCR	bisulfite genomic sequencing PCR reakcja PCR po działaniu na matrycę wodorosiarczynem sodu
CpG sites	DNA regions with a high frequency of the CG sequence regiony DNA z dużą częstością sekwencji CG
DNMTs	DNA methyltransferases metylotransferazy DNA
DREAM	digital restriction enzyme analysis of methylation analiza metylacji oparta na cyfrowych enzymach restrykcyjnych w sekwencjonowaniu nowej generacji
EDTA	ethylenediaminetetraacetic acid kwas etylenodiaminotetraoctowy
GC/MS	gas chromatography-mass spectrometry chromatografia gazowa-spektrometria mas
H_2O_2	hydrogen peroxide nadtlenek wodoru

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HDACs	histone deacetylases deacetylazy histonowe
HNPCC	hereditary non-polyposis colorectal cancer dziedziczny rak jelita grubego bez polipowatości
HPLC	high-pressure liquid chromatography wysokociśnieniowa chromatografia cieczowa
KGHM Polish Copper	Copper Mining and Metallurgical Plant, KGHM Polish Copper Kombinat Górniczo-Hutniczy Miedzi, KGHM Polska Miedź
LINE-1	long interspersed nuclear elements długie rozproszone elementy jądrowe
MeCP2	methyl-CpG binding protein 2 białko 2 wiążące się z metylowaną sekwencją CpG
MS-PCR	methylation-specific PCR reakcja PCR specyficzna dla metylacji
NCBI	National Center for Biotechnology Information Baza Narodowego Centrum Informacji Biotechnologicznej
OGG1	8-oxoguanine-DNA glycosylase glikozylaza 8-oksoguanina-DNA
PAHs	polycyclic aromatic hydrocarbons wielopierścieniowe węglowodory aromatyczne
PCR	polymerase chain reaction reakcja łańcuchowa polimerazy
PIXE	proton-induced X-ray emission emisja promieniowania rentgenowskiego wywołana przez protony
PNK T4	phage T4 polynucleotide kinase kinaza polinukleotydowa faga T4
RLGS-M	restriction landmark genomic scanning for methylation punktowa analiza restrykcyjna genomu dla poszukiwania metylacji
ROS	reactive oxide species reaktywne formy tlenu
SINE	short interspersed element krótkie rozproszone elementy
SRP	signal recognition particle cząsteczka rozpoznająca sygnał
TBE	Tris-borate-EDTA Tris-boran-EDTA
TLC	thin layer chromatography chromatografia cienkowarstwowa
Tris	tris(hydroxymethyl)aminomethane tris(hydroksymetylo)aminometan

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1. Introduction

1.1. Preface

Environmental pollution, especially due to industry, is a rising concern all over the world as it has a strong impact on our environment and health. Major air pollutants are sulphur dioxide (SO_2), nitrous oxides (NO_x), ozone (O_3) and particulates (PM2.5, PM10), while minor pollutants include heavy metals, like arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg) and nickel (Ni), as well as certain organic pollutants, like formaldehydes and dioxins. Air pollution can lead to serious health problems and even morbidity. One of the most important industrial metals is copper (Cu) (London Metals Exchange, on 10.09.2018 the copper price was 5840.50 USD per tonne). Copper production is associated with emissions of pollutants into the air, water and soil, strongly influencing human health, ecosystems, crops and infrastructures.

According to the Multilateral Investment Guarantee Agency Environmental Guidelines for Copper Smelting, principal air pollutants emitted during copper smelting are sulfur dioxide (SO_2) and particulate matter. Copper Smelters in Poland in Lower Silesia Region mainly relies on extraction from the copper ore deposits and associated copper concentrate production on-site. In the Głogów smelter/refinery, the copper is smelted using flash furnace technology leading to higher concentration of the sulfur dioxide in the off gases. Emission of sulfur dioxide can range from 4 to 2,000 kg per tonne of copper and of particulate emissions: 0.1–20 kg/t copper. Particulate matter consists mainly of copper and iron oxides, and additionally of their sulphides, sulphates, oxides, chlorides and/or fluorides. Other pollutants include arsine vapours and dioxins. Derivatives of arsenic, antimony, cadmium, lead, mercury and zinc are also detected. Wastewater from primary copper production contains dissolved and suspended solids that may include copper, lead, cadmium, zinc, arsenic and mercury as well as residues from mould release agents (lime or aluminium oxides). The major portion of the solid waste is discarded from a smelter as slag still containing 0.5–0.7% copper. The smelting process typically produces less than three tonnes of solid waste per tonne of copper produced.

The Głogów Copper Smelter is part of Kombinat Górniczo-Hutniczy Miedzi KGHM Polska Miedź (Copper Mining and Metallurgical Plant, KGHM Polish Copper) and produces the highest quality cathode copper as well as silver, gold, and concentrates of the platinum-group metals. The Głogów Copper Smelter and Refinery comprises two separate metallurgical plants: Głogów I and Głogów II. The major product is electrolytically refined 99.99% pure copper in the form of cathodes. The Precious

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Metals Plant of the Głogów Copper Smelter and Refinery produces 99.99% pure silver in the form of bars and granules, bars of gold with more than 99.95% Au content, and platinum-palladium concentrate. The products of the Głogów Copper Smelter and Refinery are of very high quality guaranteed by their internationally renowned global market brands.

The owner of the Głogów Copper Smelter and Refinery claims that they meet strict Polish and European Union environmental protection standards. It is one of the cleanest copper smelting and refining facilities in the world, even a model for other such industrial complexes all over the world. During the past 17 years (as of 2016), they spent over 450 mln PLN (150 mln USD) for environment protection and a former protection zone around the Głogów Copper Smelter has been converted into an ecological area called "Głogów Wetlands" (pol. Łęgi Głogowskie).

According to environmental studies for years 2000 through 2007 in Poland, which are presented in the National Mosaic Report (Krajowy raport…, 2010), the main source of air pollution in the Lower Silesia region is the anthropogenic emission of pollutants from industrial activities, the municipal sector and communication. One of the most important sources of air pollution in the area is Power Plant Turów SA in Bogatynia (part of the Polish Energy Group, Polska Grupa Energetyczna, PGE) followed by KGHM Polish Copper with its Głogów I Copper Smelter. Many environmental actions have been undertaken in order to improve air quality in the region. The Głogów Copper Smelter has modernised old furnaces and constructed new ones, equipping all of them with new installations for purifying exhaust gases.

The study of soil, carried out in 2000–2007 in areas directly threatened by pollution confirmed that limit values were exceeded particularly for zinc, lead, cadmium and benzo(a)pyrene, and in the vicinity of the Głogów Copper Smelter the levels of cadmium, lead and zinc. In 2000 through 2007, many actions were undertaken to protect the ground surface in the region of Lower Silesia. In KGHM Polish Copper facilities, the amount of sewage discharged was reduced thanks to the treatment of on-site parts and recycling of wastewater for technological processes.

The influence of environmental pollution may be evaluated using animal models. The animal model is a living animal used in research and studies of a human disease for a better understanding of the disease without the risk of harming a human being during the research process. Many drugs, treatments and cures for human diseases have been developed with the use of animal models. Animal models in biomedical research can be used to study: (1) normative biology or behaviour; (2) spontaneous or induced pathological processes; and (3) phenomena which resemble the same phenomena in humans or other animal species in one or more respects. The most commonly used rodent models are not always appropriate as they do not always accurately mimic the human disease. In response to these problems, a new initiative was launched within the COST Action – the longest-running European framework supporting trans-national cooperation among researchers, engineers and scholars across Europe to study the large models of which Poland is a member. The COST Action BM1308: "Sharing advances on

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large animal models" (SALAAM) emphasises the need for non-rodent animal models, like pigs, small ruminants and rabbits, which mimic the aspects of human anatomy and physiology more closely. In my studies, I decided to work on a cattle model as we have been faced with health problems of a cattle herd living in the vicinity of the Głogów Copper Smelter. A similar group of animals bred in a pollution-free environment did not have health problems, so I hypothesised that the health problems observed in animals bred close to the smelters could be associated with the environmental pollution. I focused on the analysis of genetic material of these animals to study the influence of the environment on DNA methylation and oxidation. Thanks to a retrospective study design (the animals were born between 1992 and 1998 (mainly 1997–1998) and blood samples were collected in August 1998 or 1999), the two groups of animals can be considered as a "spontaneous" or "natural" model in which naturally occurring animal diseases or conditions correspond to the same diseases or conditions in humans. Of note, the animals from the experimental group lived in the area polluted by the Głogów Copper Smelter before its modernisation as the Głogów Lead Plant I was rebuilt in the years 2000–2001. It is also worth emphasising that using only animals already living in the two different environments without constituting any additional groups solely for research is in accordance with the 3Rs rule of animal research (Replacement, Reduction, Refinement) which requires that any scientist planning to use animals in their research demonstrate why there is no alternative, and that the number of animals used and any suffering caused, should be kept to a minimum.

1.2. Methylation of DNA

DNA methylation is a physiological modification of a DNA molecule by transfer of a methyl group from the S-adenosylmethionine position to the C5 position of deoxycytidine residues. DNA methylation occurs in a majority of organisms at different levels of evolution, even in such distant species like *Escherichia coli* and *Homo sapiens*, however with different mechanisms and roles (Moore et al., 2013; Jeltsch and Jurkowska, 2014; Vandegehuchte and Janssen, 2014). So far, it has been most extensively studied in bacteria in which methylation occurs in adenine and cytosine, and is associated with DNA replication. It is generally assumed that the main function of methylation in bacteria is protection against introduction of foreign genetic information into their DNA (Noyer-Weidner and Trautner, 1993). Another role of methylation in Prokaryotes is controlling the replication accuracy (Cooper et al., 1993).

In Eukaryotes, DNA methylation is an epigenetic DNA modification which influences cell functions by changing gene expression associated with the covalent linking of the methyl group catalysed by DNA methyltransferase. In DNA of mammalian cells, only cytosines located 5' from guanosines in CpG dinucleotides undergo methylation (Bird, 2007; Moore et al., 2013; Jeltsch and Jurkowska, 2014). In a majority of cases, methylation of CpG islands in the DNA sequence reflects the information written in

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the genome. However, it may also happen to occur as a random event due to a methylase error. Methylation is one of the mechanisms allowing regulation of gene activity. It is also involved in the organization of the mammalian genome affecting chromatin structure, gene imprinting, chromosome X inactivation, control of immunological reactions and the regulation of gene expression during development (Bestor and Tycko, 1996). In higher eukaryotes, methylation involves the DNA cytosine-5'-methyltransferase (EC 2.1.137) which catalyses the transport of the methyl group from S-adenosyl--L-methionine to the fifth carbon of the cytosine residue in the dinucleotide 5'-CpG-3' sequence (Smith, 1994; Jeltsch, 2002).

CpG sites do not occur frequently in the genome and have a tendency to form groups in fragments exceeding 500 base pair (bp) referred to as CpG islands (Takai and Jones, 2002). CpG islands are found in the entire genome and are frequently associated with the gene promoter region. The level of DNA methylation is maintained by a methyltransferase whose activity is linked with cell development and increases markedly during cell growth in the S phase of the cell cycle (Szyf et al., 1991; Chen et al., 2007). During DNA replication, the parental strand remains methylated and DNA methyltransferase, which shows affinity to partially methylated DNA, methylates new DNA strand soon after replication (Adams, 1990). This mechanism assures inheritance by progeny strands of the methylation pattern which is both tissue- and gene-specific. The distribution of methylated cytosine in the entire mammalian genome in the CpG dinucleotides is not random and the majority of CpG-dinucleotide-rich sequences occur in the 5' region of many genes (Bird, 1986). Regions of CpG islands present in structures of many genes that are essential for the maintenance of cell metabolism (housekeeping genes) remain non-methylated in appropriate conditions in mature cells with the exception of the inactivated X chromosome (Klose and Bird, 2006). A majority of CpG sequences in CpG islands is not methylated in young, healthy individuals, whereas CpG sequences found outside islands are mostly methylated. When CpG sequences situated within the promoter's CpG island are methylated, the chromatin from this region is thickened and gene expression is limited or does not take place (Jones and Baylin, 2002). Differences in the methylation pattern between tissues become fixed in the course of early development as a result of a *de novo* combination of demethylation and methylation (Szyf et al., 1991).

De novo methylation process is not a simple reaction, but it is a result of interaction of many factors including: presence of the pre-programmed specific DNA-binding protein distinguishing between methylated and unmethylated DNA, DNA signalisation and changes in the conformation of particular genes as well as the availability of DNA methyltransferase (Adams, 1990; Szyf, 1991; Moore et al., 2013; Jeltsch and Jurkowska, 2014; Vandegehuchte and Janssen, 2014). Relatively little is known about the changes of methylation patterns during the development and aging. It is believed that changes of gene expression may alter the gene methylation pattern or they can be regulated by these changes (Bestor and Tycko, 1996). For example a fragment containing the intronic kappa chain transcriptional enhancer of the germline V kappa promoter and the

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nearby matrix attachment region plays a dual role in regulating B cell differentiation by inducing demethylation and by promoting tissue-specific transcription (Lichtenstein et al., 1994). The enhancer causes regional demethylation depending on the cell origin and stage of differentiation, with tissue-specific transcription being induced by demethylation. Moreover, the Sp1 factor and H1 histones presumably take part in protecting CpG islands against *de novo* methylation, which in turn supports the hypothesis about the existence of a local, intrinsic signal which marks this region off in the genome (Santoro et al., 1995).

Hypermethylation may occur in aging processes and the acquired pattern of changed methylation may contribute to an individual predisposition to carcinogenesis (Issa, 2000; Ahuja and Issa, 2000). Some researchers reported an increased methylation in normal tissues with age as well as enhanced methylation levels during tumour development. Comprehensive studies of tumour cells indicate that the changed methylation patterns of tumour suppressor genes result in defective cell growth. The knock-down of tumour suppressor gene expression can constitute one of two events from the Knudson's two-hit (damage) hypothesis, resulting in disturbances in cell development, which induces tumour-promoting mutations (Jones and Laird, 1999). Numerous investigations aim at clarifying the importance of methylation in the process of carcinogenesis and mutual interactions of epigenetic and genetic changes. It has been shown that for some genes, changes in the expression due to methylation are similar to those induced by mutations, suggesting that epigenetic and genetic inactivation of gene expression may have the same effect in the cell (Hedenfalk et al., 2001). Methylation may also contribute to the occurrence of genetic changes. For instance, hypermethylation of the human homologue of the *MutL* gene (*hMLH1*) is observed in patients with microsatellite instability (Herman et al., 1998). In addition, *MLH1* methylation was also discovered in the colon of patients with colorectal cancer (Nakagawa et al., 2001). In normal tissues, the methylation levels are very low compared to those of the same genes in tumours cells. Methylation patterns may be specific both for the tumour stage and tumour type (Paz et al., 2003). Altered methylation patterns which are frequently observed in tumour cells are associated with modified gene expression which often leads to an increased activity of oncogenes and silencing of tumour suppressor genes in the process of carcinogenesis (Counts and Goodman, 1995a).

Increased expression of DNA methyltransferase is observed at early and late stages of tumour development, which suggests that this enzyme participates in the formation of the abnormalities in DNA methylation pattern in tumour cells (El-Deiry et al., 1991). It is hypothesised that the changed methylation pattern may contribute to pathogenesis, the associated molecular mechanism in tumours, however, remains unknown (Bestor and Tycko, 1996). Increased methylation of CpG islands observed in tumour cells (Makos et al., 1992) and in immortalised cells (Antequera et al., 1990) suggests that hypermethylation may contribute to increasing the malignancy of tumours by inactivation of tumour suppressor genes (Sapienza, 1991) or by continuous silencing of genes associated with development and differentiation (Rideout et al., 1994). The proto-on-

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cogene expression increased by hypermethylation can also contribute to malignancy by conferring to cells a capacity of unrestricted growth.

Developing models for studies of *de novo* methylation molecular mechanisms is complicated by the fact that this process does not occur in mammalian cell cultures. Research using mammalian embryo tumour cells indicates that some genes contain an intrinsic regulatory element which is essential for developing a specific methylation pattern in the *cis* position (Mummaneni et al., 1993). This is in agreement with the hypothesis that the centre of the *de novo* methylation site acts as a signal for methylation along with the proteins specifically expressed in a given phase of the embryonal development (Szyf, 1991). The newly synthesised heterochromatic DNA can also be associated with a gradual loss of gene expression co-dependent with tumour development. DNA methyltransferases precisely recognise the new heterochromatic DNA which undergoes a *de novo* methylation. The appearance of a single-stranded DNA with a defined conformation can provide a signal for the initiation of DNA methylation during replication or repair (Christman et al., 1995). It is assumed that the diversity of mechanisms associated with the increase of tumour malignancy can contribute to the development of an abnormal methylation pattern observed in different stages of tumour development in humans. These mechanisms can include a change in chromatin structure indirectly inducing changes of the methylation patterns, changes of expression of genes coding DNA binding proteins which take part in methylation and demethylation processes (Adams, 1990) as well as changes in the methyltransferase activity (El-Deiry et al., 1991). Additionally, the expression of proto-oncogenes, such as v-Ha-*ras*, can also influence the methylation process by switching on the signal leading to a global genome demethylation (Szyf et al., 1995).

Mammalian DNA may undergo *de novo* methylation and demethylation randomly or in conjunction with carcinogens or mutations leading to long-term gene expression changes (Bestor and Tycko, 1996; Ducasse and Brown, 2006). Two hypotheses have been put forward to explain the association of DNA methylation with carcinogenesis. One of them suggests that DNA methylation contributes to the process of carcinogenesis through changes in gene expression (Counts and Goodman, 1995a; Holliday, 1994), while the other stresses the importance of cytoplasm methylation for the development of mutations. These two hypotheses are not mutually exclusive. The first of them assumes that the two following opposing mechanisms are possibly involved: (1) inactivation or silencing of tumour suppressor genes, or regulation of activity of genes by regional hypermethylation (Hang et al., 1997; Herman et al., 1998); (2) enhanced proto-oncogene expression as a result of reduced methylation (Hanada et al., 1993). The second model suggests that methylated cytosine has an increased sensitivity to deamination which occurs at low S-adenosyl-L-methionine level, leading to the occurrence of C > T transitions (Laird and Jaenish, 1994; Yang et al., 1995). Mutations play a key role in carcinogenesis (Greenblatt et al., 1994) and their impact depends on the tumour stage and the tissue type (Counts and Goodman, 1995a). Carcinogenesis is a complex, multi-phase process and DNA methylation may be involved in this process

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via genotoxic and epigenetic processes, i.e. by mutagenesis and changes in gene expression (Counts and Goodman, 1995a).

Epigenetics studies gene expression changes in the course of development which do not result from changes in the gene nucleotide sequence (Holliday, 1994). As it was already mentioned, DNA methylation may constitute an epigenetic mechanism of gene expression regulation by changing the chromatin structure (Rideout et al., 1994; Szyf et al., 1995) and/or by preventing the binding of direct-acting factors with methylated cytosine or with proteins mediating their binding with methylated cytosine (Lewis et al., 1992; Meehan et al., 1992). Upon analysis of the complex multi-stage process of carcinogenesis which is dependent on many factors, the involvement of DNA methylation seems quite probable (Counts and Goodman, 1995b). Although it has been demonstrated that DNA hypomethylation facilitates abnormal gene expression which promotes carcinogenesis, the inhibition of the methyltransferase activity is also associated with the prevention of tumour development and inhibition of the tumorigenic process, which argues against the oncogenic effect of DNA methylation (Laird et al., 1995; MacLeod and Szyf, 1995). Reduced DNA methylation levels may result in the inhibition of the tumour formation by a mechanism which affects directly methyltransferase, decreasing a possibility of the C > T transition occurrence and preventing incorrect DNA repair. However, the fact that transformation can be reversed by inhibiting the DNA methyltransferase activity indicates the existence of mechanisms other than mutagenesis which are associated with methylation because mutations are irreversible (MacLeod and Szyf, 1995).

Whereas mutagenesis takes part in the initiation and later stages of carcinogenesis, non-mutagenic events are also involved in carcinogenesis. Increased expression of given proto-oncogenes is associated with tumour development (Counts and Goodman, 1995a), while hypomethylation with increased gene expression (Razin and Kafri, 1994; Ferguson et al., 1995). Furthermore, the level of S-adenosyl-L-methionine is linked with transcription of c-*myc*, c-Ha-*ras* and c-Ki-*ras* at early stages of liver cancer (Simile et al., 1994). Regional DNA hypermethylation contributes to carcinogenesis by silencing tumour suppressor genes (Herman et al., 1998) which is not always accidental and hypermethylation inside intron sequences is essential for the gene expression. Genetic and epigenetic mechanisms of DNA changes can substantially influence tumour development depending on the stage of carcinogenesis, the gene involvement and the tissue type.

1.3. Regulation of DNA methylation

DNA methylation is the only known modification of a DNA molecule which occurrs physiologically in a majority of organisms on various levels of evolution (Jeltsch et al., 2007; Moore et al., 2013; Jeltsch and Jurkowska, 2014; Vandegehuchte and Janssen, 2014). Numerous studies were carried out in bacteria in which methyla-

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tion takes place on adenine and cytosine and is associated with gene regulation, DNA repair and cell cycle control. A group of DNA methyltransferases (DNMTs) participates in the methylation process. Bacterial endonucleases distinguish endogenous and foreign DNA on the basis of its methylation pattern. DNA which does not contain methyl groups is hydrolysed by nucleases (Noyer-Weidner and Trautner, 1993). In eukaryotes, methylation is associated with cytosine residues (5mC) and the occurrence of characteristic CpG sequences. In bacteria, the main role of methylation is to ensure cell protection against the inclusion of foreign DNA, whereas in eukaryotes, the methylation process cooperates with other epigenetic modifications in regulating gene expression and chromatin structure (Jeltsch et al., 2007). Thus, the methylation function appears similar in eukaryotes and prokaryotes. Both in humans and in rodents, methylation can also be a defence mechanism against viral infections as viral particles may be methylated, which leads to silencing of viral genes (Kisseljova et al., 1998). A similar mechanism may silence transgenes in mice (Sasaki et al., 1993) and, therefore, the entire system which methylates the cell DNA, and recognises and eliminates foreign DNA seems to be evolutionarily conservative. Methylation of eukaryotic DNA takes place immediately after replication as concomitant events (Araujo et al., 1998).

Genome regions which contain methylated cytosine are usually transcriptionally inactive. The *de novo* methylation taking place during embryogenesis is associated with the activity of DNMT3a and DNMT3b enzymes (Robertson et al., 1999; Xie et al., 1999; Jeltsch and Jurkowska, 2014). *DNMT1* and *DNMT2* genes take part in the maintenance of a particular level of methylation in eukaryotic cells. Studies on mice in which the *DNMT1* gene has been switched off showed that it is essential for the embryo survival. Demethylation can occur in organisms only if there is a mechanism which allows removal of methyl groups (Bhattacharya et al., 1999).

Another mechanism of regulating gene transcription by methylation is through methylation-dependent proteins, e.g. the MeCP1 protein which binds with at least seven methylated CpGs and the MeCP2 protein which binds with one methylated CpG, both efficiently inhibiting gene transcription. An entire family of proteins with such properties has been described (Hendrich and Bird, 1998). The DNA methyltransferase (e.g. *Hpa*II methylase) participates directly in the CpG sequence mutagenesis by enzymatic deamination of cytosine and proceeds through a C > U > T pathway in the absence of S-adenosylmethionine as well as the decrease in the level of repair enzymes (Shen et al., 1992). Methyltransferase activity can also be affected by bioactive food constituents. An increased concentration of selenium, a potential anticarcinogenic agent, results in a decreased DNMT1 activity and a decreased DNMT1 protein expression *in vitro*, while selenium shortage leads to an increased expression of this protein.

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1.4. Methods of methylation analysis

Although 5-methylcytosine (5mC) was the first modified base known and it was discovered almost 50 years ago, its exact function and significance in gene expression control has not yet been fully elucidated. This can be at least partly attributed to both absence and limitations of the techniques used for its detection in specific DNA fragments. Among the existing methods for the analysis of DNA methylation we can distinguish genome-wide analysis and specific loci analysis. They usually rely on methylation-specific or methylation-dependent restriction enzymes, antibodies or methyl-binding proteins, chemical-based enrichment, or bisulfite conversion (Tost, 2016). General in genome-wide DNA methylation studies, the global level of cytosine methylation in the genome is measured using methods like chromatography, methyl-specific volumetric measurements, the digital restriction enzyme analysis of methylation (DREAM), or cytosine-extension assay (Cottrell, 2004; Jelinek and Madzo, 2016; Bilichak and Kovalchuk, 2017).

Many laboratory techniques were elaborated for the analysis of gene-specific methylation. A majority of early investigations on methylation was based on the use of methyl-sensitive restriction enzymes for DNA hydrolysis followed by the detection of the expected fragments by Southern blot or DNA amplification in the polymerase chain reaction (PCR). However, the application of methods based on the degradation of the phosphodiester bond at the cytosine residue employing methyl-sensitive restriction enzymes or reactive reagents, such as hydrazine, which can distinguish cytosine from its methyl derivative was restricted to determining the methylation status of defined potentially methylated sites, while the remaining positions of methylated cytosine remained undetected.

At present, the methods which are considered to be most effective and universal and so are the most frequently used are those based on a reaction of the analysed DNA with sodium bisulphite, such as methylation-specific PCR (MS-PCR) or sequencing of DNA fragments PCR-amplified on a template which had been treated by sodium bisulphite (BS-PCR) (Cottrell, 2004). Both methods make use of the ability of sodium bisulphite to efficiently convert cytosine in single-stranded DNA into uracil at conditions in which 5mC remains unchanged. Cytosine deamination by sodium bisulphite occurs in the following steps: (1) binding of the sodium bisulphite to the 5,6-cytosine double bond; (2) hydrolytic deamination leading to the formation of a cytosine sulphate and uracil sulphate derivatives; and (3) removal of the sulfone group in alkaline conditions, which results in a conversion of these derivatives into uracil (Fig. 1).

Sodium bisulphite reacts with the cytosine in the form of a free base, a nucleoside, a nucleotide or an oligonucleotide. The reaction is highly single-strand-specific. The first stage of the reaction: formation of the cytosine sulphate derivative, is reversible. The formation of adducts is controlled by pH, the concentration of sodium bisulphite and temperature. The reaction occurs at low pH, while high pH favours the reverse reaction. During the second stage, the cytosine sulphate derivative undergoes

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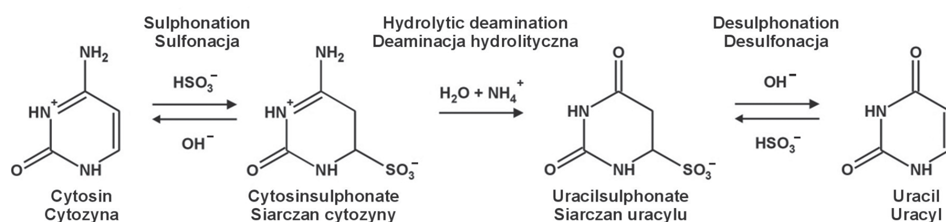

Fig. 1. Conversion of cytosine into uracil in a reaction with sodium bisulphite

Rys. 1. Konwersja cytozyny do uracylu podczas reakcji z wodorosiarczynem sodu

a hydrolytic deamination to uracil. This is catalysed by basic substances (sulphate, bisulphate, acetate ions). While sulphatation is favored by acidic pH, the reverse reaction becomes irreversible at pH above 7.0. The third stage involves an alkaline treatment in order to remove sulfone adducts. Although 5mC can also react with sodium bisulphite, the reaction is very slow and the equilibrium favours cytosine.

Following the treatment with sodium bisulphite, DNA serves as a template in a PCR reaction using specific primers to obtain fragments derived from each strand in which thymine and uracil residues are amplified as thymine, and only 5mC as cytosine. In PCR specific for methylated sites, two sets of primers, each containing at least one CpG site in their sequence, are used. One set is designed in such a way that the cytosine at the CpG site is recognised as cytosine, whereas the second one with the assumption that the cytosine was changed into thymine. Two parallel amplification reactions give one PCR product. If the product is obtained with the first set of primers, it indicates cytosine methylation and if it is obtained in a reaction with the second set of primers, it means that the cytosine is not methylated. In the BSP method, the primers are designed in such a way that there are no CpG sites in their sequence and, therefore, all cytosines are recognised as thymines. After the PCR reaction, the DNA fragment is sequenced. Additionally, primers are designed for the amplification of the same DNA fragment on a matrix which had not been treated with bisulphite. The recognition of sequences of both fragments allows for a comparative analysis based on the estimation of the occurrence of CpG sites present in the amplified fragment. The methods based on DNA treatment with sodium bisulfite allow for a methylation profile analysis of a single gene with about 200 pg of genomic DNA which corresponds to approximately 100 cells.

Moreover, in order to identify unknown sites susceptible to methylation or CpG islands in the genome, several methods have been developed for the analysis of "wild-type" genomes, such as the restriction landmark genomic scanning for methylation (RLGS-M) and CpG island microarray (Beck and Rakyan, 2008).

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1.5. Oxidative DNA damage

The impact of single factors of environmental contamination has been extensively discussed in scientific literature (Sato and Aoki, 2002). Epidemiological studies including evaluations of environmental contamination levels, carried out in urban areas and industrial regions revealed a negative influence exerted on health by dust (Pope, 2000; Utell and Frampton, 2000) and gaseous pollutants (Lippmann et al., 2000; Petroeschevsky et al., 2001). Moreover, a simultaneous contamination by fine-size dust and ozone has been shown to have an additive effect (Oberdörster, 2001). Even metals which are essential to build important components of the organism may catalyse reactive oxide species (ROS) generation from various organic carcinogens, resulting in oxidative DNA damage and additional source of metals can be found in the environment as water, soil or air pollutants (Oikawa and Kawanishi, 1998; Lloyd and Philips, 1999; Kawanishi et al., 2002; Faucher et al., 2012; Jena, 2012). Oxidative stress, local and systemic, especially in the lung tissue, may be the main factor responsible for increased mortality (Avissar et al., 2000; MacNee and Donaldson, 2000), leading not only to the activation of the nuclear transcription factor NFκB (Jiménez et al., 2000) and the inflammatory response of the respiratory airways but also to such changes in the cardiovascular system as hypercoagulability associated with leukocyte activation and release of cytokines (Pope, 2000; Utell and Frampton, 2000). Another important factor is the presence of xenooestrogens, chemical compounds affecting the organism in a way similar to the action of oestrogens, caused by the level of urbanization (Baccarelli et al., 2000; Tsuji, 2000) and cigarette smoking (Meek and Finch, 1999), which cause disturbances in the hormone management in living organisms. Epidemiological studies reveal that food products containing phytoestrogens can protect against many chronic diseases as well as unfavourable environmental conditions (Polkowski and Mazurek, 2000; Wiseman, 2000). Emissions from vehicles, especially exhaust fumes from diesel engines, power plants, industrial activities, tobacco smoking as well as burning of fossil fuels are among the main sources of mutagens in the environment (Schoket, 1999). In order to assess mutagenicity of contaminants present in the air, a number of epidemiological studies and experiments estimating DNA adduct levels in mammalian cells have been conducted (Hemminki, 1997; Lewtas et al., 1997).

DNA adducts are useful biomarkers of exposure to mutagens that can be found in the environment. In accordance with epidemiological studies which indicate that contamination levels are associated with an increased risk of tumours (Hemminki and Pershagen, 1994; Törnqvist and Ehrenberg, 1994), also DNA adducts are used to assess the risks of cancer occurrence. In biological systems, DNA is repaired by specific repair enzymes, which allows the cells to recover their normal functions. Lack of DNA repair or incorrect process can result in the occurrence of mutations, namely in substitutions of one base by another or in deletions, leading to carcinogenesis.

Reactive oxygen species (ROS) produced due to various environmental factors can exert a negative influence on the health of living organisms. Reactive oxygen species

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cause oxidative damage and may result in binding of peroxide groups to fats (Halliwell and Gutteridge, 1984; Boyd and McGuire, 1991) as well as in the formation of oxidized nucleotide derivatives, which, most probably, contributes to the initiation and maintenance of carcinogenesis (Kasai et al., 1987; Kamiya et al., 1992; Cerutti, 1994; Faucher et al., 2012; Jena, 2012; Dizdaroglu, 2015). DNA oxidative damage plays an important role in aging processes as well as in the development of various diseases, including cancer (Ames et al., 1993). Free oxygen radicals produced by normal oxygen metabolism attack bases in the DNA, thus contributing to the development of oxygen adducts and it is possible that the full spectrum of oxidative lesions in endogenous mammalian DNA exceeds 100 different types, of which 8-hydroxyguanine (8-oxoG) is one of the most abundant (Fig. 2) (Croteau and Bohr, 1997). In nuclear and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG or 8OHdG) or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG or 8oxodG) is one of the predominant forms of free radical-induced oxidative lesions, and has therefore been widely used as a biomarker for oxidative stress and carcinogenesis.

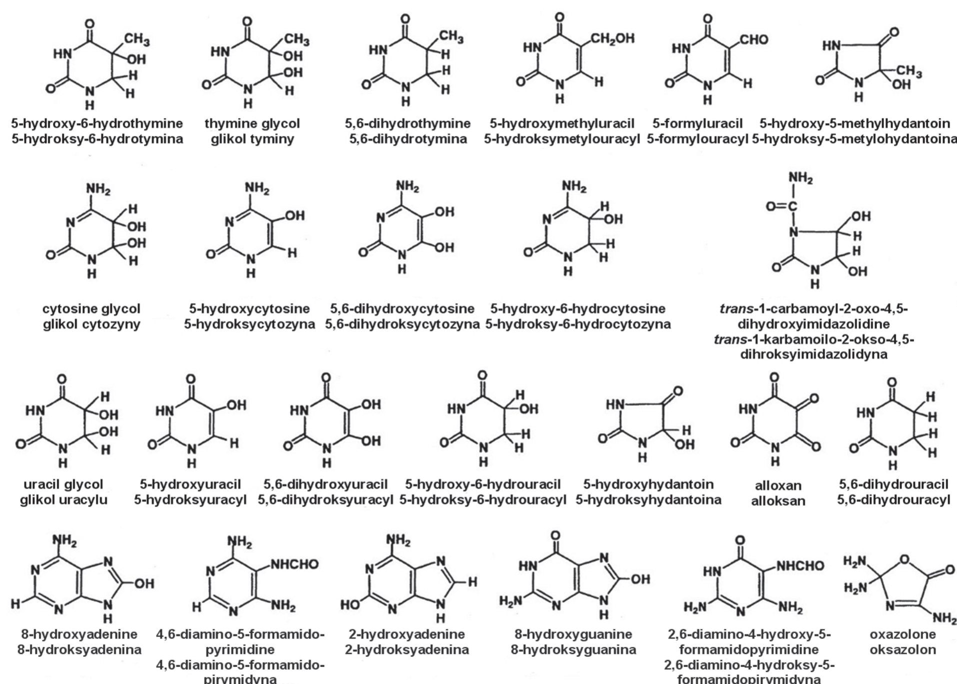

Fig. 2. Products of interactions between DNA bases, reactive oxygen and free radical species (Cooke et al., 2003)

Rys. 2. Produkty interakcji zasad DNA z reaktywnym tlenem i wolnymi rodnikami (Cooke i in., 2003)

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The European Standards Committee for Oxidative DNA Damage resolved in 1997 methodological problems with quantitative measurement of 8-hydroxy-2'-deoxyguanosine (8OHdG) or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). The hydroxyl radical acts on deoxyguanosine inducing the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG). The tautomeric form of 8OHdG is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), and in the scientific literature both names (8OHdG and 8-oxodG) are used for the same compound (Kasai, 1997; ESCODD, 2000; Valavanidis et al., 2009). 8-hydroxyguanine (8-oxoG) is repaired by excision of bases or nucleotides (Croteau and Bohr, 1997), whereas unrepaired 8-oxo2dG induces G to T transversions (Cheng et al., 1992). Elevated 8-hydroxydeoxyguanosine (8-OHdG) levels were reported in DNA isolated from lungs of rodents which were either administered exhausts of diesel fuel directly into the windpipe or intratracheally injected with diesel exhaust particles (Ichinose et al., 1997; Aoki et al., 2001; Sato et al., 2001; Sato and Aoki, 2002).

Research indicated that ROS generated by solid particles of diesel fuel fumes lead to carcinogenesis combined with the formation of 8-oxo2dG (Osawa et al., 1995). 8-oxo2dG was also identified in the leukocytes of workers exposed to the influence of asbestos (Marczynski et al., 2000) as well as in the urine of patients suffering from lung cancer, which indicates the formation of DNA oxidative damage due to environmental exposures (Erhola et al., 1997).

Hydrogen peroxide (H_2O_2) as well as copper(I) (Cu(I)) cause DNA damage in the thymine residue adjacent to the guanine residue, especially in 5'-GTC-3'. Exogenous reductants, such as ascorbate, glutathione, and a reduced form of NADPH, generate H_2O_2 in the presence of Cu(II) and lead to similar types of DNA damage. Typical antioxidants exert either a very minor or no preventive effect against damage caused by copper, which appears to indicate that copper does not lead to the development of free radicals but it may affect DNA in a way similar to that of hydroxide radicals. Some researchers claim that copper hydroperoxide [Cu(I)OOH] is produced in a reaction of H_2O_2 with Cu(I).

Nevertheless, the effect of free radicals on DNA damage associated with the presence of copper cannot be ruled out. Copper(II) preferentially binds to the charged phosphate group of the guanine N7 atom. This may be the source of interpretations that the Cu(II) copper binding to specific sites in DNA is reduced to Cu(I) copper allowing it to react with H_2O_2, leading to the development of the DNA-Cu(I)OOH complex. This complex may be associated with the release of a hydroxide radical which attacks DNA constituents neighbouring the site of copper binding to DNA before it is decomposed by a catalase. For this reason, Cu(II) site-specific DNA damage can be pre-conditioned by Cu(II) binding sites in DNA (Kawanishi et al., 2001).

The presence of Cu(I) causes specific damage in the double-stranded DNA, at 5'G in 5'-GG-3' and 5'-GGG-3' sequences (Kawanishi et al., 1999). It has been demonstrated that the level of 8-oxo2dG is higher in double-stranded DNA than in single-stranded DNA, which indicates that the helical structure plays an important role in the formation of DNA damage. The oxidised form of 8-oxo2dG (probably guanidinohydantion)

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may lead to G to T and G to C transversions (Duarte et al., 1999). 2,2-diaminooxazolon, which is an unstable piperidine product of guanine oxidation, may induce G to C transversions (Kino et al., 1998). Site-specific DNA damage caused by reactive oxygen species may result in the appearance of tandem CC to TT mutations (Newcomb et al., 1999).

It is assumed that telomere shortening is most likely associated with cell aging processes and oxidation influence. In normal conditions, the telomere length in human fibroblasts is shortened by approximately 50–200 base pairs per cell division. Some researchers reported an increased rate of telomere shortening as a result of an oxidative stress in human fibroblasts (von Zglinicki et al., 1995). However, the mechanism of this process is still under investigation. Experiments focused on elucidation of the acceleration mechanism of telomere shortening are linked with investigations on oxidative, site-specific DNA damage as one of important factors. H_2O_2 together with Cu(II) cause DNA damage, switching on the 8-oxo2dG formation specifically in the 5'-GGG-3' sequence situated within the characteristic telomere sequence (5'-TTAGGG-3') (Oikawa and Kawanishi, 1999). UV radiation in combination with riboflavin also causes specific formation of 8-oxo2dG in the 5'-GGG-3' sequence in telomeres (Kawanishi et al., 1999). Human 8-oxo2dG DNA glycosylase introduces chain breaks in two-stranded oligonucleotide specifically at the 8-oxo2dG residue (Roldán-Arjona et al., 1997). Therefore, the formation of 8-oxo2dG in the 5'-GGG-3' triplet contained in the telomere sequence resulting from oxidative stress may co-participate in the acceleration of the telomere shortening. Consequently, site-specific DNA damage may play a substantial role not only in carcinogenesis but also in the process of cell aging.

A majority of oxidative damages result in a wide range of chromosomal irregularities causing DNA replication blockage and extensive cytotoxicity. A mutation can occur as a result of an erroneous DNA repair or incorrect replication of DNA sequences which had been destroyed during chromosome reorganisation, and can cause improper repair of broken strands (Bohr et al., 1995). This kind of genetic modifications can result in permanent DNA damage and in the initiation of cell populations which do not undergo repair and overcome cytotoxicity in a process leading to the development of progeny cells.

8OHdG and 7,8-dihydro-8-oxoguanine, which form due to reactive oxygen species, are critical DNA damages (Minowa et al., 2000), highly mutagenic, and lead to the appearance of GC to TA transversions. A mutation is formed when polymerase replicates DNA containing 8-oxoguanine (8-oxoG) or 8OHdG and inserts adenosine against modified guanine (Shibutani et al., 1991; Arnett et al., 2005).

Environmental pollution is strongly associated with the development of modern civilization. Emissions of pollutants into the environment are caused by the recovery of natural raw materials, development of road networks and urbanisation processes, development of industry as well as intensive agricultural exploitation. The impact of substances which contribute to the environmental pollution has been one of the focal points of intensive scientific research for many years and at present these investigations are carried out at a molecular level.

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1.6. Relationship between methylation and oxidation of DNA

There are hypotheses that oxidation damage, in addition to inducing DNA alterations, can also contribute to carcinogenesis *via* epigenetic mechanisms, mainly DNA methylation (Cerda and Weitzman, 1997). The redox signalling and oxidative stress may influence the phenotype of cells by changing the function of histones and DNA modifying enzymes (Mikhed et al., 2015). Oxidative DNA damage causes microsatellite instability (Jackson et al., 1998). Oxidants are capable of inducing changes in the DNA of oncogenes and tumour suppressor genes. Hydroxide radicals can activate oncogenes, causing point mutations in the sequence of GC bases (Jackson, 1994). Moreover, the exposure of cells which contain a mutated or deleted *p53* gene to hydroxide radicals inactivates or inhibits the G1 phase of the cell cycle, thus reducing the possibilities of DNA repair. Increased frequency of replication errors may disturb the process of correct DNA replication, leading to the activation of oncogenes and inactivation of tumour suppressor genes in newly-divided cells, ultimately resulting in carcinogenesis (Jackson, 1994). Cytotoxicity caused by oxidative factors may also lead to the initiation of tumours due to the depletion of the proper cell population, resulting in a clonal proliferation of more resistant newly-developed cells, which increases the probability of the mutation occurrence.

The second mechanism influenced by oxidation damage and leading to carcinogenesis is associated with modifications of gene expression. Epigenetic changes in gene expression can stimulate cell growth and division signals, and thus cause the formation of a tumour (Crawford et al., 1995). Chromosome rearrangements associated with incorrect repair of a DNA strand with gaps lead to gene amplifications, aneuploidy, changes in gene expression and loss of heterozygosity, and as a result to accelerated development of the cancer disease (Bohr et al., 1995). It has been shown that reactive oxygen species can activate the transcription of genes responsible for the activation of cell growth and proto-oncogenes like c-*fos*, c-*jun* and c-*myc*. The fact that proto-oncogene activity is stimulated by oxidation stress emphasises the significance of oxidation in gene expression changes and tumour formation. In addition, oxidants can affect gene expression via oxidative-reduction mechanisms which can regulate protein-protein and protein-DNA interactions (Crawford et al., 1995). This mechanism can regulate the activity of the transcription factors like apurinic/apyrimidinic endonuclease 1 (AP-1) and nuclear factor NF-κB affecting the expression of genes associated with cell divisions (Vile et al., 1995; Mikhed et al., 2015). Investigations indicate that oxidants, irrespective of their origin, specifically modify DNA methylation of many gene families. The mechanism responsible for the methylation changes by oxidative agents is not known but it has been suggested that single-stranded DNA induces *de novo* methylation and it seems possible that single strand breaks developed as a result of oxidative stress may contribute to substantial modifications of DNA methylation levels in cell lines (Smith, 1994). Surprisingly, the same mechanism which changes methylation in specific sites is

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probably responsible also for the occurrence of global changes. The substitution of guanine by 8-hydroxyguanine inhibits methylation of the adjacent cytosine and binding of the methyltransferase to DNA. Experimental studies suggest that oxidative damage can possibly influence DNA methylation in fast-growing cells. Changes in the methylation pattern lead to abnormal gene expression and promotes a selective growth of some cell populations, which, in turn, may result in carcinogenesis. Little is known so far about the effectiveness of the mechanisms of 8-hydroxyguanine repair which developed in methylated CpG islands as well as about prevention of abnormal methylation. CpG sites may constitute "hot spots" for oxidative damage resulting from ineffective repair mechanisms, which can lead to the occurrence of mutations and abnormal methylation patterns associated with carcinogenesis.

1.7. *Alu* sequences

Alu sequences, approximately 300 bp long and made of two similar fragments of poly(A), occur in mammalian genomes. In the human genome, they appear in intron and inter-gene sequences in numbers of about 900 000 per haploid genome. The left *Alu* sequence segment contains an intrinsic polymerase RNA III promoter with poly(A) repeats at the 3' end. The evolutionarily younger part of the *Alu* family is surrounded by microsatellite sequences, i.e. poly(A) and dinucleotide (CA), (AG), (TA) or (CG). The *Alu* sequences' function has not been recognised yet despite the fact that the structural homologue of the *Alu* sequence, the human 7SL RNA sequence, constitutes a part of the signal recognition particle (SRP), mediating the transport of secretory proteins in the endoplasmic reticulum. *Alu* sequences can spread in the genome through transcription in which polymerase RNA III takes part as well by the phenomenon of transposition, through reverse RNA transcription and integration of the developed DNA into chromosomes. *Alu* sequences can be analysed with the *Alu*-PCR method in which DNA fragments are amplified between the terminal parts of the 3' segments of the neighboring *Alu* sequences arranged in opposite directions (Zietkiewicz et al., 1992). Only one primer is used in this method, which substantially simplifies the reaction.

Alu elements are members of the short interspersed element (SINE) family of mobile DNA elements. Although a majority of genomic *Alu* integrations occur into noncoding sequence and have no phenotypic effect, occasionally new integrates disrupt gene expression and function, and have been implicated in occurrence of human diseases, including cancer. *Alu* elements integrate primarily through an endonuclease-dependent mechanism called target site-primed reverse transcription. Occasionally, genomic integration occurs through an alternative endonuclease-independent pathway. DNA inverted repeats are hotspots of genomic instability caused by their ability to fold into hairpin- or cruciform-like DNA structures interfering with DNA replication and other genetic processes, and the most frequently occurring long inverted repeats in the human genome are inverted *Alu* repeats (Voineagu et al., 2008). *Alu* elements are

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considered to be one of the major factors leading to genomic instability by *de novo* insertion, insertion-mediated genomic deletion, and recombination-associated genomic deletion in humans (Kim et al., 2016). *Alu* elements are associated with approximately 0.1% of human genetic disorders. The recombination between *Alu* elements in human gene regions may disrupt gene function, subsequently leading to human diseases.

Recent studies connect methylation of *Alu* elements with transcriptional inactivation and inhibition of retrotransposition (Udomsinprasert et al., 2016). Hypomethylation probably facilitates genomic instability by retrotransposition of transposable elements, dysregulation of DNA repair genes and changing the expression of different genes. *Alu* and long interspersed nuclear elements (LINE-1) elements may be influenced by reactive oxygen species inducing oxidative stress. One of the oxidased bases: the 8-hydroxy-2'-deoxyguanosine (8oxo2dG), interferes with the ability of DNA to function as a substrate for DNA methyltransferases (DNMTs), leading to global DNA hypomethylation and subsequent genomic instability (Ziech et al., 2011; Udomsinprasert et al., 2016).

1.8. Effects of heavy metals on living organisms

Living organisms require varying amounts of heavy metals: iron (Fe), cobalt (Co), copper (Cu), manganese (Mn), molybdenum (Mo), and zinc (Zn) are necessary for the correct functioning of human organisms. However, their excessive levels can be damaging to the organism. Also other heavy metals, such as mercury (Hg), plutonium (Pu), and lead (Pb), are toxic metals and their accumulation in bodies of animals over time can cause serious illness. Still, certain elements that are normally toxic are, for certain organisms or under certain conditions, beneficial. Examples include vanadium (V), tungsten (W), and even cadmium (Cd).

Heavy metal toxicity can result in damaged or reduced mental and central nervous function, lower energy levels, and damage to blood composition, lungs, kidneys, liver and other vital organs (Table 1). Long-term exposure may result in slowly progressing physical, muscular, and neurological degenerative processes that mimic the Alzheimer's disease, Parkinson's disease, muscular dystrophy or multiple sclerosis (Duffus, 2002; Abdul-Wahab and Marikar, 2012).

Heavy metals have an effect on the stability of colloids which is relevant to eukaryotic cells. Some of them are dangerous for health or for the environment (e.g. mercury, cadmium, lead, chromium), some may cause corrosion (e.g. lead), and some are harmful in other ways (Hogan, 2011). Within the European Union, there are eleven elements of the highest concern, the emissions of which are regulated. These are: arsenic, cadmium, cobalt, chromium, copper, mercury, manganese, nickel, lead, tin and thallium. Some of these elements are actually necessary for humans in small amounts (cobalt, copper, chromium, manganese, nickel), while others are carcinogenic or toxic, affecting among others the central nervous system (manganese, mercury, lead, arsenic), the kid-

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Table 1. The most common heavy metal pollutants and their effect on human health
Tabela 1. Najczęstsze zanieczyszczenia metalami ciężkimi i ich wpływ na zdrowie człowieka

Pollutants Zanieczyszczenia	Major source Główne źródła	Effect on human health Wpływ na zdrowie człowieka
Arsenic Arsen As	pesticides, fungicides, metal smelters pestycydy, fungicydy, huty metali	bronchitis, dermatitis, poisoning zapalenie oskrzeli, zapalenie skóry, zatrucia
Cadmium Kadm Cd	welding, electroplating, pesticides, fertilizers, Cd and Ni batteries, nuclear fission plants spawanie, galwanizacja, pestycydy, nawozy, baterie typu Cd i Ni, rozszczepienia jądrowe	renal dysfunction, lung diseases, including lung cancer, bone defects (osteoporosis), increased blood pressure, kidney damage, bronchitis, gastrointestinal disorders, bone marrow disorders, different cancers niewydolność nerek, choroby płuc, w tym rak płuc, uszkodzenia kości (osteoporoza), podwyższone ciśnienie krwi, uszkodzenia nerek, zapalenie oskrzeli, choroby układu pokarmowego, choroby szpiku kostnego, różne nowotwory
Chromium Chrom Cr	mines, mineral sources kopalnie, źródła mineralne	damage to the nervous system, fatigue, irritability uszkodzenie układu nerwowego, zmęczenie, drażliwość
Copper Miedź Cu	mining, pesticide production, chemical industry, metal piping górnictwo, produkcja pestycydów, przemysł chemiczny, rury metalowe	anaemia, liver and kidney damage, stomach and intestinal irritation anemia, uszkodzenia wątroby i nerek, drażliwość żołądka i jelit
Lead Ołów Pb	paint, pesticides, cigarette smoking, automobile emission, mining, burning of coal farby, pestycydy, palenie papierosów, emisje samochodowe, górnictwo, spalanie węgla	mental and other developmental retardation in children, fatal infant encephalopathy, congenital paralysis, sensor neural deafness, acute or chronic damage to the nervous system, epilepsy, damage of the liver, kidney and gastrointestinal tract upośledzenie umysłowe i inne opóźnienia rozwojowe u dzieci, śmiertelna encefalopatia u dzieci, porażenie wrodzone, głuchota czuciowo-nerwowa, ostre lub przewlekłe uszkodzenie układu nerwowego, epilepsja, uszkodzenie wątroby, nerek i przewodu pokarmowego
Magnesium Magnez Mg	welding, fuel additives, ferromanganese production spawanie, dodatki do paliw, produkcja żelazomanganu	inhalation or contact causes damage to the central nervous system wdychanie lub kontakt powoduje uszkodzenie ośrodkowego układu nerwowego

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Table 1 cont. – Tabela 1 cd.

Pollutants Zanieczyszczenia	Major source Główne źródła	Effect on human health Wpływ na zdrowie człowieka
Mercury Rtęć Hg	pesticides, batteries, paper industry pestycydy, baterie, przemysł papierniczy	tremors, gingivitis, minor psychological changes, acrodynia characterised by pink hands and feet, spontaneous abortion, damage to the nervous system, poisoning drżenie, zapalenie dziąseł, drobne zmiany psychologiczne, akrodynia charakteryzująca się różowymi dłońmi i stopami, spontaniczne poronienia, uszkodzenie układu nerwowego, zatrucie
Zinc Cynk Zn	refineries, brass manufactures, metal plating, plumbing rafinerie, produkcja wyrobów z mosiądzu, powlekanie metalem, hydraulika	zinc fumes damage the skin and the nerve cell membranes opary cynku uszkadzają skórę i błony komórek nerwowych

neys or liver (mercury, lead, cadmium, copper), or skin, bones, or teeth (nickel, cadmium, copper, chromium). The standards of soil quality and ground quality according to Polish law are presented in Table 2.

Heavy metal pollution can arise from many sources but most commonly it is due to industrial purification of metals, e.g. copper smelting and preparation of nuclear fuels. According to Polish Regulations (Rozporządzenie…, 2016), installations for the production and processing of metals, like those for roasting or sintering metal ores, including sulphide ore, could cause with high probability pollution of land. Types of activities that are likely to cause historical pollution of the surface of the earth, together with an indication of examples of pollution for these activities, is set out in Annex 2 to the Regulation. Metals and metalloids like arsenic, bar, cadmium, chromium, cobalt, copper, lead and mercury, molybdenum, nickel, tin, zinc are examples for those of pollution and their level should be analysed now or in the past for identification of contaminated sites.

Electroplating is the primary source of chromium and cadmium. Cadmium, lead and zinc are released in tiny particulates as dust from rubber tires on road surfaces. Small size allows these toxic metals to rise on the wind to be inhaled, or transported on the soil surface or onto edible plants. Through precipitation or by ion exchange into soils and muds of their compounds, heavy metal pollutants can accumulate in the environment, which can have severe negative effects on the environment. Unlike organic pollutants, heavy metals do not decay and thus pose a different kind of challenge for remediation. Plants, mushrooms or microorganisms are occasionally successfully used to remove some heavy metals such as mercury. Plants which have capacities of metal

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Table 2. Limit values for concentration of soil and land pollutants (mg/kg dry mass)
Tabela 2. Limity stężeń zanieczyszczeń gleb i gruntów (mg/kg suchej masy)

Pierwiastek chemiczny Chemical element	Maximum concentrations permitted by the regulations (mg/kg dry mass) Maksymalne stężenie dopuszczone w rozporządzeniach (mg/kg suchej masy)			
	2002–2016 Rozporządzenie… (2002)		since 2016.09.05 – od 5.09.2016 r. Rozporządzenie… (2016)	
	soil class – klasa gleby			
	A	B	I	II
Arsenic – Arsen	20	20	25	10–50
Cadmium – Kadm	1	4	2	2–5
Lead – Ołów	50	100	200	100–500
Magnesium – Magnez				
Mercury – Rtęć	0.5	2	5	2–5
Zinc – Cynk	100	300	500	300–1000
Chromium – Chrom	50	150	200	150–500
Copper – Miedź	30	150	200	100–300

A – (1) land properties belonging to the area protected under the provisions of the Act – Water Law; (2) areas protected under the provisions of the nature conservation regulations if maintaining the current level of soil contamination does not pose a risk to human health or the environment.

B – grounds classified as agricultural lands up to 0–0.3 m below the ground level.

I – urban area.

II – agricultural lands up to 0–0.25 m below the ground level.

A – (1) nieruchomości gruntowe wchodzące w skład obszaru poddanego ochronie na podstawie przepisów ustawy Prawo wodne; (2) obszary poddane ochronie na podstawie przepisów o ochronie przyrody, jeżeli utrzymanie aktualnego poziomu zanieczyszczenia gruntów nie stwarza zagrożenia dla zdrowia ludzi lub środowiska.

B – grunty zaliczone do użytków rolnych do głębokości 0–0,3 m pod poziomem terenu.

I – tereny zabudowy mieszkaniowej.

II – tereny rolnicze do głębokości 0–0,25 m poniżej poziomu terenu.

hyper-accumulation concentrate heavy metals in their biomass and so can be used to remove them from soils. There are known examples where the plants growing on mining tailings were burned for recovery of heavy metals accumulated in them.

Harmful effects of heavy metals on living organisms were already widely recognised in ancient Rome. However, a global contamination of the environment with heavy metals started at the beginning of the last century as a result of a rapid development of metallurgy for military purposes and led to the deterioration of living conditions and reduction of yields of many crop plants. In addition, the value in use of agricultural and forest products decreased and the ecological function of the soil-plant cover deteriorated. The most important disasters caused by heavy metals are listed in Table 3.

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Table 3. The most important disasters caused by heavy metals
Tabela 3. Najważniejsze katastrofy spowodowane przez metale ciężkie

Year Rok	Site Miejsce	Details Dane szczegółowe
1932	Minamata Bay, Japan Zatoka Minamata, Japonia	Sewage containing mercury was released by Chisso's chemicals works into Minamata Bay in Japan. Mercury accumulates in sea creatures, eventually leading to mercury poisoning in the population. Due to the consumption of mercury contaminated fish, the first cases of mercury poisoning appear in the population of Minamata Bay in 1952, bringing over 500 fatalities ("the Minamata Syndrome"). Since then, Japan has the most strict environmental laws in the industrialised world. Ścieki zawierające rtęć zostały uwolnione przez zakłady chemiczne Chisso w zatoce Minamata w Japonii. Rtęć gromadzi się w organizmach morskich, prowadząc ostatecznie do zatrucia rtęcią w populacji. Ze względu na spożycie ryb zanieczyszczonych rtęcią, pierwsze przypadki zatrucia rtęcią pojawiają się w populacji zatoki Minamata w 1952 r., przynosząc ponad 500 ofiar śmiertelnych („zespół Minamata"). Od tego czasu Japonia ma najbardziej surowe przepisy dotyczące ochrony środowiska w uprzemysłowionym świecie.
1986	Sandoz warehouse, Switzerland Magazyn Sandoz, Szwajcaria	Water used to extinguish a major fire at Sandoz agrochemical storehouse carried approximately 30 tonnes of fungicides containing mercury into the Upper Rhine. Fish were killed over a stretch of 100 km. The shock drives many European Environment Agency projects forwards. Woda użyta do gaszenia dużego pożaru w magazynie agrochemicznym Sandoz wprowadziła około 30 ton fungicydów zawierających rtęć do górnego Renu. Ryby zostały zabite na odcinku 100 km. Szok przyczynił się do rozwoju wielu projektów Europejskiej Agencji Ochrony Środowiska.
1998	Coto de Donana Nature Reserve, Spain Rezerwat Przyrody Coto de Donana, Hiszpania	Toxic chemicals in water from a burst dam belonging to a mine contaminated the Coto de Donana Nature Reserve in Southern Spain. Approximately 5 mln cubic metres of mud containing sulphur, lead, copper, zinc and cadmium flew down the Rio Guadimar. Experts estimate that the Europe's largest bird sanctuary as well as Spain's agriculture and fisheries will suffer permanent damage from the pollution. Toksyczne chemikalia w wodzie z zapory po przerwaniu zapory należącej do kopalni zanieczyściły rezerwat przyrody Coto de Donana w południowej Hiszpanii. Do Rio Guadimar wpłynęło ok. 5 mln m^3 szlamu zawierającego siarkę, ołów, miedź, cynk i kadm. Eksperci szacują, że największy w Europie rezerwat ptaków, a także rolnictwo i rybołówstwo w Hiszpanii poniosą trwałe szkody spowodowane zanieczyszczeniem.

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Table 3 cont. – Tabela 3 cd.

Year Rok	Site Miejsce	Details Dane szczegółowe
2011	Dow Industries, Midland, Texas, USA Dow Industries, Midland, Teksas, USA	Community members filed suit against Dow and three other companies for contaminating groundwater there with hexavalent chromium. Barred from use in the European Union because of its toxicity, hexavalent chromium is a known carcinogen. The United States Environmental Protection Agency Hazard Report states that exposure, including through contaminated drinking water "may produce effects on the liver, kidney, gastrointestinal and immune systems" (EPA…, 1998). Członkowie społeczności złożyli pozew przeciwko firmie Dow i trzem innym firmom za zanieczyszczenie wód gruntowych sześciowartościowym chromem. W Unii Europejskiej wprowadzono zakaz jego stosowania ze względu na toksyczność, sześciowartościowy chrom jest znanym czynnikiem rakotwórczym. W raporcie o zagrożeniach Agencji Ochrony Środowiska Stanów Zjednoczonych stwierdzono, że narażenie, w tym przez zanieczyszczoną wodę pitną, „może wywoływać skutki w wątrobie, nerkach, układzie pokarmowym i układzie odpornościowym" (EPA…, 1998).

Regions characterised by increased heavy metal content can also be found in Poland and one of these regions is the area adjacent to the Głogów Copper Mining-Smelting Plant. Therefore, it appears interesting to compare DNA methylation in cattle of identical breed living in this region and with that in animals kept in the region free of heavy industry. Genome regions containing methylated cytosine are usually transcriptionally inactive. Since DNA methylation is a reversible process which is independent of DNA sequence, it was recognised as an epigenetic mechanism of gene regulation. The analyses presented in this dissertation are of considerable importance because, at present, it is difficult to find areas which have been contaminated by heavy metals and are still used for rearing animals. Therefore, the two groups of animals under study constituted a unique model. In addition, I also succeeded in securing a group of control animals which were relatively closely related to the experimental animals, which allowed to eliminate the influence of the genetic background and so further enhanced the attractiveness of the model, rule out various reservations associated with the fact that such animals were equipped in different gene pools. Since the analysis of epigenetic changes can also be considered as somewhat analogous to those on humans, the experiments linking the environment with DNA methylation changes and their role in the development of human tumours are beginning to gain increasingly practical implications.

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2. Objectives of the study

The hazards of heavy metals for living organisms were known already in ancient Rome. However, global environmental contamination by heavy metals started in the beginning of the fifteenth century and was a consequence of the massive development of steel production for military purposes. This has resulted in a deterioration of living conditions and crop yields, decreased the value in use of agricultural and forestry lands, and worsened the ecological function of soil and vegetation cover. In our country, there are regions with a high contamination with heavy metals, one of which is the area near the Głogów Copper Smelters (part of KGHM Polish Copper).

The overall objective of this study was to determine associations between the state of environment in which farm animals live and develop, and their health condition on a DNA level. To this effect, I analysed DNA changes in the Limousin cattle living in the vicinity of a copper smelter.

The first specific objective of the study was to collect information on the content of heavy metals in the vicinity of copper smelters and to compare them with the current standards. This was planned to be achieved through my own research and based on literature data on pollution in the neighbourhood of the two Głogów smelters.

The second aim of this study was to collect blood samples from Limousin cattle reared under the influence of the pollution from the smelters and from cattle in a region free from such a contamination. For this purpose, I sought to establish two groups of animals (the experimental group and the control group) that would encompass closely related individuals (half-sisters). The main goal was to implement molecular testing in order to compare the levels of DNA methylation and oxidation as well as the profiles of *Alu* sequences between the two groups of animals.

The last aim of my study deals with the impact of these DNA changes on the phenotype and functional characteristics of the animals. This may, in a long run, contribute to understanding similar mechanisms of pollution-induced changes in humans.

3. Material and methods

3.1. Animals and biological material

The analysis of methylation was conducted in two groups of beef cattle living in Poland in extremely different environmental conditions, each made up of 50 individuals. The farm on which the experimental animals were kept is situated in Skidniów located in the Kotla commune. According to Grzebisz et al. (1997), 44% of land in this area belongs to the 4th productivity class, while soils of the 3rd and 5/6th class take up 56% of the commune area. The majority of winds are westerly and their average speed is 2 m/s, which favours the contamination fallout in areas closest to the Głogów Copper Smelters. The distribution of the smelter emitters in the area of the Żukowice commune has a direct impact on agricultural activities carried out in the Skidniów farm. The farm is separated from the Smelter only by a narrow land strip of a sanitary protection zone and the Odra River. All grassland and arable land areas are exposed to a direct negative influence of the Smelters and this negative impact concerns the soil, air and water environments. The area on which the animals from the experimental group were kept can, therefore, be considered as an area particularly suitable for carrying out comparative studies on the effects of environmental contamination.

Despite the fact the soil quality index in this area is higher than the average for the country (domination of the 3rd and 4th soil productivity class), soil contamination with heavy metals and its considerable acidification have a negative effect on animal organisms through contaminated feed. The cattle were fed mainly roughages produced on the farm (fresh forage during the grazing season, and silages, hay silages, and straw during the remaining period). Concentrates derived in 95% from cereals produced in the neighbourhood of Skidniów and were supplemented with rapeseed meal and meat-and-bone meal obtained from outside the region. The rearing system adopted on the farm involves outdoor management system, with animals remaining indoor, in a simple cowshed, only for two to four weeks after calving. Feeding also takes place outdoor. This long period of outdoor management creates favourable conditions for the dust-gaseous fallout to affect the animals.

The control group comprised 50 animals of the beef type kept in cowsheds which belong to the Sława farm (LubuskieVoivodeship). More than 50% of the area in the Sława commune is forested and it also boasts to have the largest lake in the region (812 ha) as well as a number of smaller lakes connected by channels. Another important asset of the commune lands is that there is practically no industry. In comparison with the region around Skidniów, the soil environment here is significantly poorer, with the

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5th and 6th class soils which occupy over 60% of arable lands. There are practically no soils of the 3rd class or higher. The results achieved by farmers here, both in plant and animal production, are better than expected based on quality index of the soils.

The feeding system of ruminants employed on the Sława farm does not differ much from the one used in Skidniów. The main bulky feeds comprise silage from maize and beet leaves as well as forages from meadows and arable lands. Concentrates are manufactured in the farm's feed mixing plant, using raw material from the farm fields. In addition, the diets were supplemented with silages obtained from the farm distillery as well as from straw. The animals are kept in typical drive-through cow-houses and from April to October they remain outdoor on pastures where they were also fed. The animals kept on this farm are characterised by high daily body weight gains, favourable food conversion ratios as well as an overall good health condition. A part of the heifers born on the farm in recent years was used to enlarge the farm's beef cattle herd and another part was sold to neighbouring farms specialising in beef cattle breeding. Some animals from Sława were also sold to the farm in Skidniów. The group of control animals from Sława used in this study included the progeny of mothers of the black and white lowland breed mated with Limousin breed fathers. The localisation of the Skidniów and Sława farms is shown in Figure 3.

Table 4. Summary of the Limousin blood share in animals from the control and experimental group

Tabela 4. Zestawienie udziału krwi limousine u zwierząt w grupie kontrolnej i badanej

Cattle breed – Rasa bydła	Share Udział (%)	Number of animals – Liczba zwierząt	
		control animals zwierzęta kontrolne	experimental animals zwierzęta badane
Limousin – Limousine	100	21	1
Limousin/Black and White Limousine/czarno-biała	87.5/12.5	0	1
Limousin/Black and White Limousine/czarno-biała	75/25	19	34
Limousin/Black and White Limousine/czarno-biała	50/50	3	10
Limousin/Black and White/Simmental Limousine/czarno-biała/simental	50/25/25	6	1
Limousin/Black and White/Simmental Limousine/czarno-biała/simental	50/12.5/37.5	0	1
Limousin/Black and White/Charolais Limousine/czarno-biała/charolaise	50/25/25	1	2

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Fig. 3. Geographical location of the farms in Skidniów and Sława (indicated with arrows) from which the biological material for research was collected (Krupiński, 2003)

Rys. 3. Geograficzna lokalizacja gospodarstw w Skidniowie i Sławie (wskazano strzałkami), z których pobrano materiał do badań (Krupiński, 2003)

The herd of animals kept on the Skidniów farm was intended as a response to the market demand for young beef and an example of utilization of arable lands excluded from cultivation, and of degraded meadows and pastures as a source for feeds for ruminants. The analysed group ("experimental group") comprised 49 cows and heifers, and one bull of the meat breed with a 50% to 100% proportion of the Limousin breed blood and an admixture of blood of the lowland black and white breed. Similarly, the control material from the Sława farm comprised mature and young animals of beef breeds whose blood contained from 50–100% of the Limousin breed blood. Detailed characteristics of all animals are shown in Table 4.

Noteworthy, both groups of animals included the progeny of the Limousin Jaśmin bull (20 animals in the experimental group and 15 among controls) and this consanguinity allowed, very important and interesting, from a scientific point of view, comparisons. Another scientifically interesting subgroup is the progeny of the Jasny bull (16 animals only in the experimental group), giving a possibility to analyse animals with a similar genetic background. The biological material obtained from the experimental group animals can also serve for other studies, providing an unique model for investigations not only on other animals but also on humans.

3.2. DNA isolation

For molecular analysis, 10 ml of peripheral blood were collected on EDTA and DNA was isolated by the proteinase K method. In order to perform lysis, 10 ml of blood was mixed with 30 ml buffer containing: 155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM Tris-HCl (pH 7.4) and then it was incubated for 30 min at 0°C. Blood lysates were centrifuged at 3000 rpm at 4°C for 10 min and the obtained pellet was suspended in the lysis buffer (10 ml), and centrifuged again. This operation was repeated three times.

The leukocyte pellet was treated with 5 ml of SE buffer containing: 75 mM NaCl, 1 mM Na_2EDTA (pH 8.0), 25 µl proteinase K (10 mg/ml) and 250 µl SDS (20%), and incubated for 16 h at 55°C. The obtained lysate was then mixed with 750 µl of 6 M NaCl for 15 s and centrifuged at 3600 rpm at 20°C for 15 min. DNA was precipitated using two volumes of ethanol, washed with 70% ethanol and dissolved in 500 µl of water. All DNA samples were kept at 4°C until the completion of the planned experiments and then stored at –20°C.

3.3. Global DNA methylation analysis

Thin layer chromatography (TLC) was used to evaluate the global methylation of leukocyte DNA. The analysis was conducted in two steps: a two-dimensional thin layer chromatography followed by a radioactivity measurement on scanner

Typhoon 8600 (GE Healthcare) as previously described (Phillips, 1997). The DNA enzymatic hydrolysis was conducted at 37°C for 6 h in 10 μl of the reaction mixture containing: 1–3 μg DNA, 0.02 units of micrococcal nuclease and 0.001 units of spleen phosphodiesterase in 20 mM sodium succinate, pH 6.0 containing 10 mM $CaCl_2$. One fifth (2 μl) of the hydrolysed material was freeze-dried and suspended in 3 μl of a solution containing 10 mM bicine-NaOH buffer (10× buffer: 100 mM bicine, 100 mM $MgCl_2$, 100 mM DTT, 10 mM spermidine), pH 9.6, 0.15 mM ATP, 1.5 units of PNK T4 (polynucleotide kinase, phage T4) and 1 μCi of γ^{32}P-ATP. The sample was then incubated at 37°C for 45 min. After the reaction, the excess of radioactivity was removed by adding 3 μl apyrase at a concentration of 10 units/ml in 10 mM bicine-NaOH buffer, pH 9.6, and the sample was incubated at 37°C for 30 min. Next, 2.8 μl of 0.1 M ammonium acetate (pH 5.3) and 0.2 μl nuclease P_1 (1 mg/ml) were added to the reaction mixture in order to hydrolyse 3' phosphates. The entire mixture was then incubated at 37°C for 30–60 min. The separation in the first direction (Cellulose Avicel, Schleicher & Schuell or DC-Cellulose Merck) was conducted in the isobutyric acid:ammonium hydroxide:water solution (at a volumetric proportion of 66:1:33). The separation in the second direction was performed in propanol:ammonium sulphate: 0.1 M phosphate buffer, pH 6.8 (at a volumetric-gravimetric-volumetric proportion of 2:60:100) as described elsewhere (Słomski, 2008). Quantitative analyses were carried out using a scanner of radioactivity and fluorescence Typhoon 8600 (GE Healthcare). The plates were placed in a cartridge equipped with screens exciting radioactivity for 1–5 h and scanned at a 100 μm resolution using the manufacturer's software (Typhoon Scanner Control). The quantitative evaluation was carried out using the ImageQuant 5.1 programme. Methylation levels were measured using the *R* coefficient as a ratio of spot intensities for single nucleotides according to the following formula:

$$5\ \text{mC} / (5\ \text{mC} + \text{C} + \text{T}) \times 100.$$

3.4. Methylation analysis of selected genes

Based on the literature review and data contained on the website of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov, last accessed 8.8.2017), I selected two genes that may be responsible for DNA repair in cattle for the analysis of the promoter methylation status: nudix hydrolase 1 (*Nudt1*) and apurinic/apyrimidinic endodeoxyribonuclease (*Apex1*).

The *Nudt1* gene, also denoted as *Nudt*, *Nudix* or *LOC 525496*, is homologous to the *hMTH1* gene (8-oxo-dGTPase). It encodes a protein called related to 7,8-dihydro-8-hydroxyguanine triphosphatase (8OHdGTPase). This protein hydrolyses oxidized purine nucleotide triphosphates (8OHdGTP, 2OHdATP) to monophosphates, which prevents their incorporation into the DNA structure at replication and transcription. *Apex1*, also denoted as *Apex1*, *Apen*, *Bap1* or *Ref-1*, encodes a multifunctional DNA repair enzyme, *Apex1*, which has both an AP endonuclease

and a purine/pyrimidine lyase (AP lyase) activity and plays an important role in DNA repair processes.

The sequences of the selected genes were analysed for the content of CpG islands in the promoter region. The primers were designed using programmes Methprimer and Primer III, and the results were verified through a detailed analysis of the selected sequence. The nucleotide sequence of the *Apex1* gene fragment containing the promoter is presented in Figure 4 and analysis of CpG islands in Figure 5, and of the *Nudt1* gene fragment in Figure 6 and 7, respectively.

Methylation of selected fragments of promoters of these genes was conducted by methylation-specific PCR (MSP). This technique involves a denaturation of DNA and its modification with sodium bisulfite followed by a PCR amplification using two pairs of primers: one pair specific for methylated DNA and the other one for unmethylated DNA. After treatment with sodium bisulfite, all cytosines which were not methylated become converted to uracil which is read as thymine in a PCR reaction. For the analysis of the methylation status of the promoter of the *Apex1* gene, a fragment comprising 255 bp was selected, and for the *NudtI* gene – a fragment encompassing 297 bp. The sequences of the designed primers are shown in Table 5.

The sequences of the selected genes were analysed for the occurrence of recognition sites for restriction enzymes using the Restriction Mapper software which automatically generates a list of hydrolysing enzymes for the analysed sequence. Twenty microliters of DNA were then hydrolysed with a restriction enzyme (1 unit/μl) in the appropriate buffer, in a total volume of 30 μl. Samples were incubated for 5 h at 37°C. The reaction was stopped by placing the samples on ice at 4°C. The following restriction enzymes were used: *Apa*I (GGG*CC↓*C), *Pvu*II (CAG↓*CTG) and *Xba*I (T↓*CTAGA) (Sigma Aldrich).

For the modification of DNA with sodium bisulfite, 1 μg of DNA was hydrolysed with restriction enzymes in a total volume of 21 μl of the TE buffer. Then, the DNA was added to 4 μl of freshly prepared 10 N NaOH and incubated 15 min at 50°C in a water bath. Next, 50 μl of 2% low melting agarose were added. DNA-agarose beads were formed by pipetting 12.5 μl of this mixture in a 2-ml tube with 300 μl of mineral oil which had been cooled by placing at 0°C for 30 min. The beads were left in the tube with oil on ice for at least 30 min and 500 μl of the mixture of sodium bisulfite (2.5 M sodium bisulfite in 100 mM hydroquinone) were added to the tubes protected from light and incubated at 50°C in a laboratory oven for 4 h. Afterwards, the oil layer was removed and the solution was washed with 1 ml of the TE buffer, pH 8.4. DNA immobilized on agarose was desulfurized by a 30-minute incubation in 500 μl of 0.2 M NaOH. The incubation was stopped by washing with 1 ml of TE buffer.

Before amplifying the DNA by PCR, the beads were washed with water. Polymerase chain reaction was performed using primers specific for sodium bisulfite-treated DNA and for untreated DNA. Each reaction contained 2 μl of DNA, 2 μl of 10 × PCR buffer, 1 μl forward primer (10 pmol), 1 μl reverse primer (10 pmol), 1 μl of 5 mM dNTPs, 0.2 μl Taq polymerase (5 U/μl) and 12.8 μl of water. Polymerase chain reaction

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5’–TCGACCCACGGATCGAATTCAAGTCTCCTGTACTGCAGGCAGATTCTTTAC
CACTGAGCCACCTGGGAAGCCCTTTTTACCGTTACATAATCTCAGAGAGTAGGAT
CAAAAATTAGGAGGCACAATGCGCACTCTTCACCAACGTTTAGAAAGATGGCAGGG
GACGCTGTGCGGAAAAGTGCGCCACGAGTTACAAATCCCCAAGTCCTCACCTGTGC
CGGGAGGCGTGACGTAAGTCCGCCGCGGGTTCGCCAGTACTTTGCCATCCCGTAC
CACTCCCACGCCAATCTTGTTGGCGCTGCCTTCAAAACCCAGCACCGCCGGCATG
GCTCCGAGCTAGCACTGGAGCCAAGGAGCCCTCACCAGTCCGCGCTGGGACGCA
GACTCCGG**GTTGCCGAGTAGGAGGATGAGCCAGTGGGTGCTAAGCAGCCGCGC**
CAACCAGAACAACAGCTACCAGAAAATTCCCATAGGTAGCGCGCCGACGCCGGTC
CCAACCTCTATCGTTACGACGACCCATCAAGACCCCTTCGAGGGCCTGTTCGCT
TAACTGCCGGGAACGCTGAGCCGGCGTGTGCTCCTTAAATTCCTATCCGTTCCTCT
GCTTCTCTCCCATGGTCCAGCCCAGCCTTTCCCCTGATTTCGTTCTCTCTTACACA
GCTTTAGGATCTCTAGTCAGGGTGGCAGAAAGAGTTGTGAATCTGGCATATTGATAAA
CAGCCCAAGAATGGAGGTTGGGACCTTCAATGTCCCGGAGTTCACGAGAAGGGCGG
GAAGCTTTCGGTCACGTGGTACAACTAGACCAATGACCCTTATTCCTCCGCCCCTG
GAAGAGCATCTCAGATCATGTGACCACGCCACTTACCCACGTGGGGCACAGCGTGCTC
CATTCTTTGTGCTGGGTTAAGGAGGAGGCATACAGGGGCCGCAGCAGGTCAGCTAAA
GGGTATGTGTTGTTTTGCCCATCTTAGCAGCTTCTCTGGACGTCTGATCCGCAAGGC
CAG–3’

Fig. 4. Complete nucleotide sequence of the *Apex1* gene promoter. Fragment of *Bos taurus* breed Hereford chromosome 10, Bos_taurus_UMD_3.1, (AC_000167.1) encompassing a 1001-base pair region from base 26709023 to 26710023. The gene fragment analysed is written in bold and CpG sites are underlined

Rys. 4. Pełna sekwencja nukleotydowa promotora genu *Apex1*. Fragment chromosomu 10 *Bos taurus* rasy Hereford, Bos_taurus_UMD_3.1 (AC_000167.1), obejmujący region 1001 pz od nukleotydu 26709023 do 26710023. Analizowany fragment genu pogrubiono, a miejsca CpG podkreślono

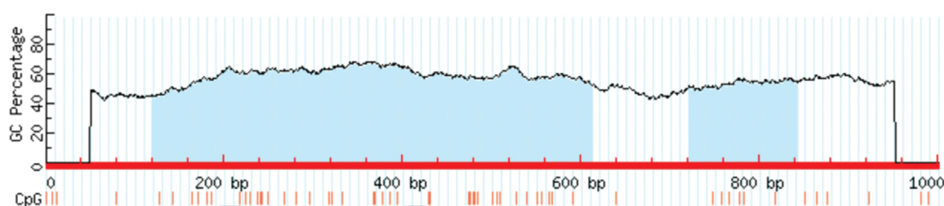

Fig. 5. Prediction of the CpG island occurrence in the *Apex1* gene promoter using program MethPrimer (Li and Dahiya, 2002). Two CpG islands were found in a 1001-base pair sequence, one of 495 bp (119–613) and another one of 123 bp (721–843). The analysed fragment (391–645 bp) is located within the main part of the first CpG island

Rys. 5. Przewidywanie wysp CpG w promotorze genu *Apex1* z zastosowaniem programu MethPrimer (Li and Dahiya, 2002). Wykryto dwie wyspy CpG obejmujące 495 pz (119–613) i 123 pz (721–843) w 1001-nukleotydowej sekwencji. Analizowany fragment (391–645 pz) jest lokalizowany w głównej części pierwszej wyspy CpG

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5’–CTTACCTCCACCGCTTTGGCAGGCAGCACAGGGTCTGACCAACCTGTCA
CATTTCTTGATAAAATTGAACTTACCTTGTGTGTAACTGAGAAGGGCAATTTTCAA
GCCTCTTTGGAAAGTGGGGCTTTTGAGTTTATTGAGTGCCATGGGTTATCTCTT
TAACTCTCAGAACAACCCTACAAAGTATCTACATTCTTCTGACAAAAAGAAAAC
AAAAAACTATTATTAGCCCCATGGCTCCCAGGCAAAGTTCCCGCAGCATGAAAACAT
GTTGA**CGAGGATCCGGAGACAATACTTTGGAACTGCTGCCGTGAAAAGGCGTAAG**
GAGCCTAGAAAGCGGGCACAGTGTCCTGGGCGCCGAGTTAACTGCAGCG
GTCAGCGCAGGGGTGGAGAGTGGAGCCGCACACCCCGCGTCCGCCTGCTGTGGGCG
GCTGACCGCCAGCCCCCGCCCACGGCCAGGGTCCAGCCACACGGGGCGCTCGT
GTCCTGACAGCGGCTTCAGGCCAGCGGTGCTCTGCAGCCGACACGCCTCCAC
CAGCAGCGCCCCTCCTGCCCCTAAAGGCCTGTCCAGCCGCCCGCCCGCCGCCGCTC
CTCCGGAGCCTCGGCCGGTCCGAGCACGGACGGTCCGCTCCAGTCACCTCGC
CGGGGCTGCTCCGACCCCGTTCCGGGCCAGGACGAGGTAAGCTCTGAGCCCCCTTC
CGGACCCGAAAAACGGGGCCGCCGTCTGGCCACTCCGCCTCCCGCACACATGCAG
TAGGTGCTCCATTAATTCCCACTGAGCGCCAGCCGCCGCGCCCTCCGACCTCCCACC
CCAGCCGCCTCACCACTCACCCCGCCATCGACGCGGACCCGCTCCGGCAGTCGCCAC
CGGAAGTACCG–3’

Fig. 6. Complete nucleotide sequence of the *Nudt1* gene. Fragment of *Bos taurus* nudix (nucleoside diphosphate linked moiety X)-type motif 1 (Nudt1), transcript variant X3, mRNA (XM_002703156.3) encompassing a 2047-base pair region from base 438 to 1316. The analysed gene fragment is written in bold and CpG sites are underlined

Rys. 6. Pełna sekwencja nukleotydowa promotora genu *Nudt1*. Fragment motywu 1 typu nudiks (ang. *nucleoside diphosphate linked moiety X*) (Nudt1) dla *Bos taurus*, wariant X3 transkryptu, mRNA (XM_002703156.3), obejmujący region 2047 pz od nukleotydu 438 do 1316. Analizowany fragment genu pogrubiono, a miejsca CpG podkreślono

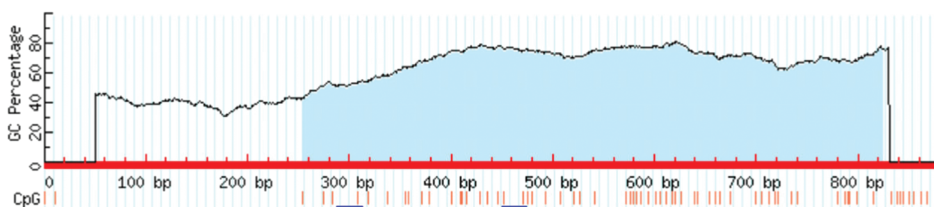

Fig. 7. Prediction of the CpG island occurrence in the *Nudt1* gene promoter using program Methprimer (Li and Dahiya, 2002). One CpG island was found in a 879-base pair sequence, encompassing 571 bp (254–824). The analysed fragment (276–572 bp) is situated within the CpG island

Rys. 7. Przewidywanie wysp CpG w promotorze genu *Nudt1* z zastosowaniem programu MethPrimer (Li and Dahiya, 2002). Wykryto jedną wyspę CpG obejmującą 571 pz (254–824) w 879-nukleotydowej sekwencji. Analizowany fragment (276–572 pz) jest lokalizowany w obrębie wyspy CpG

Table 5. Sequences of primers used for amplification of the *Apex1* and *Nudt1* genes promoters by PCR and methylation-specific PCR (MSP)

Tabela 5. Sekwencje starterów wykorzystanych do amplifikacji promotorów genów *Apex1* i *Nudt1* w reakcji PCR i PCR specyficznej dla metylacji (MS-PCR)

Method Metoda	Primer Starter	Primer sequence Sekwencja startera		Amplicon (bp) Amplikon (pz)
PCR	APEX I	F	GCCAACCAGAACAACAGCTA	173
		R	AGCAGAGGAACGGATAGGAA	
PCR	NUDT I	F	CCTAGAAAGCGGGCACAGT	237
		R	CTGGACAGGCCTTTAGGG	
MSP	APEX1M/2M	F	CGGTTTTAATTTTTATCGTTACGAC	100
		R	AATTTAAAAAACACACGCCGA	
MSP	APEX1U	F	TGGTTTTAATTTTTATTGTTATGATGA	101
		R	AAATTTAAAAAACACACACCAAC	
MSP	APEX2U	F	TGGTTTTAATTTTTATTGTTATGATGA	100
		R	AATTTAAAAAACACACACCAAC	
MSP	NUDT1M	F	GATAATATTTTGGAATTGTTGTCGT	187
		R	CCGTATAACTAAACCCTAACCGTA	
MSP	NUDT1U	F	GATAATATTTTGGAATTGTTGTTGT	188
		R	CCCATATAACTAAACCCTAACCATA	
MSP	NUDT 2M	F	GGAGATAATATTTTGGAATTGTTGTC	185
		R	TAACTAAACCCTAACCGTAAACGAA	
MSP	NUDT 2U	F	GATAATATTTTGGAATTGTTGTTGT	182
		R	TAACTAAACCCTAACCATAAACAAA	

F – forward, R – reverse.

was performed under the following conditions: initial denaturation at 94°C for 5 min, 35 cycles of DNA amplification (denaturation at 94°C for 45 s, annealing of primers at a 46–66°C temperature gradient for 90 s, and synthesis at 72°C for 100 s), final synthesis at 72°C for 5 min. The results were verified by electrophoresis in a 2% agarose gel with ethidium bromide (0.5 μg/ml) in TBE buffer, pH 8.5, at 100V for 20 min. 5 μl of

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the product were added to 5 μl of loading solution (0.025 g of bromophenol blue, 4 g of sucrose, distilled water to a volume of 10 ml).

All DNA samples were subjected to sodium bisulfite conversion followed by two parallel PCR reactions with primers encompassing sequences within which at least two CpG islands occurred. In the first reaction, the primers specific for unchanged cytosine within the CpG islands were used and so the fragments in which the cytosine within the CpG island was methylated were amplified. The primers used in the second reaction were specific for sequences within which all the cytosines were changed to thymines.

As described in the literature on sodium bisulfite DNA treatment methods, hydrolysing DNA with restriction enzymes to shortest possible fragments as an initial step, increases the efficiency of bisufite conversion as shorter DNA fragments are more susceptible to denaturation into a single-stranded structure, which determines the success of bisulfite conversion. The analysis of sites recognized by restriction enzymes within the analysed fragment using the Restriction Mapper software demonstrated that the sequence of *Apex1* gene is recognized by three restriction enzymes: *Apa*I, *Xba*I and *Pvu*I. These enzymes were used for DNA hydrolysis prior to bisulfite treatment, however, the best results were obtained using the *Apa*I enzyme (Broszkiewicz, 2009).

The analysis was performed once whenever a PCR product allowing to determine the methylation status of the amplified fragment was obtained in one reaction. The analysis was repeated if no PCR product was obtained or determining the methylation status was not possible. For some DNA samples, no PCR products at all or no products of the expected sizes were obtained after two reactions.

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3.5. DNA oxidation analysis

The analysis of DNA oxidation in the two groups of animals, was carried out by high-pressure liquid chromatography (HPLC) of DNA hydrolysed to single nucleotides. To this effect, 25 µg DNA dissolved in 100 µl of 0.01 M sodium acetate (pH 4.5) were incubated with 10 µl of micrococcal nuclease (1 U/µl) for two hours at 37°C. Then, 10 µl of 0.1 M Tris (pH 7.5) and 5 µl of alkaline phosphatase (1 U/µl) were added and the samples were incubated at 37°C for 1 h. Afterwards the ratio of 8-hydroxydeoxyguanosine (8-oxo2dG) to non-oxidased form of 2-deoxyguanosine (2dG) was determined using HPLC system with a Gynkotek autosampler and the Hypersil BDSTMC18 column with a pre-column (Thermo Hypersil). 8-oxo2dG and 2dG were separated on a 4.6 × 150 mm column. The mobile phase contained the phosphate buffer (pH 5.0) and 20% methanol at a 35:65 ratio. The flow rate was 1 ml/min. Quantities of 8-oxo2dG and 2dG eluted from the column were determined using the Coul Array electrochemical system and the obtained data were analysed using the Coul Array software for Windows (ESA Inc.). The retention time of 2dG was 6.7 min when the measurements were taken at 800 mV and for 8-oxo2dG it was 9 min at 400 mV.

3.6. DNA fingerprinting by *Alu*-PCR

The *Alu*-profiles of DNA were determined by *Alu*-PCR using the Cy5-AGC GAGACTCCG primer. Each PCR reaction was performed in 20 µl containing 1 µl DNA, 1 µM primer, dNTPs (200 µmols each), and 2 µl 1× buffer Taq (50 mM KCl, 10 mM Tris-HCl, pH 8.9, 1.5 mM $MgCl_2$), and one unit of the Taq polymerase which was added following the initial 7-minute denaturation at 94°C. Each sample was amplified in duplicate. The amplification conditions were as follows: initial denaturation for 7 min at 94°C, 27 amplification cycles (denaturation: 94°C for 30 s, annealing: 50°C for 45 s, synthesis: 72°C for 2 min), and final synthesis for 7 min at 72°C. The PCR products were then separated in a 5% non-denaturing polyacrylamide gel for 10 h at 1500 V using the ALFExpress system. The appearance of additional DNA fragments, disappearing of the existing fragments, or changes in the band intensity were considered as indicative of changes in *Alu*-PCR DNA profiles.

3.7. Statistical analyses

Statistical analyses were performed using the Microsoft Office Excel and Statistica 10.0 (StatSoft Inc., 2011). The results for qualitative scale variables were presented as crude numbers (N) and percentages. For quantitative variables, the following basic descriptive statistics parameters were calculated: arithmetic mean, median,

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standard deviation, and minimum and maximum values. The normal distribution of quantitative variables was verified by the Shapiro-Wilk test.

As the assumptions for using parametric methods to verify hypotheses were not met (lack of normal distribution for almost all variables), the following non-parametric methods were used: the Mann-Whitney U test, the chi-square test with the Yate's correction for prevention of overestimation of statistical significance for small data, and the Spearman's rank correlation test, with the assumed significance level (α) of 0.05. *P*-values below 0.05 were considered as indicative of a statistical significance.

4. Results

4.1. Animal characteristics

Beef cattle breeding have a relatively short history in Poland due to a lack of tradition in this field in our country. However, political changes that took place in 1989 resulted in considerable changes in some feeding habits leading to, among others, a significant increase of demand for culinary beef in the general population and as a consequence to the appearance of breeders who became interested in this type of cattle production. Still, as this is relatively new in our country, it is important to support Polish breeders when they try to compete with farmers from Western Europe who have a long tradition and more experience of such a production.

The analyses presented in this dissertation were carried out on material obtained from the Skidniów farm (the experimental group) and from the farm in Sława (control group). The animals living in the Skidniów farm were exposed to a wide range of environmental factors associated with proximity to smelter emissions. In contrast, the control animals were bred in a pollution-free zone. Over 50% of the Sława commune is forested and due to its exceptional natural values, and a zone of protected landscape was here established, especially in view of the fact that there is very little industry in this area. The feeding systems employed on the two farms were identical. Main roughages were obtained from the fields and meadows belonging to the farms, while concentrates were manufactured in the farms' feed mixing plants. On both farms, animals were kept in typical drive-through cow-houses and paddocks, with feeding and water supply provided outdoor.

The biological material for the analyses was peripheral blood collected from 50 animals from the experimental group and 50 animals from the control group. An interesting subgroup comprised the progeny of Jaśmin bull (i.e. half-sisters), including 20 individuals in the experimental group and 15 animals in the control group (Table 6). Animals from both the experimental and the control group remained on their respective farms for at least six months. No phenotypic changes were observed in animals from the control group, whereas the progeny of animals from the experimental group exhibited some developmental abnormalities which manifested as vision defects (complete blindness of all newborn calves), low body weight gains and visible changes in the skeleton (rickets).

Methylation levels of DNA isolated from animal leukocytes were measured using a two-dimensional thin layer chromatography (TLC). The obtained autoradiographs were analysed with a Typhoon 8600 radioactivity scanner, which allowed a quantitative

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Table 6. Basic characteristics, cytosine methylation and oxidation levels of the DNA from leukocytes of animals from the experimental and control groups

Tabela 6. Podstawowa charakterystyka, poziom metylacji cytozyny i oksydacji DNA leukocytów zwierząt z grupy eksperymentalnej i grupy kontrolnej

Number Numerr	Age (days) Wiek (dni)	Father Ojciec	Mother Matka	Global methylation Metylacja całkowita	Global oxidation Oksydacja całkowita	*Apex1* Specific methylation Metylacja specyficzna	*Nudt1* Specific methylation Metylacja specyficzna
1	2	3	4	5	6	7	8
Control animals – Zwierzęta kontrolne							
1	396	Jaśmin/LMS	50LMS-50CB	0.70	10.4	M	ND
2	395	Sten/LMS	50LMS-50CB	0.65	22.9	ND	M
3	233	Jaśmin/LMS	50LMS-50CB	1.00	20.8	M	ND
4	213	Jonasz/LMS	LMS	1.20	7.1	M	M
5	275	Dialog/LMS	LMS	0.70	111.3	M	ND
6	326	Jaśmin/LMS	50LMS-50CB	0.65	19.3	M	ND
7	394	Satyr/LMS	LMS-50CB	0.85	45.2	NM	ND
8	514	Jaśmin/LMS	50CB-50SIM	0.90	41.2	ND	NM
9	186	Jonasz/LMS	LMS	1.10	125.4	ND	ND
10	393	Sven/LMS	50CB-50LMS	0.90	237.1	M	NM
11	283	Jaśmin/LMS	50CB-50SIM	0.75	17.1	ND	NM
12	195	Insolet/LMS	LMS	0.95	5.3	NM	M
13	457	John/LMS	50LMS-50CB	1.25	52.6	ND	NM

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Table 6 cont. – Tabela 6 cd.

1	2	3	4	5	6	7	8
14	220	Jonasz/LMS	LMS	1.40	82.8	M	ND
15	280	Jaśmin/LMS	50LMS-50CB	1.55	11.4	M	M
16	389	Jaśmin/LMS	50LMS-50CB	0.95	5.3	NM	ND
17	284	Jaśmin/LMS	50SIM-50CB	0.85	8.3	M	M
18	208	Jonasz/LMS	LMS	1.90	22.4	ND	ND
19	456	Star/LMS	50LMS-50CB	1.20	22.6	M	ND
20	556	Goliat/LMS	50CH-50CB	2.10	7.5	ND	ND
21	397	Jaśmin/LMS	50SIM-50CB	1.55	4.1	M	NM
22	154	Dialog/LMS	LMS	0.80	31.4	ND	ND
23	524	Jonasz/LMS	LMS	2.00	34.8	M	ND
24	266	Jonasz/LMS	LMS	0.70	55.2	M	M
25	642	Goliat/LMS	50SIM-50CB	0.70	20.0	M	M
26	465	Dialog/LMS	LMS	1.80	27.0	M	NM
27	316	Ilion/LMS	LMS	1.15	29.5	M	ND
28	233	Insolet/LMS	LMS	1.25	19.3	M	ND
29	213	Jonasz/LMS	LMS	0.85	11.2	M	M
30	505	Jaśmin/LMS	50SIM-50CB	1.25	17.9	M	ND
31	233	Jonasz/LMS	LMS	1.80	16.3	M	M

Table 6 cont. – Tabela 6 cd.

1	2	3	4	5	6	7	8
32	659	Jonasz/LMS	LMS	0.75	134.6	M	ND
33	275	Dialog/LMS	LMS	0.80	5.0	M	ND
34	297	Jonasz/LMS	LMS	0.75	13.7	ND	NM
35	287	Jonasz/LMS	LMS	0.55	13.6	ND	NM
36	250	Dialog/LMS	LMS	0.60	10.0	NM	NM
37	287	Jaśmin/LMS	50LMS-50CB	0.70	12.6	M	M
38	179	Insolet/LMS	LMS	1.00	2.5	M	NM
39	550	Fredo/LMS	LMS	0.65	26.1	NM	ND
40	516	Jaśmin/LMS	50LMS-50CB	1.25	50.2	M	M
41	395	Jaśmin/LMS	50LMS-50CB	2.40	943.6	M	NM
42	555	Goliat/LMS	CB	1.50	1 397.4	M	NM
43	563	Goliat/LMS	50LMS-50CB	0.95	170.6	NM	NM
44	553	Goliat/LMS	CB	1.10	405.3	M	ND
45	555	Goliat/LMS	CB	1.20	430.5	NM	ND
46	555	Goliat/LMS	50LMS-50CB	1.30	706.1	ND	ND
47	487	Jaśmin/LMS	50LMS-50CB	0.95	34.4	NM	M
48	556	Goliat/LMS	50LMS-50CB	1.15	34.2	M	ND
49	487	Goliat/LMS	50LMS-50CB	2.50	604.8	M	NM
50	504	Jaśmin/LMS	50LMS-50CB	1.75	45.5	NM	ND

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Table 6 cont. – Tabela 6 cd.

1	2	3	4	5	6	7	8
Experimental animals – Zwierzęta badane							
51	597	Jaśmin/LMS	50LMS-50CB	0.40	71.3	NM	M
52	719	Fort/LMS	50LMS-50CB	0.25	263.3	M	NM
53	627	Jaśmin/LMS	50LMS-50CB	0.45	41.8	M	NM
54	845	Jaśmin/LMS	50LMS-50CB	0.45	46.4	M	ND
55	652	Jaśmin/LMS	50LMS-50CB	0.50	52.2	M	ND
56	599	Jaśmin/LMS	50LMS-50CB	0.15	72.5	M	M
57	645	Jaśmin/LMS	50LMS-50CB	0.70	323.9	ND	ND
58	858	Ilion/LMS	50CB-50CB	0.25	52.7	M	ND
59	755	Jaśmin/LMS	50LMS-50CB	0.85	37.8	M	ND
60	592	Jaśmin/LMS	50CB-50CB	0.65	62.2	M	ND
61	594	Jaśmin/LMS	50CB-50CB	0.30	415.4	M	ND
62	650	Jaśmin/LMS	50CB-50CB	1.10	ND	ND	ND
63	460	Jaśmin/LMS	50LMS-50CB	0.90	ND	ND	ND
64	460	Jaśmin/LMS	50LMS-50CB	1.15	ND	ND	ND
65	596	Jaśmin/LMS	50LMS-50CB	0.35	66.4	M	ND
66	596	Jaśmin/LMS	50LMS-50CB	0.25	ND	NM	ND
67	502	Ilion/LMS	50LMS-50CB	0.40	139.3	ND	ND

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Table 6 cont. – Tabela 6 cd.

1	2	3	4	5	6	7	8
68	499	Jaśmin/LMS	50LMS-50CB	0.70	323.9	M	ND
69	594	Jaśmin/LMS	50CH-50CB	0.65	53.7	M	NM
70	395	Jasny/LMS	50LMS-50CB	1.10	133.0	M	ND
71	497	Ilion/LMS	50LMS-50CB	0.55	18.8	ND	ND
72	597	Jaśmin/LMS	50LMS-50CB	0.90	239.4	ND	M
73	505	Ilion/LMS	50LMS-50CB	0.85	ND	ND	ND
74	390	Jasny/LMS	50LMS-50CB	0.75	1 830.0	NM	ND
75	393	Jasny/LMS	75LMS-25CB	0.60	173.4	ND	ND
76	495	Ilion/LMS	50LMS-50CB	0.75	20.3	M	ND
77	501	Ilion/LMS	50LMS-50CB	0.90	308.9	M	M
78	505	Jasny/LMS	50LMS-50CB	0.95	4 392.8	NM	ND
79	386	Jasny/LMS	50LMS-50CB	0.90	82.9	ND	ND
80	335	Jasny/LMS	50LMS-50CB	0.65	35.0	ND	ND
81	598	Jaśmin/LMS	50sim-50CB	1.20	888.9	M	M
82	262	Jasny/LMS	50LMS-50CB	1.25	2 596.5	NM	ND
83	264	Jasny/LMS	50LMS-50CB	1.00	2 088.2	ND	ND
84	290	Hubert/LMS	CB	0.85	206.1	ND	NM
85	264	Jasny/LMS	50LMS-50CB	0.70	358.0	ND	ND
86	262	Jasny/LMS	50LMS-50CB	0.50	468.4	ND	ND

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Table 6 cont. – Tabela 6 cd.

1	2	3	4	5	6	7	8
87	331	Jasny/LMS	50LMS-50CB	0.40	51.6	M	ND
88	337	Jasny/LMS	50LMS-50CB	0.30	349.4	NM	NM
89	265	Jaśmin/LMS	50LMS-50CB	0.35	93.5	NM	NM
90	331	Jasny/LMS	CB	0.25	415.0	NM	ND
91	262	Jasny/LMS	50LMS-50CB	0.40	460.0	M	NM
92	262	Jasny/LMS	50CH-50CB	0.70	90.0	NM	M
93	597	Jasny/LMS	75SIM-25CB	0.65	41.8	NM	NM
94	262	Jaśmin/LMS	50LMS-50CB	0.70	107.0	M	ND
95	524	Ilion/LMS	CB	0.55	148.9	ND	ND
96	305	Hubert/LMS	CB	0.70	367.3	ND	ND
97	509	Hubert/LMS	CB	0.50	126.2	ND	NM
98	2423	Dalos/LMS	CB	0.45	383.9	ND	NM
99	1767	Sten/LMS	CB	0.70	28.5	M	ND
100	865	Jonasz/LMS	LMS	0.40	186.9	ND	ND

Methylation values are expressed in per cent using the *R* coefficient as a ratio of the 5'-methylcytosine (5mC) to the global content of 5'-methylcytosine (5mC), cytosine (C) and thymine (T) (5mC / (5mC + C + T) × 100). Global oxidation values are expressed as ratio of 8-hydroxydeoxyguanosine (8-oxo2dG) to non-oxidased form of 2-deoxyguanosine (2dG) (8-oxo2dG/dG×10^{-5}). LMS – Limousin, CB – Black and White, CH – Charolais, SIM – Simmental, M – methylated, NM – not methylated, ND – not determined.

Poziom metylacji określono w procentach, w postaci współczynnika *R*, jako stosunek zawartości 5'-metylocytozyny (5mC) do całkowitej zawartości 5'-metylocytozyny (5mC), cytozyny (C) i tyminy (T). Poziom oksydacji określono jako stosunek 8-hydroksydeoksyguanozyny (8-oxo2dG) do nieutlenionej formy 2-deoksyguanozyny (2dG) (8-oxo2dG / dG × 10^{-5}). LMS – limousine, CB – czarno-biała, CH – charolaise, SIM – simental, M – metylowany, NM – niemetylowany, ND – nie określono.

analysis. The content of 5'-methylcytosine resulting from methylation was determined in relation to the total amount of 5'-methylcytosine, cytosine and thymine. Representative results are presented in Figure 8.

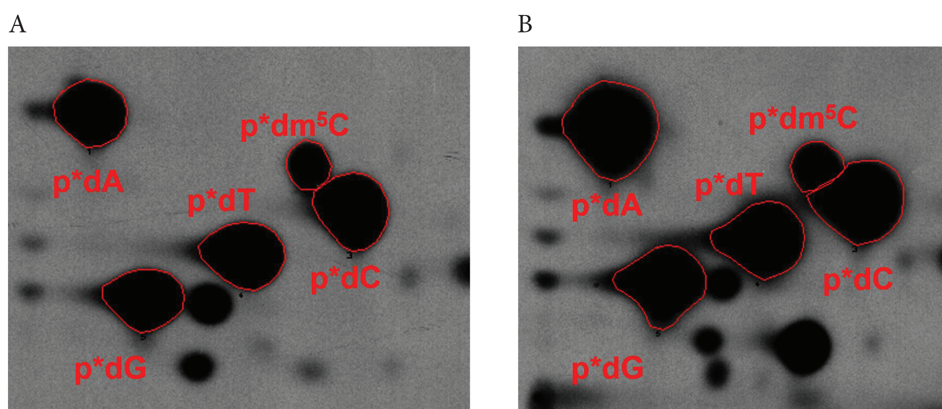

Fig. 8. Evaluation of DNA methylation levels in subject no. 89 in the experimental group descending from bull Jaśmin (A) and no. 8 from the control group descending from bull Jaśmin (B). TLC plates were dried and inserted into cassettes equipped with radioactivity sensitive screens for 2 h. The screens were scanned on a Typhoon 8600 scanner with a resolution of 100 microns using manufacturer's software. Quantitative analysis was performed using the ImageQuant software. Cytosine methylation level was estimated as 0.35% (A) and 0.9% (B) 5 mC / (5 mC + C + T), respectively

Rys. 8. Ocena poziomu metylacji DNA u osobnika 89 z grupy badanej po buhaju Jaśminie (A) i u osobnika 8 z grupy kontrolnej po buhaju Jaśminie (B). Płytki TLC suszono i umieszczano w kastetach z wrażliwym na radioaktywność ekranem przez 2 h. Ekrany skanowano na skanerze Typhoon 8600 z rozdzielczością 100 mikronów z wykorzystaniem oprogramowania producenta. Ilościową analizę przeprowadzono z zastosowaniem oprogramowania ImageQuant. Poziom metylacji cytozyny określono na poziomie 0,35% (A) i odpowiednio 0,9% (B) 5 mC / (5 mC + C + T)

Most animals were born in 1997 or 1998 year, and blood samples were collected in 1998 or 1999. The average age of animals was 556.38 days for the experimental group and 382.22 days for controls. The differences in age between the experimental and the control group were found to be statistically significant as shown by the Mann-Whitney U test ($Z = -3.38$; P-value = 0.0007). The age median values and arithmetic averages were significantly higher for the experimental group (Table 7). Similarly, Jaśmin progeny was older than other animals in both groups ($Z = 3.58$; P-value = 0.00034).

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Table 7. Age distribution in experimental and control animals
Tabela 7. Rozkład wieku u zwierząt badanych i kontrolnych

Animals Zwierzęta	*M*	Me	Min	Max	SD	*Z*	*P*-value
Experimental Badane *N* = 50	556.38	503.50	262	2 423	364.41	–3.38	0.0007
Control Kontrolne *N* = 50	382.22	393.50	154	659	141.04		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *Z* – value of the Mann-Whitney U test statistics (for the size of both groups *N* > 20), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *Z* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup *N* > 20), *N* – liczba zwierząt.

4.2. Specific methylation of the promoters of *Apex1* and *Nudt1* genes

Examples of the results obtained by a PCR amplification of sodium bisulfite converted DNA are shown in Figure 9. I noticed that the reaction efficiency was lower when template DNA having been subjected to the sodium bisulfite conversion was used. A clear band of about 173 bp was obtained, which corresponds to the expected product size. It was accompanied by a non-specific product of less than 100 bp. When using the first set of primers to analyse methylation, a product of the expected size (100 bp) was obtained only in the reaction with primers specific for methylated sequences in the two replications, whereas reaction with primers specific for unmethylated DNA yielded no product in all replicates, which confirms that the analysed fragment is methylated. In a reaction with the second set of primers, the obtained product did not correspond to the expected size of about 100 bp, which makes the methylation status of the fragment impossible to determine.

Interpretable results were obtained for the methylation analysis in 68 subjects for the *Apex1* gene promoter and in 42 subjects for *Nudt1* gene promoter, with the complete analysis (methylation status determined for both genes) successfully completed for 33 animals. Table 8 summarizes the test results.

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Table 8. Methylation status of promoter regions of the *Apex1* and *Nudt1* genes
Tabela 8. Status metylacji regionów promotorowych genów *Apex1* i *Nudt1*

ID	Control animals – Zwierzęta kontrolne		ID	Experimental animals – Zwierzęta badane	
	Apex1	*Nudt1*		*Apex1*	*Nudt1*
1	M	ND	51	M	M
2	ND	M	52	M	M
3	M	ND	53	M	M
4	M	M	54	M	ND
5	M	ND	55	M	ND
6	M	ND	56	M	M
7	NM	ND	57	ND	ND
8	ND	NM	58	M	ND
9	ND	ND	59	M	ND
10	M	NM	60	M	ND
11	ND	NM	61	M	ND
12	NM	M	62	ND	ND
13	ND	NM	63	ND	ND
14	M	ND	64	ND	ND
15	M	M	65	M	ND
16	NM	ND	66	M	ND
17	M	M	67	ND	ND
18	ND	ND	68	M	ND
19	M	ND	69	M	M
20	ND	ND	70	M	ND
21	M	NM	71	ND	ND
22	ND	ND	72	ND	M
23	M	ND	73	ND	ND
24	M	M	74	M	ND
25	M	M	75	ND	ND
26	M	NM	76	M	ND
27	M	ND	77	M	M

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Table 8 cont. – Tabela 8 cd.

ID	Control animals – Zwierzęta kontrolne		ID	Experimental animals – Zwierzęta badane	
	Apex1	*Nudt1*		*Apex1*	*Nudt1*
28	M	ND	78	M	ND
29	M	M	79	ND	ND
30	M	ND	80	ND	ND
31	M	M	81	M	M
32	M	ND	82	M	ND
33	M	ND	83	ND	ND
34	ND	NM	84	ND	M
35	ND	NM	85	ND	ND
36	NM	NM	86	ND	ND
37	M	M	87	M	ND
38	M	NM	88	M	M
39	NM	ND	89	M	M
40	M	M	90	M	ND
41	M	NM	91	M	M
42	M	NM	92	M	M
43	NM	NM	93	M	M
44	M	ND	94	M	ND
45	NM	ND	95	ND	ND
46	ND	ND	96	ND	ND
47	NM	M	97	ND	M
48	M	ND	98	ND	M
49	M	NM	99	M	ND
50	NM	ND	100	ND	ND

M – methylated, NM – not methylated, ND – not determined.

M – metylowany, NM – niemetylowany, ND – nie określono.

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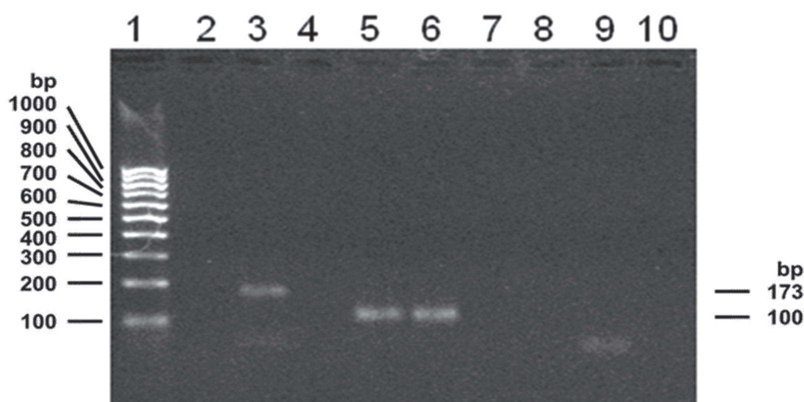

Fig. 9. An example of an electrophoretic separation of PCR products from the second MS-PCR reaction for *Apex1* gene promoter. PCR reaction was performed on DNA hydrolysed with *Apa*I enzyme and converted with sodium bisulfite. Lanes: 1 – 100 bp PCR Low Ladder, 2 – negative control (no DNA), 3 and 4 – primer APEX1 specific for not converted DNA, 5 and 6 – primer APEX1M specific for methylated DNA (product size: 100 bp), 7 and 8 – primer APEX1U specific for unmethylated DNA, 9 – primer APEX2M specific for methylated DNA, 10 – primer APEX2U specific for unmethylated DNA

Rys. 9. Przykładowy rozdział elektroforetyczny produktów PCR drugiej reakcji MS-PCR dla promotora genu *Apex1*. Reakcję PCR prowadzono na DNA hydrolizowanym enzymem *Apa*I i poddanym konwersji wodorosiarczynem sodu. Tory: 1 – marker wielkości 100 bp PCR *Low Ladder*, 2 – kontrola negatywna (bez DNA), 3 i 4 – startery APEX1 specyficzne dla DNA niepoddanego konwersji, 5 i 6 – startery APEX1M specyficzne dla metylowanego DNA (wielkość produktu 100 pz), 7 i 8 – startery APEX1U specyficzne dla niemetylowanego DNA, 9 – startery APEX2M specyficzne dla metylowanego DNA, 10 – startery APEX2U specyficzne dla niemetylowanego DNA

4.3. Global methylation and oxidation

The Shapiro-Wilk test showed statistically significant deviations from the normal distribution for almost all analysed variables, like age, global methylation and global oxidation, for all animals together and separately for the experimental group and the control group. Therefore, the data were further analysed using the non-parametric Mann-Whitney U test and non-parametric (Spearman) correlation. The results are shown in Figure 10 and Table 9.

A comparison of the global methylation levels between the experimental group and controls showed a statistically significant difference ($Z = 5.76$; P-value = 0.0000). The control group was characterised by a higher median and arithmetic mean of global DNA methylation. A comparison of the global oxidation levels of the two groups also showed a statistically significant difference ($Z = -5.07$; P-value = 0.0000), with the me-

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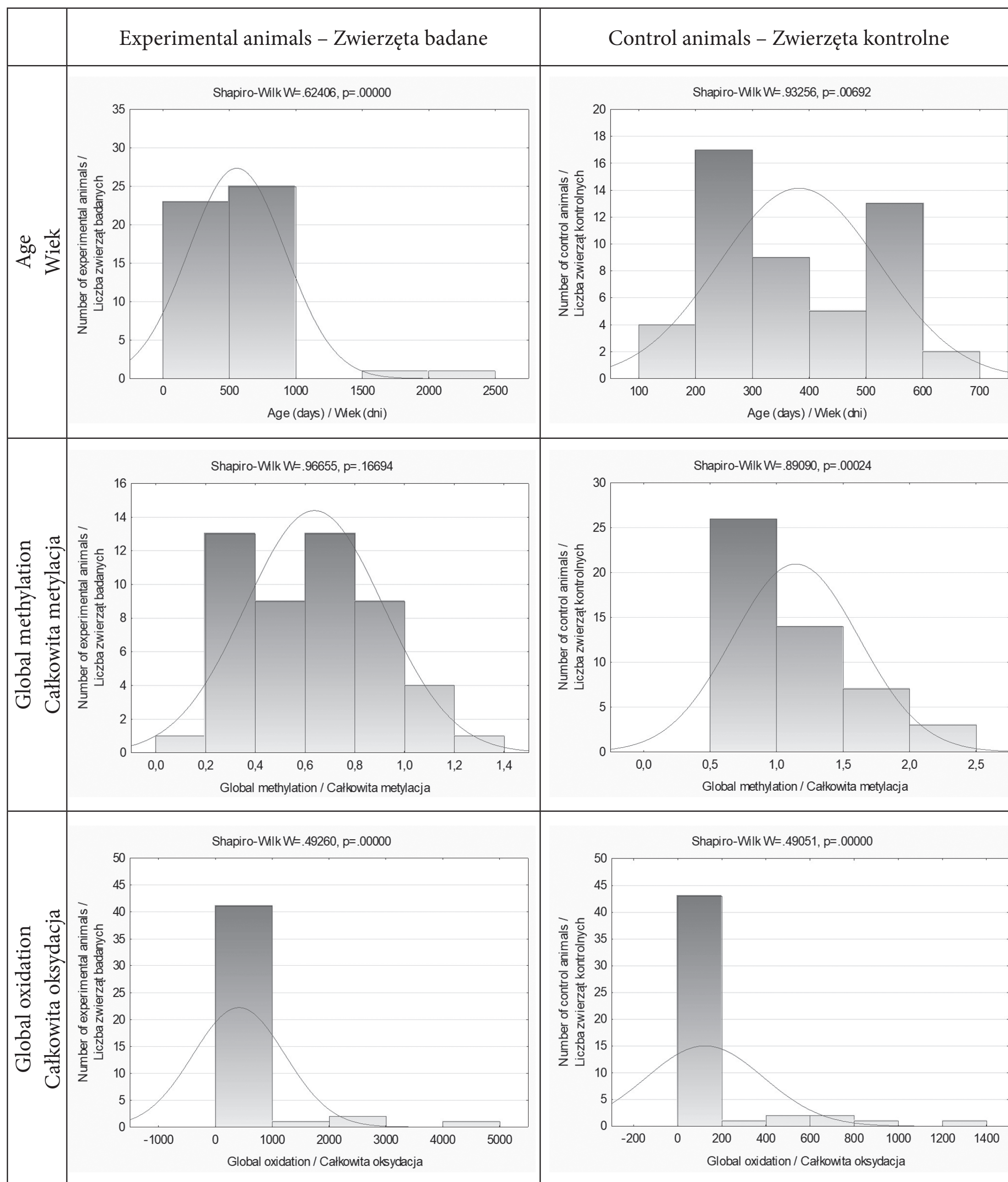

Fig. 10. The analysis of age, global methylation and global oxidation distribution in all animals and in the analysed groups. The expected normal distribution is marked with the line

Rys. 10. Analiza rozkładu wieku, całkowitej metylacji i całkowitej oksydacji u wszystkich zwierząt i w analizowanych grupach. Oczekiwany rozkład normalny zaznaczono linią

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dian and arithmetic mean values of global DNA oxidation being higher for the experimental group (Table 10 and Fig. 11).

Table 9. The analysis of age, global methylation and global oxidation distribution in all animals and in the analysed groups

Tabela 9. Analiza rozkładu wieku, całkowitej metylacji i całkowitej oksydacji u wszystkich zwierząt i w analizowanych grupach

Variable Zmienna	All animals Wszystkie zwierzęta		Experimental animals Zwierzęta badane		Control animals Zwierzęta kontrolne	
	W	*P*-value	*W*	*P*-value	*W*	*P*-value
Age Wiek	0.65	0.0000	0.62	0.0000	0.93	0.0069
Global methylation Całkowita metylacja	0.91	0.0000	0.97	0.1669	0.89	0.0002
Global oxidation Całkowita oksydacja	0.44	0.0000	0.49	0.0000	0.49	0.0000

W – the Shapiro-Wilk test's statistics.

W – wartość statystyki testu Shapiro-Wilka.

Table 10. A comparison of global methylation and oxidation distribution in the experimental and control animals

Tabela 10. Porównanie rozkładu całkowitej metylacji i oksydacji u zwierząt badanych i kontrolnych

Animals – Zwierzęta	*M*	Me	Min	Max	SD	*Z*	*P*-value
Global methylation – Całkowita metylacja							
Experimental Badane	0.64	0.65	0.15	1.25	0.28	5.76	0.0000
Control Kontrolne	1.15	1.0	0.55	2.50	0.48		
Global oxidation – Całkowita oksydacja							
Experimental Badane	415.85	139.30	18.80	4 392.80	808.39	–5.07	0.0000
Control Kontrolne	123.67	24.50	2.50	1 397.40	265.62		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *Z* – the Mann-Whitney U test statistics (for the size of both groups $N > 20$), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *Z* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup $N > 20$), *N* – liczba zwierząt.

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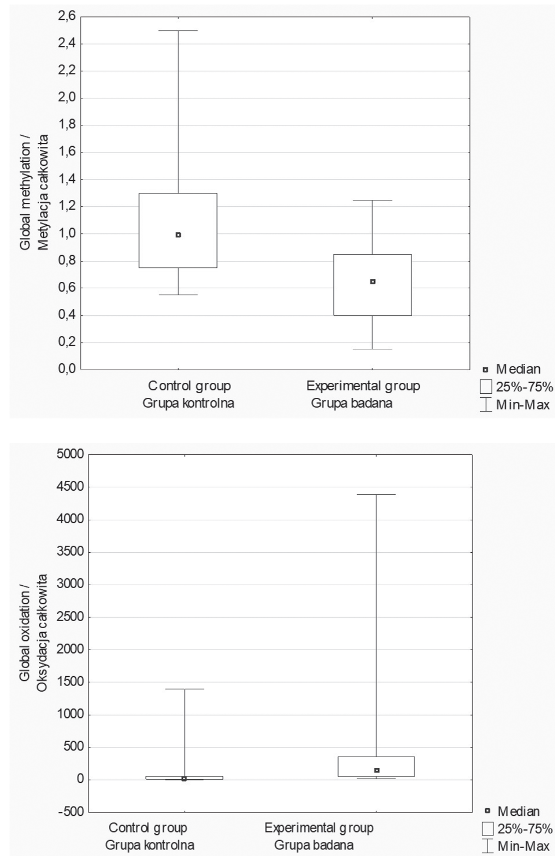

Fig. 11. A comparison of global methylation (up) and oxidation (down) distribution in the control and experimental animals

Rys. 11. Porównanie rozkładu całkowitej metylacji (góra) i oksydacji (dół) u zwierząt kontrolnych i badanych

A comparison of the frequency of methylation of the *Apex1* and *Nudt1* gene promoters between the two groups showed no statistically significant differences (*P*-value = 0.2544), even though the percentage of cattle with methylation of the *Apex1* gene was slightly higher in the control group. The frequency of the *Nudt1* gene promoter methylation did not significantly differ between the experimental and control animals (*P*-value = 0.8186; Table 11).

Both global methylation and global oxidation were significantly associated with age in all animals (Table 12). Age and global methylation correlated negatively but the association was weak (*R* = –0.23; *P*-value = 0.0201). For age and global oxidation, the association strength was similarly low but the correlation was positive (*R* = 0.25; *P*-value = 0.0135; Fig. 12).

Table 11. Methylation status of the *Apex1* and *Nudt1* gene promoters in the experimental and control group

Tabela 11. Status metylacji promotorów genów *Apex1* i *Nudt1* w grupie badanej i kontrolnej

Methylation Metylacja	*Apex1*				*Nudt1*			
	experimental animals zwierzęta badane		control animals zwierzęta kontrolne		experimental animals zwierzęta badane		control animals zwierzęta kontrolne	
	N = 30		*N* = 38		*N* = 16		*N* = 26	
	N	%	*N*	%	*N*	%	*N*	%
Unmethylated Brak metylacji	10	33.3	8	21.1	10	62.5	14	53.8
Methylated Metylacja	20	66.7	30	78.9	6	37.5	12	46.2
Chi-square test Test Chi-kwadrat	χ^2 = 1.3, df = 1, *P*-value = 0.2544				χ^2 = 0.05, df = 1, *P*-value = 0.8186			

N – number of animals, df – degrees of freedom.

N – liczba zwierząt, df – liczba stopni swobody.

Table 12. A correlation analysis of age and global methylation, and age and global oxidation in all animals

Tabela 12. Analiza korelacji między wiekiem zwierząt a całkowitą metylacją oraz wiekiem a całkowitą oksydacją u wszystkich zwierząt

Animals – Zwierzęta	Age (days) and global methylation Wiek (dni) i całkowita metylacja		Age (days) and global oxidation Wiek (dni) i całkowita oksydacja	
	R Spearman's rank	*P*-value	*R* Spearman's rank	*P*-value
All Wszystkie	–0.23	0.0201	0.25	0.0135
Experimental Badane	–0.22	0.1237	–0.35	0.0189
Control Kontrolne	0.16	0.2734	0.44	0.0014

When considering control animals only, no correlation was found between age and global methylation levels (*R* = 0.16; *P*-value = 0.2734) and significant moderately strong positive correlation between age and global oxidation appeared (*R* = 0.44; *P*-value = = 0.0014). Global oxidation increased with the age of animals. In the experimental group, I found no correlation between age and global methylation (*P*-value = 0.1237) and a statistically significant moderately strong negative association between age and global oxidation (*P*-value = 0.0189, Fig. 12).

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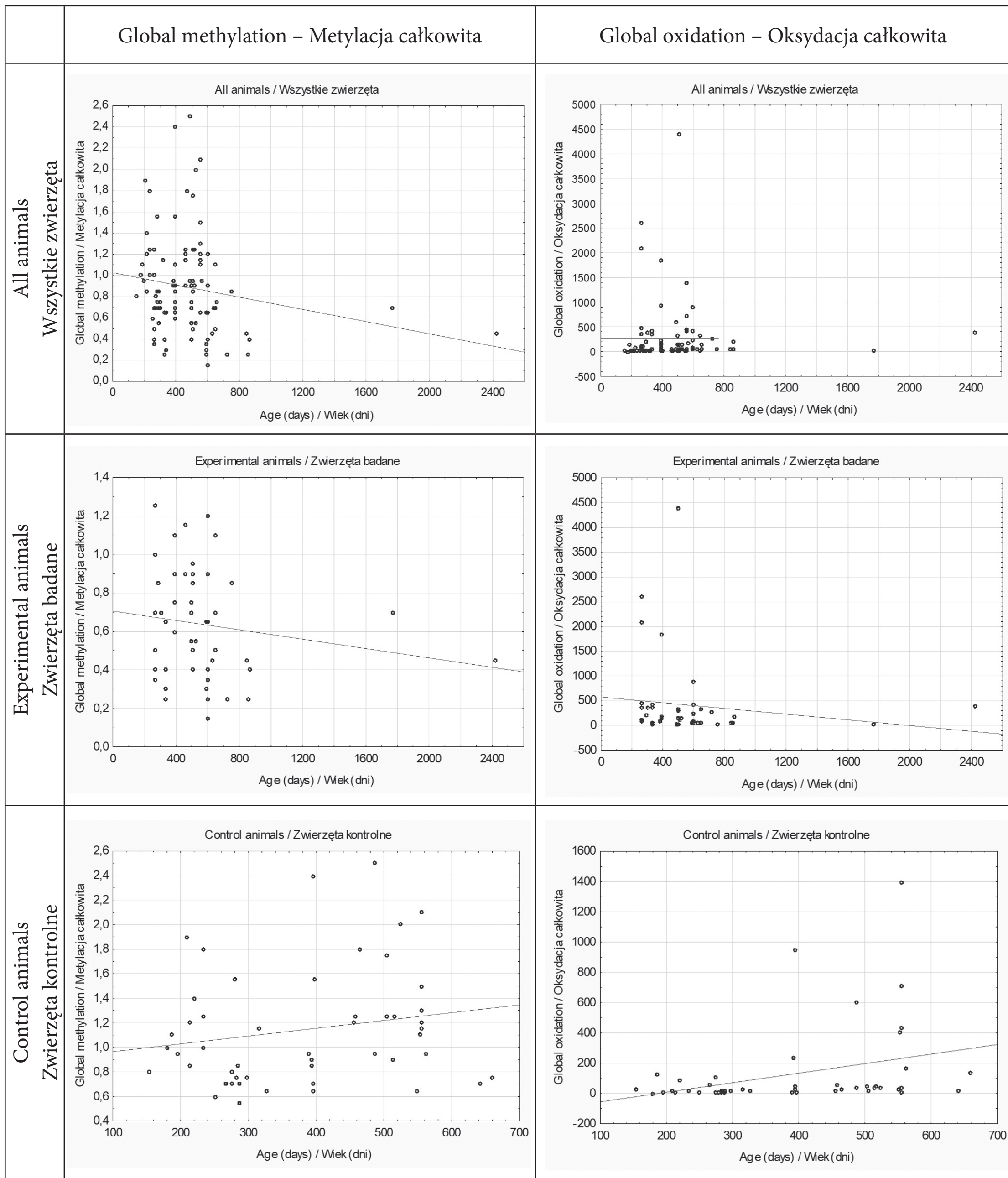

Fig. 12. A graphical distribution of methylation (left) and oxidation levels (right) in comparison with age for all animals, the control animals and the experimental animals

Rys. 12. Graficzny rozkład poziomu metylacji (z lewej) i oksydacji (z prawej) w porównaniu z wiekiem dla wszystkich zwierząt, zwierząt kontrolnych i zwierząt eksperymentalnych.

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Table 13. A correlation analysis between global methylation and global oxidation levels
Tabela 13. Analiza korelacji między poziomem całkowitej metylacji i całkowitej oksydacji

Animals – Zwierzęta	Global methylation and global oxidation Całkowita metylacja i całkowita oksydacja	
	R Spearman's rank	*P*-value
All – Wszystkie	–0.15	0.1593
Experimental – Badane	0.19	0.2033
Control – Kontrolne	0.23	0.1052

As a next step, a correlation analysis between global methylation and global oxidation for all animals, control animals and experimental animals was performed but no correlation was found (Table 13).

4.4. Analysis of Jaśmin progeny

Then, the analysis of methylation and oxidation levels in the progeny of bull Jaśmin was performed in both groups, comprising 35 daughters of Jaśmin bull. There was no difference between global methylation or oxidation levels in the progeny of Jaśmin bull as compared to other animals (*P*-value = 0.5583 and 0.1672, respectively (Table 14).

Table 14. Analysis of global methylation and global oxidation level in the Jaśmin bull progeny
Tabela 14. Analiza poziomu całkowitej metylacji i całkowitej oksydacji u potomstwa buhaja Jaśmina

Animals – Zwierzęta	*M*	Me	Min	Max	SD	*Z*	*P*-value
Global methylation – Całkowita metylacja							
Jaśmin progeny – Potomstwo Jaśmina *N* = 35	0.85	0.75	0.15	2.4	0.47	–0.58	0.5583
Other animals – Pozostałe zwierzęta *N* = 65	0.91	0.8	0.25	2.5	0.47		
Global oxidation – Całkowita oksydacja							
Jaśmin progeny – Potomstwo Jaśmina *N* = 31	133.5	46.4	4.1	943.6	232.9	–0.38	0.1672
Other animals – Pozostałe zwierzęta *N* = 64	324.4	82.85	2.5	4 392.8	711.0		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *Z* – the Mann-Whitney U test statistics (for the size of both groups *N* > 20), *N* – number of animals.
M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *Z* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup *N* > 20), *N* – liczba zwierząt.

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4.5. Analysis of Jasny progeny

The experimental group included 16 daughters of Jasny. The difference between global methylation levels in these animals as compared to those in other animals from the experimental group was borderline significant (U = 478.5; P-value = 0.0691). However, global oxidation levels were significantly higher among the Jasny bull progeny than in other cattle (U = 253.5; P-value = 0.0002; Table 15).

Table 15. Global methylation and global oxidation level in the Jasny bull progeny
Tabela 15. Analiza poziomu całkowitej metylacji i całkowitej oksydacji u potomstwa buhaja Jasnego

Animals – Zwierzęta	*M*	Me	Min	Max	SD	*U*	*P*-value
Global methylation – Całkowita metylacja							
Jasny progeny Potomstwo Jasnego N = 16	0.6938	0.675	0.25	1.25	0.289	478.5	0.0691
Other animals Pozostałe zwierzęta N = 84	0.9292	0.85	0.15	2.5	0.4822		
Global oxidation – Całkowita oksydacja							
Jasny progeny Potomstwo Jasnego N = 16	847.9	353.7	35	4 392.8	1242.8	253.5	0.0002
Other animals Pozostałe zwierzęta N = 79	143.4	45.5	2.5	1 397.4	239.2		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups $N < 20$), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup $N < 20$), *N* – liczba zwierząt.

4.6. Specific methylation in Jaśmin and Jasny progeny

Afterwards, an analysis of the methylation status of the *Apex1* and *Nudt1* promoters for Jaśmin progeny compared to other animals was performed. The differences between the two groups were not statistically significant for any of these two genes (P-value = 0.4304 for *Apex1* promoter and 0.2915 for *Nudt1*; Table 16).

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Table 16. A comparison of methylation of the *Apex1* and *Nudt1* gene promoters in the Jaśmin bull progeny and other animals

Tabela 16. Porównanie metylacji promotorów genów *Apex1* i *Nudt1* u potomstwa buhaja Jaśmina i u pozostałych zwierząt

Variable – Zmienna	Jaśmin progeny Potomstwo Jaśmina		Other animals Pozostałe zwierzęta	
	N	%	*N*	%
Apex1 methylation – Metylacja *Apex1*				
Unmethylated – Brak metylacji	6	21.4	12	30.0
Methylated – Metylacja	22	78.6	28	70.0
Total – Razem	28	100.0	40	100.0
Chi-square test – Test Chi-kwadrat	χ^2 = 0.6, df = 1, *p* = 0.4304			
Nudt1 methylation – Metylacja *Nudt1*				
Unmethylated – Brak metylacji	7	43.8	17	65.4
Methylated – Metylacja	9	56.3	9	34.6
Total – Razem	16	100.0	26	100.0
Chi-square test – Test Chi-kwadrat	χ^2 = 1.1, df = 1, *p* = 0.2915			

N – number of animals, df – degrees of freedom.

N – liczba zwierząt, df – liczba stopni swobody.

Specific methylation of the *Apex1* gene promoter was significantly lower for the Jasny bull progeny compared to other animals (*P*-value = 0.0028). For the methylation of the *Nudt1* gene promoter, no statistically significant differences between the Jasny progeny and other animals were found. All results are shown in Table 17.

Table 17. A comparison of methylation of the *Apex1* and *Nudt1* gene promoters in the Jasny bull progeny and other animals

Tabela 17. Porównanie metylacji promotorów genów *Apex1* i *Nudt1* u potomstwa buhaja Jasnego i u pozostałych zwierząt

Variable – Zmienna	Jasny progeny Potomstwo Jasnego		Other animals Pozostałe zwierzęta	
	N	%	*N*	%
Apex1 methylation – Metylacja *Apex1*				
Unmethylated – Brak metylacji	7	70.0	11	19.0
Methylated – Metylacja	3	30.0	47	81.0
Total – Razem	10	100.0	58	100.0
Chi-square test – Test Chi-kwadrat	χ^2 = 8.9, df = 1, *p* = 0.0028			

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Table 17 cont. – Tabela 17 cd.

Variable – Zmienna	Jasny progeny Potomstwo Jasnego		Other animals Pozostałe zwierzęta	
	N	%	*N*	%
Nudt1 methylation – Metylacja *Nudt1*				
Unmethylated – Brak metylacji	3	75.0	21	55.3
Methylated – Metylacja	1	25.0	17	44.7
Total – Razem	4	100.0	38	100.0
Chi-square test – Test Chi-kwadrat	$\chi^2 = 0.1$, df = 1, $p = 0.8199$			

N – number of animals, df – degrees of freedom.

N – liczba zwierząt, df – liczba stopni swobody.

4.7. Comparison of specific methylation with global methylation and oxidation

The *Apex1* gene promoter methylation and global methylation were not significantly associated (*P*-value = 0.1937 for all animals, 0.8772 for the experimental animals and 0.2374 for control animals; Table 18).

Table 18. A comparison of methylation of the *Apex1* gene promoter and global methylation levels in all animals, experimental animals and control animals

Tabela 18. Porównanie metylacji promotora genu *Apex1* i poziomu całkowitej metylacji u wszystkich zwierząt, zwierząt badanych oraz zwierząt kontrolnych

Variable – Zmienna	M	Me	Min	Max	SD	*U*	*P*-value
All animals – Wszystkie zwierzęta							
Apex1 methylated Metylacja *Apex1* *N* = 50	0.96	0.85	0.15	2.5	0.52	356	0.1937
Apex1 unmethylated Brak metylacji *Apex1* *N* = 18	0.77	0.725	0.25	1.75	0.40		
Experimental animals – Zwierzęta badane							
Apex1 methylated Metylacja *Apex1* *N* = 20	0.59	0.58	0.15	1.2	0.29	96	0.8772
Apex1 unmethylated Brak metylacji *Apex1* *N* = 10	0.59	0.53	0.25	1.25	0.34		

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Table 18 cont. – Tabela 18 cd.

Variable – Zmienna	M	Me	Min	Max	SD	*U*	*P*-value
Control animals – Zwierzęta kontrolne							
Apex1 methylated Metylacja *Apex1* *N* = 30	1.21	1.15	0.65	2.5	0.50	86.5	0.2374
Apex1 unmethylated Brak metylacji *Apex1* *N* = 8	1.00	0.95	0.6	1.75	0.36		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups *N* < 20), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup *N* < 20), *N* – liczba zwierząt.

There was no statistically significant correlation between the methylation of the *Apex1* gene and global oxidation for all animals (*P*-value = 0.1710) but in the experimental animals statistically significant correlation between methylation of the *Apex1* gene and global oxidation was shown (U = 47.5, *P*-value = 0.0436) and higher oxidation levels were observed in cattle with unmethylated *Apex1* gene. In controls, *Apex1* methylation did not correlate with global oxidation (*P*-value = 0.9580; Table 19).

Table 19. A comparison of the *Apex1* gene promoter methylation and global oxidation levels in all animals, experimental animals and control animals

Tabela 19. Porównanie metylacji promotora genu *Apex1* i poziomu całkowitej oksydacji u wszystkich zwierząt, zwierząt eksperymentalnych oraz kontrolnych

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
All animals – Wszystkie zwierzęta							
Apex1 methylated Metylacja *Apex1* *N* = 50	156.86	48.3	2.5	1 397.4	276.77	329.5	0.1710
Apex1 unmethylated Brak metylacji *Apex1* *N* = 17	624.00	90	5.3	4 392.8	1 206.87		
Experimental animals – Zwierzęta badane							
Apex1 methylated Metylacja *Apex1* *N* = 20	174.33	64.3	20.3	888.9	217.21	47.5	0.0436
Apex1 unmethylated Brak metylacji *Apex1* *N* = 9	1 097.81	349.4	41.8	4 392.8	1 535.36		

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Table 19 cont. – Tabela 19 cd.

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
Control animals – Zwierzęta kontrolne							
Apex1 methylated Metylacja *Apex1* *N* = 30	145.22	21.7	2.5	1 397.4	313.32	118	0.9580
Apex1 unmethylated Brak metylacji *Apex1* *N* = 8	90.96	30.25	5.3	430.5	147.52		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups $N < 20$), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup $N < 20$), *N* – liczba zwierząt.

There was no correlation between the *Nudt1* gene methylation and global methylation for all animals analysed together (*P*-value = 0.4846) or any of the two groups of animals (*P*-value = 0.1806 for the experimental animals and 0.4025 for controls; Table 20).

Table 20. A comparison of the *Nudt1* gene promoter methylation and global methylation levels in all animals, control and experimental animals

Tabela 20. Porównanie metylacji promotora genu *Nudt1* i poziomu całkowitej metylacji u wszystkich zwierząt, zwierząt kontrolnych oraz eksperymentalnych

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
All animals – Wszystkie zwierzęta							
Nudt1 methylated Metylacja *Nudt1* *N* = 18	0.91	0.88	0.15	1.8	0.39	188	0.4846
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 24	0.93	0.75	0.25	2.5	0.62		
Experimental animals – Zwierzęta badane							
Nudt1 methylated Metylacja *Nudt1* *N* = 6	0.71	0.80	0.15	1.20	0.38	17.5	0.1806
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 10	0.49	0.45	0.25	0.85	0.18		

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Table 20 cont. – Tabela 20 cd.

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
Control animals – Zwierzęta kontrolne							
Nudt1 methylated Metylacja *Nudt1* *N* = 12	1.01	0.90	0.65	1.80	0.37	67.5	0.4025
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 14	1.24	0.98	0.55	2.50	0.63		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups *N* < 20), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup *N* < 20), *N* – liczba zwierząt.

Similarly, *Nudt1* methylation did not correlate with global oxidation as shown by the Mann-Whitney U test (*P*-value for all animals = 0.1180, 0.8749 for the experimental animals and 0.1598 for controls; Table 21).

Table 21. A comparison of the *Nudt1* gene promoter methylation and global oxidation level in all animals, experimental and control animals

Tabela 21. Porównanie metylacji promotora genu *Nudt1* i poziomu całkowitej oksydacji u wszystkich zwierząt, zwierząt eksperymentalnych oraz kontrolnych

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
All animals – Wszystkie zwierzęta							
Nudt1 methylated Metylowany *Nudt1* *N* = 18	106.99	28.65	5.30	888.90	211.96	154	0.1180
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 24	231.46	73.60	2.50	1 397.40	338.69		
Experimental animals – Zwierzęta badane							
Nudt1 methylated Metylacja *Nudt1* *N* = 6	278.50	164.70	71.30	888.90	314.94	28	0.8749
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 10	201.97	166.15	41.80	460.00	154.92		

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Table 21 cont. – Tabela 21 cd.

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
Control animals – Zwierzęta kontrolne							
Nudt1 methylated Metylacja *Nudt1* *N* = 12	21.24	14.45	5.30	55.20	16.75	56	0.1598
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 14	252.52	34.10	2.50	1 397.40	430.34		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups *N* < 20), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup *N* < 20), *N* – liczba zwierząt.

Next, a possible association between methylation of the *Apex1* gene promoter and age of animals was analysed. When analysing all animals together, I found no statistically significant correlations between these two parameters (*P*-value = 0.5408). However, cattle with methylation of the *Apex1* gene turned out to be older than the cattle with unmethylated *Apex1* and this result was statistically significant (*U* = 52, *P*-value = = 0.0350). In control animals alone, I found no statistically significant differences in age with the *Apex1* methylation status (*P*-value = 0.3684; Table 22).

Table 22. Methylation of the *Apex1* gene promoter in association with age of animals
Tabela 22. Asocjacja metylacji promotora genu *Apex1* z wiekiem zwierząt

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
All animals – Wszystkie zwierzęta							
Apex1 methylated Metylacja *Apex1* *N* = 50	477.46	476.00	179.00	1 767.00	255.95	405.5	0.5408
Apex1 unmethylated Brak metylacji *Apex1* *N* = 18	424.17	438.50	195.00	597.00	140.97		
Experimental animals – Zwierzęta badane							
Apex1 methylated Metylacja *Apex1* *N* = 20	627.05	595.00	262.00	1 767.00	315.35	52	0.0350
Apex1 unmethylated Brak metylacji *Apex1* *N* = 10	414.20	363.50	262.00	597.00	145.37		

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Table 22 cont. – Tabela 22 cd.

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
Control animals – Zwierzęta kontrolne							
Apex1 methylated Metylacja *Apex1* *N* = 30	377.73	359.50	179.00	659.00	140.77	94	0.3684
Apex1 unmethylated Brak metylacji *Apex1* *N* = 8	436.63	495.50	195.00	563.00	144.12		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups $N < 20$), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup $N < 20$), *N* – liczba zwierząt.

The *Nudt1* gene promoter methylation was not associated with age (*P*-value = = 0.4688 for all animals, 0.8749 for the experimental animals and 0.1759 for control animals; Table 23).

Table 23. Correlation of the *Nudt1* gene promoter methylation with age of animals
Tabela 23. Korelacja metylacji promotora genu *Nudt1* z wiekiem zwierząt

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
All animals – Wszystkie zwierzęta							
Nudt1 methylated Metylacja *Nudt1* *N* = 18	398.06	341.00	195.00	642.00	165.15	187	0.4688
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 24	506.04	427.00	179.00	2 423.00	432.62		
Experimental animals – Zwierzęta badane							
Nudt1 methylated Metylacja *Nudt1* *N* = 6	525.67	597.00	262.00	599.00	134.84	28.5	0.8749
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 10	662.30	551.50	262.00	2 423.00	641.13		

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Table 23 cont. – Tabela 23 cd.

Variable – Zmienna	*M*	Me	Min	Max	SD	*U*	*P*-value
Control animals – Zwierzęta kontrolne							
Nudt1 methylated Metylacja *Nudt1* *N* = 12	334.25	282.00	195.00	642.00	143.39	57.5	0.1759
Nudt1 unmethylated Brak metylacji *Nudt1* *N* = 14	394.43	396.00	179.00	563.00	119.84		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups $N < 20$), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup $N < 20$), *N* – liczba zwierząt.

Methylation of the *Apex1* gene promoter did not correlate with the methylation of the *Nudt1* promoter ($\chi^2 = 0.0$; *P*-value = 1; Table 24).

Table 24. A comparison of the *Apex1* and *Nudt1* gene promoter methylation in the analysed groups

Tabela 24. Porównanie występowania metylacji promotorów genów *Apex1* i *Nudt1* w analizowanych grupach

Apex1 methylation Metylacja *Apex1*	*Nudt1* methylation – Metylacja *Nudt1*			
	unmethylated – brak metylacji		methylated – metylacja	
	N = 16		*N* = 16	
	N	%	*N*	%
Unmethylated – Brak metylacji	5	31.3	4	25.0
Methylated – Metylacja	11	68.8	12	75.0
Chi-square test – Test Chi-kwadrat	$\chi^2 = 0.0$, df = 1, *P*-value = 1			

N – number of animals, df – degrees of freedom.

N – liczba zwierząt, df – liczba stopni swobody.

4.8. Comparison of specific methylation with global methylation and oxidation in Jaśmin progeny

When analysing the Jaśmin progeny, I found statistically significant difference in global methylation between experimental and control animals ($U = 49.5$; P-value = 0.0008), with higher values for controls. Global oxidation levels in the Jaśmin progeny were significantly higher in animals from the experimental group ($U = 22$; P-value = 0.0001; Table 25). A comparison of global methylation and global oxidation levels between Jaśmin progeny from the experimental and control group is shown in Figure 13.

Table 25. A comparison of global methylation and global oxidation levels in the Jaśmin bull progeny between experimental and control animals

Tabela 25. Porównanie całkowitego poziomu metylacji i oksydacji u potomstwa buhaja Jaśmina między zwierzętami eksperymentalnymi a zwierzętami kontrolnymi

Animals – Zwierzęta	*M*	Me	Min	Max	SD	*U*	*P*-value
Global methylation – Całkowita metylacja							
Experimental Badane *N* = 20	0.64	0.65	0.15	1.20	0.31	49.5	0.0008
Control Kontrolne *N* = 15	1.15	0.95	0.65	2.40	0.49		
Global oxidation – Całkowita oksydacja							
Experimental Badane *N* = 16	181.02	71.90	37.80	888.90	224.40	22	0.0001
Control Kontrolne *N* = 15	82.81	17.90	4.10	943.60	238.59		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups $N < 20$), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup $N < 20$), *N* – liczba zwierząt.

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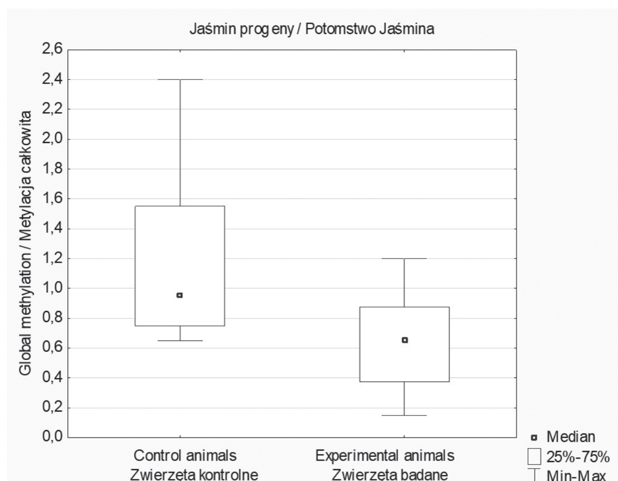


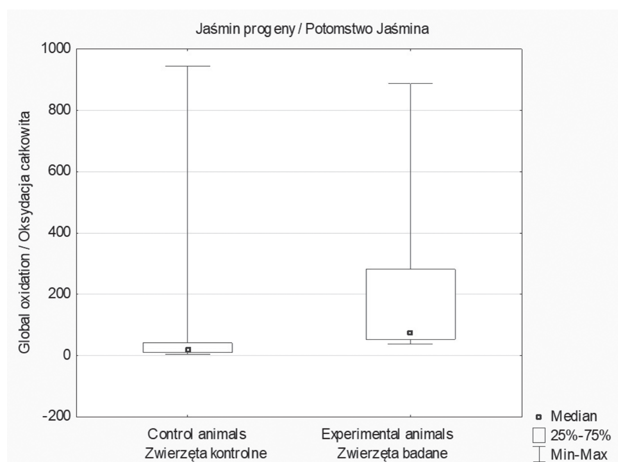


Fig. 13. A comparison of global methylation and global oxidation levels in the Jaśmin bull progeny between control and experimental animals

Rys. 13. Porównanie całkowitego poziomu metylacji i oksydacji u potomstwa buhaja Jaśmina między zwierzętami kontrolnymi a eksperymentalnymi

There was no difference in global methylation levels between Jaśmin progeny and other animals in the experimental group (U = 289.5, P-value = 0.8430) or in controls (U = 260, P-value = 0.9662; Table 26 and Figure 14).

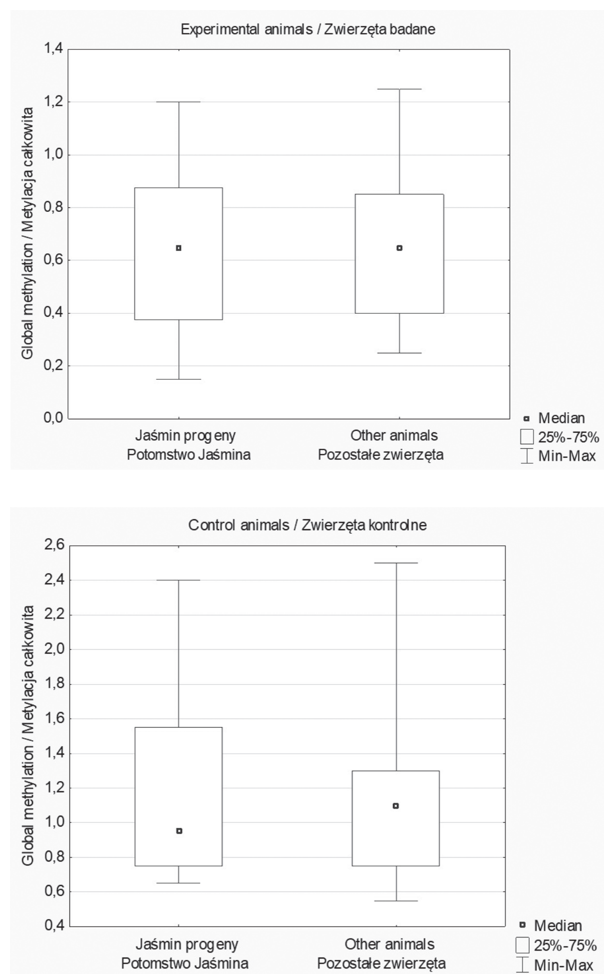
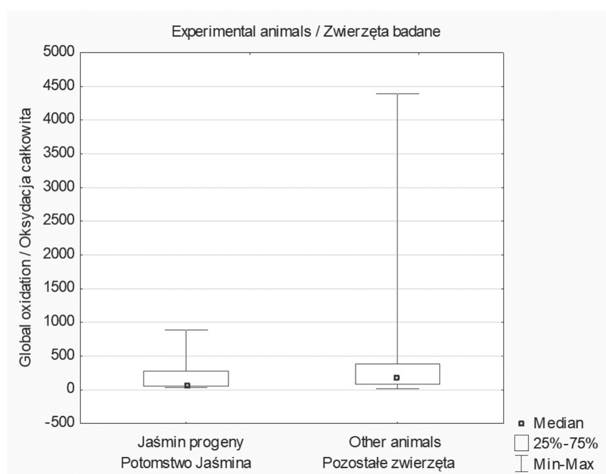


Fig. 14. A comparison of global methylation levels between the Jaśmin bull progeny and other animals in the experimental animals and in control animals

Rys. 14. Porównanie całkowitego poziomu metylacji między potomstwem buhaja Jaśmina a pozostałymi zwierzętami u zwierząt eksperymentalnych i kontrolnych



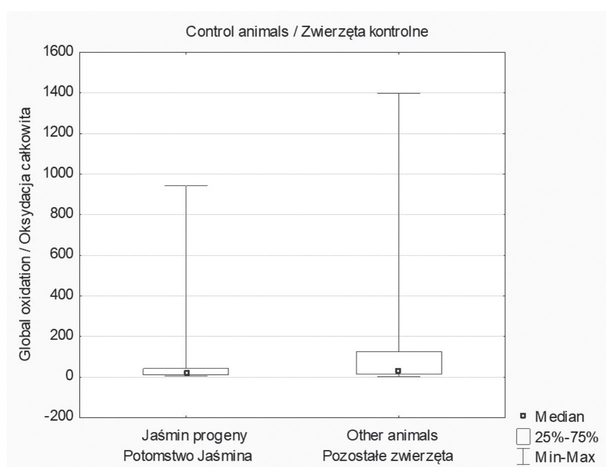


Fig. 15. A comparison of global oxidation levels between Jaśmin bull progeny and other animals in experimental and control animals

Rys. 15. Porównanie całkowitego poziomu oksydacji między potomstwem buhaja Jaśmina a pozostałymi zwierzętami u zwierząt eksperymentalnych i kontrolnych

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Table 26. Distribution of global methylation levels in Jaśmin progeny and other animals from the experimental and control group

Tabela 26. Analiza rozkładu poziomu metylacji między potomstwem Jaśmina a pozostałymi zwierzętami z grupy eksperymentalnej i z grupy kontrolnej

Animals – Zwierzęta	*M*	Me	Min	Max	SD	*U*	*P*-value
Experimental animals – Zwierzęta badane							
Jaśmin progeny Potomstwo Jaśmina *N* = 20	0.64	0.65	0.15	1.20	0.31	289.5	0.8430
Other animals Pozostałe zwierzęta *N* = 30	0.64	0.65	0.25	1.25	0.26		
Control animals – Zwierzęta kontrolne							
Jaśmin progeny Potomstwo Jaśmina *N* = 15	1.15	0.95	0.65	2.40	0.49	260	0.9662
Other animals Pozostałe zwierzęta *N* = 35	1.14	1.10	0.55	2.50	0.48		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups $N < 20$), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup $N < 20$), *N* – liczba zwierząt.

There was no difference in global oxidation levels between Jaśmin progeny and other animals in the experimental group ($U = 172.5$, *P*-value = 0.1618) or in control animals ($U = 190$, *P*-value = 0.1274; Table 27 and Figure 15).

Table 27. Distribution of global oxidation levels between Jaśmin progeny and other animals from the experimental and control group

Tabela 27. Analiza rozkładu poziomu oksydacji między potomstwem Jaśmina a pozostałymi zwierzętami z grupy eksperymentalnej i z grupy kontrolnej

Animals – Zwierzęta	*M*	Me	Min	Max	SD	*U*	*P*-value
Experimental animals – Zwierzęta badane							
Jaśmin progeny Potomstwo Jaśmina *N* = 16	181.02	71.90	37.80	888.90	224.40	172.5	0.1618
Other animals Pozostałe zwierzęta *N* = 29	545.42	186.90	18.80	4 392.80	975.21		

Table 27 cont. – Tabela 27 cd.

Animals – Zwierzęta	*M*	Me	Min	Max	SD	*U*	*P*-value
Control animals – Zwierzęta kontrolne							
Jaśmin progeny Potomstwa Jaśmina *N* = 15	82.81	17.90	4.10	943.60	238.59	190	0.1274
Other animals Pozostałe zwierzęta *N* = 35	141.18	29.50	2.50	1 397.40	277.82		

M – arithmetic mean, Me – median, Min – minimal value, Max – maximal value, SD – standard deviation, *U* – the Mann-Whitney U test statistics (for the size of both groups $N < 20$), *N* – number of animals.

M – średnia arytmetyczna, Me – mediana, Min – wartość minimalna, Max – wartość maksymalna, SD – odchylenie standardowe, *U* – wartość statystyki testu U Manna-Whitneya (dla liczebności obu grup $N < 20$), *N* – liczba zwierząt.

Global methylation and global oxidation in Jaśmin progeny seemed to weakly correlate but the results did not reach statistical significance ($R = -0.32$; *P*-value = 0.0815). For other animals, global methylation and global oxidation clearly did not correlate ($R = -0.11$, *P*-value = 0.3750; Table 28 and Figure 16).

Table 28. A correlation analysis of global methylation and global oxidation for Jaśmin progeny and other animals

Tabela 28. Analiza korelacji między całkowitą metylacją a całkowitą oksydacją u potomstwa buhaja Jaśmina a pozostałymi zwierzętami

Animals – Zwierzęta	Global methylation and global oxidation Całkowita metylacja i całkowita oksydacja	
	R Spearman's rank	*P*-value
Jaśmin progeny Potomstwo Jaśmina	–0.32	0.0815
Other animals Pozostałe zwierzęta	–0.11	0.3750

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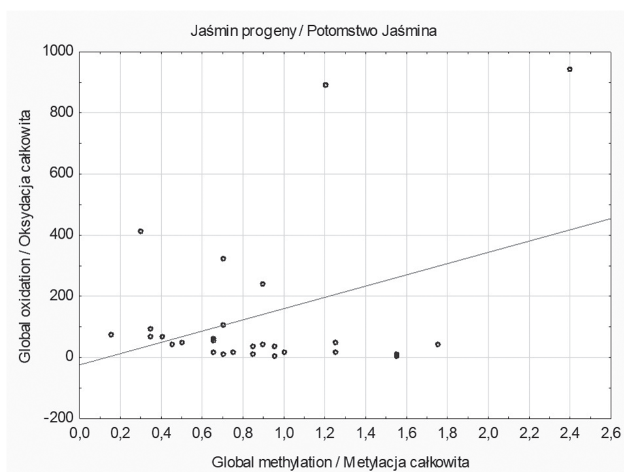

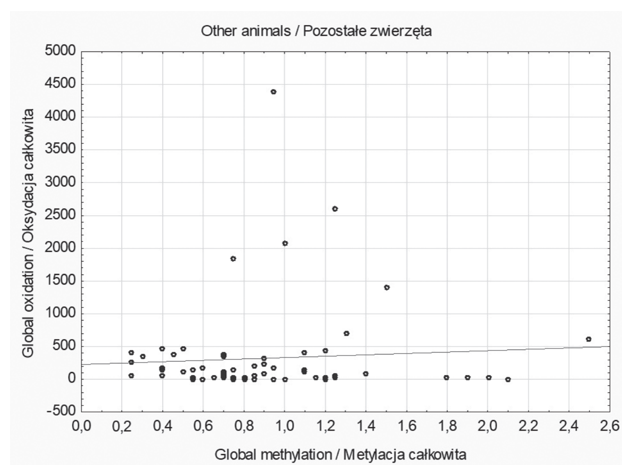

Fig. 16. A graphical analysis of a correlation between global methylation and global oxidation for Jaśmin progeny and other animals

Rys. 16. Graficzna analiza korelacji między całkowitą metylacją a całkowitą oksydacją u potomstwa buhaja Jaśmina a pozostałymi zwierzętami

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4.9. Analysis of *Alu* fragments

DNA analysis using the *Alu*-PCR method with the Cy5-AGCGAGACTCCG primer was performed for 50 animals from the experimental group and 50 controls. Each sample was amplified in duplicate. The results for all the analysed samples were almost identical but bands which would differentiate the two groups of animals could be identified. The distribution and height of the peaks among control animals was more similar as for experimental animals. Figure 17 presents three superimposed examples of the results from the experimental and control animals. Table 29 summarises the number of changes in the *Alu* fragments in cattle from the experimental and control groups. In the experimental group, out of 34 animals with changes in *Alu* profiles, 16 animals showed changes in two or more *Alu*-PCR fragments, whereas among controls only three animals had two or more changes and only one change was observed in 12 animals. The occurrence of changes in the *Alu* fragments patterns in experimental animals compared to the control group was highly significant (*P*-value = 0.0001).

Table 29. The *Alu*-PCR fragment changes in cattle reared in the Skidniów farm (experimental animals) which is situated in the vicinity of the Głogów Copper Smelters I in comparison with animals from the Sława farm (control animals)

Tabela 29. Określenie zmian fragmentów *Alu*-PCR u bydła hodowanego w gospodarstwie Skidniów (zwierzęta badane) w pobliżu Huty Miedzi Głogów I w porównaniu ze zwierzętami z gospodarstwa w Sławie (zwierzęta kontrolne)

Alu fragments Fragmenty *Alu*	Experimental animals Zwierzęta badane		Control animals Zwierzęta kontrolne	
	N	%	*N*	%
No changes in *Alu*-PCR fragments Brak zmian fragmentów *Alu*-PCR	16	32	35	70
One or more changes in *Alu*-PCR fragments Jedna lub więcej zmian fragmentów *Alu*-PCR	34	68	15	30
Razem – Total	50	100	50	100
Chi-square test Test Chi-kwadrat	χ^2 = 14.4, df = 1, *P*-value = 0.0001			

N – number of animals, df – degrees of freedom.

N – liczba zwierząt, df – liczba stopni swobody.

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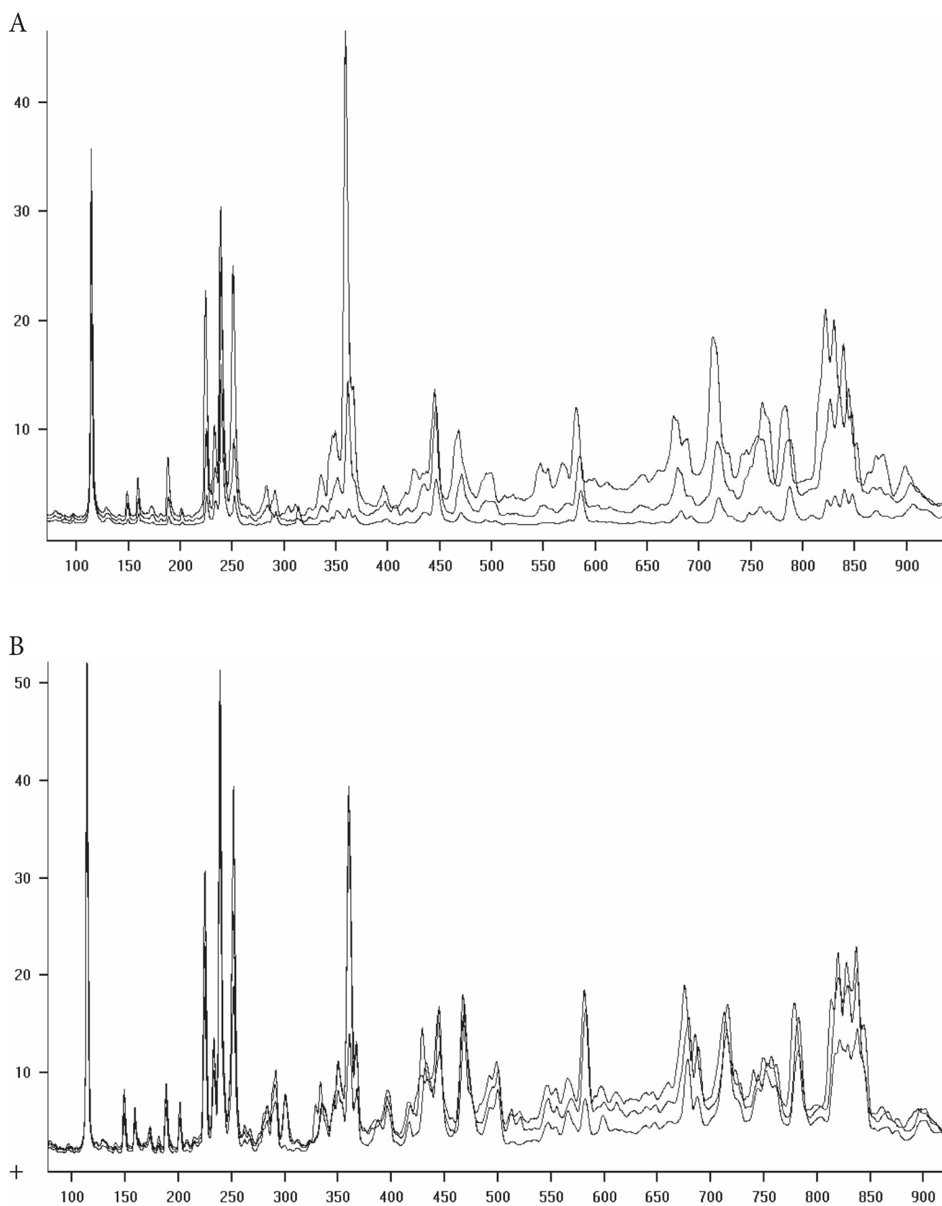

Fig. 17. Electrophoresis of *Alu*-PCR products in 5% non-denaturing polyacrylamide gel: A – superimposed electrophoregrams of *Alu*-PCR products for three experimental animals (ID 52, 59 and 60), B – superimposed electrophoregrams of *Alu*-PCR products for three control animals (25, 26 and 27)

Rys. 17. Rozdział elektroforetyczny produktów reakcji *Alu*-PCR w 5-procentowym niedenaturującym żelu poliakryloamidowym: A – nałożone na siebie elektroforegramy produktów reakcji *Alu*-PCR dla trzech zwierząt badanych (52, 59 i 60), B – nałożone na siebie elektroforegramy produktów reakcji *Alu*-PCR dla trzech zwierząt kontrolnych (25, 26 i 27)

5. Discussion

The establishment of copper industry in the Legnica-Lubin-Głogów region (part of KGHM Polish Copper) resulted in an intensive economic development which was, unfortunately, accompanied by soil degradation, increased discharge of sewage and deterioration of the aero-sanitary conditions. These phenomena also occurred in the neighbourhood of the Głogów Copper Smelters. The achieved production potentials were not accompanied by increased development of facilities necessary to protect the natural environment. On the contrary, the environment degradation was so extensive that it became necessary to establish a protection zone around the smelters with all the consequences, i.e. cessation of agricultural production, relocation of residents and farm liquidation.

Agricultural production in the regions with copper industry requires continuous monitoring of the environment, mainly for the presence of heavy metals (Pb, Cu, Zn) which are emitted by the mines, steel mills and tailing landfills. The term "heavy metals" is commonly used as a group name for metals and semimetals (metalloids) associated with contamination and potential toxicity or ecotoxicity. It is well reflected in legal regulations concerning heavy metals. However, there is no scientific chemical or toxicological data confirming that heavy metals and their compounds have highly toxic or ecotoxic properties (Duffus, 2002). Even the term "metal" is confusing in this respect as it means the pure metal and all the chemical species in which it may exist, which implies that the pure metal and all its compounds have the same physicochemical, biological, and toxicological properties. Such an assumption is of course not logical but a proper classification which would replace the confusing term "heavy metal" with some more appropriate categories is still unavailable (Duffus, 2002) and raises a lot of emotions and discussion in various publications. In this study, I have used the term "heavy metals" being fully aware of the controversy. The fact that my analyses were performed on DNA obtained from bovine peripheral blood in the absence of a direct parallel analysis of the particular pollutants in the environment and in animal bodies partially justifies this decision, especially that no specific factor that led to the occurrence of the observed differences in animal herds was indicated and my studies were conducted with an assumption of a negative impact of the pollution from the copper smelter.

Emissions from the Głogów Copper Smelters I and II considerably increased heavy metal levels, especially copper, in the soil. The accumulation of microelements in soil in quantities many times exceeding the levels found in areas not affected by industrial emissions exerts a significant impact on the development and yields of crop plants

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(Baluk et al., 1993). Heavy metals decompose in soil over a very long period of time, even up to several hundred years. Years back, in 1987, problems with pollution of agricultural lands by copper smelter were already noted (Greinert et al., 1987). Soils in the immediate vicinity of a copper smelter contained significant amounts of copper and lead. Because this area encompassed approximately 10,000 ha of rich soil, it was very important to protect it from pollution so that it can serve for agricultural purposes but this implied a strict necessity to reduce the smelter emissions. Some cases of poisoning or complete soil degradation connected with a substantial decrease or even lack of crops have already been noted. However, the greatest problem for agriculture was not just soil contamination with heavy metals but also precipitation of industrial dust on plants leading occasionally to burning of plant. In the eighties, the problem of animal or even human health was of course not mentioned in Poland but now we can imagine that such pollution associated with dust and crop destruction could have had a very strong influence on livestock living in such a polluted area. The authors of the above-cited study (Greinert et al., 1987) only suggest the need to reduce the emissions of particulate matter and SO_2 by copper smelters, and possible evacuation of people living in the most polluted areas.

The effect of emissions from the Głogów Copper Smelters on soil acidification and accumulation of copper and lead was also described by Grzebisz et al. (1997). In addition, the soil structure was found to deteriorate (decrease of the organic matter content in humus horizons), and agricultural production declined. The Legnica and Głogów industrial region was recognised in 1992 as the largest region of ecological hazard in Poland (GUS, 1992). Fifty percent of contamination of the entire area come from emissions of the Głogów Copper Smelters. Now, years after modernisation and pro-ecological investments, the Głogów Copper Smelter/Refinery meets strict Polish and European Union environmental protection standards. In the former protection zone around the facilities, the KGHM Polish Copper prepared the "Głogów Wetlands" as a new habitat for plants and animals.

The Voivodship Inspectorate of Environmental Protection in Wrocław prepared an evaluation of the degree of soil contamination in Lower Silesia in 2008 for areas at a direct pollution risk (Wojewódzki Inspektorat…, 2009). In the area around the Głogów Copper Smelter I and II, including Skidniów, measures were performed. As a result, four sites with excessive benzo(a)pyrene concentrations were identified (0.038–0.059 mg/kg as compared to the permitted maximum of 0.03 mg/kg for B class grounds), one site with excessive concentration of copper (375 mg/kg against the allowed limit of 150 mg/kg), and one site with an overdose of lead (133 mg/kg against the permitted 100 mg/kg). Inspectorate analysed also farmlands in Skidniów, were experimental group of animals was kept. All analysed soil parameters in these farmlands did not exceed the applicable standards. Soil samples were alkaline in Skidniów. The evaluation of the cadmium, nickel, lead, chromium and zinc content in soils based on the Institute of Soil Science and Plant Cultivation – State Research Institute (IUNG) classification showed natural content levels for most heavy metals (grade 0) for this site but an unnaturally high content

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of copper (grade I) was observed (Wojewódzki Inspektorat…, 2009). Sulphate sulphur levels in vicinity of Głogów Copper Smelter I and II ranged from 0.8 mg to 8.7 mg per 100 g and for Skidniów increased level was found (grade IV, content anthropogenic). The soil analyses were performed about 10 years after the biological analyses of cattle presented in this dissertation (animals born mainly between 1997–1998, blood collection 1998 and 1999) but even if major pollution has now been substantially limited by improving the safety of copper smelters, heavy metals are still present in the environment and animals living in the area may be influenced by this pollution.

It is estimated that in areas of increased industrial dust emissions, on average 70 to 90% of the plant metal content comes from atmospheric sources (Rosada and Przewocka, 2015). Heavy metals may be excessively deposited in the crops, and then in the tissues and organs of farm animals as well as humans, causing adverse physiological effects (Dobrzański et al., 2003). Analyses of the content of heavy metals (lead, copper and zinc) in vegetable fodder in the area influenced by the copper industry of KGHM Polish Copper conducted in 1998, 2000 and 2002 showed that the organs which accumulate nutrients (storage roots, storage stems, storage leaves, fruit and seeds), forming the edible parts of a plant, usually contain fewer metals than roots, leaves and sprouts. A significant decrease in lead content was observed in particular in wheat and grass, whereas increased concentrations of copper and zinc was found in potato tubers and in the meadow grass. Meadow grasses are used as good indicators of environmental contamination and are also directly used as cattle feed. The observed levels of contaminants in plants were well within the standards for feed (Dobrzański et al., 2003) and did not represent an ecotoxicological threat to animals and humans.

Decrease of industrial contamination according to decrease of production and improving of environment protection does not solve problems with pollutants. It is necessary to conduct such tests of vegetable fodder regularly, especially in areas prone to contaminations and good example could be another heavily contaminated region in Poland – Upper Silesia. Upper Silesia is a region with a historically very intensive industrial activity and, as a consequence, the environment which has been significantly contaminated by different harmful pollutants, including heavy metals. Evaluation of the content of the heavy metals such as cadmium, lead and zinc in soils of Upper Silesia, with a particular emphasis on the areas around buildings where children playgrounds and other recreational areas are located was performed. The content of heavy metals like cadmium, lead and zinc was analysed in 76 soil samples using optical emission spectrometry with inductively coupled plasma. Despite significant reduction in the emission of heavy metals into the environment, heavy polluted areas still contain high concentrations of cadmium, lead and zinc and it is necessary to take preventive actions to reduce exposure to metals (Dziubanek et al., 2012).

The Institute of Plant Protection – the National Research Institute (IOR–PIB) in Poznań is involved in the analyses of the effects of industrial pollution on the environment and in the identification of efficient methods of reclaiming agricultural lands in cooperation with the Głogów Copper Smelter. The agricultural lands (6,600 ha) includ-

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ed the protection zone of the plant and the areas located on the left and right bank of the Odra River. Data obtained in years 2012 through 2014 showed that in the analysed soil only copper concentration exceeded permissible standards (>150 mg/kg d.m.) set by the Regulation of the Minister of Environment of September 9, 2002 (Rozporządzenie…, 2002, no. 165, item 1359) on soil quality standards and land quality standards. This was probably a result of the unfavourable impact of the wind in this region, carrying the emitted metallurgical dust. In view of the frequency of the winds, the soil pollution soil is higher in the south-east and east direction but concentrations of lead, zinc, cadmium and arsenic did not exceed acceptable standards (Rosada and Przewocka, 2015). The modernisation of the copper production technology significantly limited environmental pollution levels but the main problem in the soil is the presence of heavy metals from earlier periods of high emission. Historical heavy metals pollution is still present in our environment and may influence not only health of animals living in polluted environment but also may influence human health.

Agricultural areas affected by the emissions from the Głogów Copper Smelters have been in the centre of interest of various research institutes for a number of years. At present, although smelters meet the world standards with regard to the techniques employed in their technological processes, they are still perceived as sources of ecosystem pollution. In case of the discussed areas, their small distance from the emitters of contaminants increase the probability of pollution with emissions from smelters. This refers, in particular, to agrocenoses situated along the line of winds prevailing in this region. Only several years ago, the agricultural lands affected by emissions from the Głogów Copper Smelters were considered as a region of ecological disaster. Quantities of metal-bearing gasses and dusts exceeded many times the permissible standards. Until 2001, the area of the protection zone of the Smelters was quite large, covering 2,840 ha. However, during the last decade, emissions of toxic metal-bearing gasses and dusts were dramatically reduced. Nevertheless, in recent years, average amounts of copper were as follows: 0.078 mg/kg in soil solution and exchangeable copper, 11.76 weakly bound to specific sites, 69.4 organically bound, 26.2 in oxide-occluded materials and 14.1 in the residual copper fraction. However, the concentration of soil solution and exchangeable copper was extremely small in comparison with the amounts of copper in other forms and constituted only 0.06% of the total fractionated copper. This means that in the top (0–20 cm) soil layer, about 234 g/ha of copper was potentially available to crops (Grzebisz et al., 1997).

It is commonly recognised that the maximum level of copper in soil should not exceed 100 mg Cu/kg and when this level is exceeded, agricultural production should be abandoned. In the discussed region, within the radius extending from 1.3 to 9 km from the copper smelter, Grzebisz et al. (2001) found copper concentrations ranging from 20 to 400 mg Cu/kg, while Rosada (2005): from 42 to 298 mg Cu/kg. The presence of soil and plant contamination exceeding acceptable standards as a result of industrial activities was confirmed by studies carried out by Zwozdziak and Zwozdziak (1985), Monkiewicz (1988), as well as Rosada (2006).

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Soil heavy metal content depends on the distance from the emitters of contaminants as well as on the level and type of organic fertilisation (Monkiewicz, 1988; Pilc et al., 1999; Rosada, 2005). Keeping animals in an environment contaminated with emissions from a smelter results in elevated contents of lead in their blood as well as lead, copper and zinc in their soft tissues. Longer stays in the neighbourhood of a smelter leads to pathological changes in kidneys and liver (Monkiewicz, 1988). Moreover, milk yields of cows are unsatisfactory and even though no apparent poisoning occurs, the results of haematological and biochemical examinations confirm damage of the hematopoietic system.

It is well known that the toxicity of heavy metals is associated mainly with the generation of reactive oxygen species (ROS) via Fenton and Haber-Weiss reactions (Aseervatham et al., 2013; Jazvinšćak Jembrek et al., 2014; Jing et al., 2016). Reactive oxygen species are chemically reactive molecules containing oxygen and may interfere with the antioxidant enzyme activities, leading to oxidative damage of membrane lipids, proteins or nucleic acids (Jing et al., 2016). Heavy metals induce changes of enzyme activity and DNA strand breaks (Uriu-Adams and Keen, 2005; Jing et al., 2016). Copper can disturb the intracellular redox balance, induce oxidative stress, and subsequently cause irreversible damage of cells, leading to a variety of diseases. It was shown that a short-term exposure of single cells to copper (10 or 50 μM) does not induce statistically significant changes in average ROS generation, consumption of glutathione and oxidative DNA damage but the observation of such changes suggests the influence of metals on cells, emphasising the need for environmental risk assessment of copper pollution (Jing et al., 2016).

It has been suggested that reactive oxygen species may influence DNA methylation patterns, leading to pathophysiological changes and even to carcinogenesis (Wu and Ni, 2015). The oxidative stress caused by ROS may be associated with individual life style factors like smoking, inflammation and cell metabolism or with environmental factors connected with air pollution, UV light or radiation. Reactive oxygen species levels, most likely hydrogen peroxide from NADPH oxidases Duox1/2 and upon dismutation of Nox2 (CYBB)-derived superoxide, may be downregulated by DNA hypermethylation and histone hypoacetylation at the genomic level. Generation of high levels of ROS by these sources may result from increased DNA methyltransferase (DNMT) and histone deacetylases (HDACs) activity, which in turn may "silence" the DUOX1/2 and CYBB genes (Mikhed et al., 2015).

It is widely recognised that epigenetic alterations can be used as indicators of susceptibility to environmental exposures and are studied in association with disease outcomes. Epigenetics is defined as the concepts of molecular modifications to DNA and/or chromatin in the absence of any alteration to the underlying DNA sequence. The deregulation of epigenetic mechanisms is considered as a major cause of cancer as well as hereditary and neurodegenerative diseases. Reactive oxygen species can be generated by industrial pollutants as a result of the activity of the Głogów Copper Smelters. Epigenetic changes found in my study involved regional CpG island hyper-

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methylation in the promoter region of tumour suppressor genes and global genomic hypomethylation. Oxidative stress induced by reactive oxygen species leads to formation of an oxidased DNA nucleotide: 8-hydroxy-2'-deoxyguanosine (8-OHdG) or even GC to TA transversions (Mikhed et al., 2015). 8-OHdG can interfere with the ability of DNA to function as a substrate for the DNA methyltransferases (DNMTs), leading to global DNA hypomethylation and subsequent genomic instability. Oxidative conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC, another form of DNA oxidation, not analysed in my study) under oxidative stress conditions changes the DNA methylation pattern by suppressing the activity of the methyltransferase DNMT1 and the methyl-CpG binding protein 2 (MeCP2) (Mikhed et al., 2015; Wu and Ni, 2015). Reactive oxygen species may also influence site-specific hypermethylation by up-regulating the expression of DNA methyltransferases (DNMTs) or the formation of new DNMT complexes.

Alterations of DNA methylation patterns induced by reactive oxygen species are thought to be involved in malignant transformation and also the progression of tumours. Judging from my experimental work the reactive oxygen species induced by pollutions from Głogów Copper Smelters caused different health problems in grazing beef cattle living in the vicinity of this major pollution emitter. Lack of observation of cancer cases may be connected with short time of living animals in this environment but longer stay may influenced even human health.

Oxidative stress conditions may significantly increase the levels of 8-OHdG in human DNA. Such oxidation may occur also in guanine in CpG dinucleotides and the methylation of cytosine in the 5' position to 8-OHdG may interfere with efficient repair of this lesion (Pfeifer, 2006). Guanine in CpG dinucleotide can be easily oxidised to 8-hydroxy-2'-deoxyguanosine (8-OHdG) or 8-oxoguanine (oxoGua). 8-oxo-7,8-dihydroguanine may cause G → T transversions. This modification can be removed by base excision DNA repair (BER) mechanism with specific 8-oxoguanine-DNA glycosylase (OGG1) generating an apurinic- or apyrimidinic- (AP) site. All AP sites are then processed by apurinic/apyrimidinic endonuclease 1 (APEX1/APE-1), leaving clean 3' and 5' ends that allow DNA polymerase β (Polβ) and DNA ligase I (Lig1) or DNA ligase IIIα to insert and ligate the appropriate base (Kasymov et al., 2013; Mikhed et al., 2015; Fleming and Burrows, 2017; Ba and Boldogh, 2018). APEX1 protein has very unique functions in the nucleus, because it has both repair and redox regulatory domains. The redox function of this protein is a new evolutionary addition found only in mammals, while the repair function is conserved through evolution. The APEX1 endonuclease is an essential member of nucleotide excision repair, performing cleavage of the flanking DNA ends to prepare them for polymerase and ligase activities (Mikhed et al., 2015). It was shown that the methylation of the adjacent cytosine abolished stimulation of OGG1 by repair endonuclease APEX1 (Kasymov et al., 2013; Wu and Ni, 2015). The exonuclease activity of APEX1 may remove methylated cytosine present in the 5' position to 8-OHdG, providing a possible mechanism for DNA demethylation coupled with oxidative damage repair. Formation of 8-oxo-7,8-dihydroguanine (8-oxoG) dur-

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ing oxidative stress leads to toxicological effects but also influenced embryonic growth. Short term stresses like oxidative stress may allow DNA modification for adaptation and responding to changes in similar way as 5mC is important for longer time of response. This may indicate contribution of oxidation of guanine base to epigenetic changes of DNA and serving as epigenetic mark (Fleming and Burrows, 2017; Ba and Boldogh, 2018).

As a model for analysing the influence of environment on living organisms, two groups of beef cattle were selected – one was bred in Skidniów in the vicinity of the Głogów Copper Smelter (experimental group) and the other in environmentally clean area in Sława (control group). All animals were of Limousin origin and both groups included half-sisters, the progeny of Jaśmin bull and additionally experimental group included larger group of Jasny bull progeny. Cattles were bred in a similar way with respect to the time the animals spent outdoor and to the use of local grasslands. However, farm owners from Skidniów noticed health problems in their animals, leading to lower weight gains and consequently loss of farmers' profits from beef cattle breeding. Therefore, diseases which occurred among animals reared in the Skidniów farm called for special attention. Phenotype changes in mature cattle's were barely noticeable in contrast to calves and the serious problem of mature animals concerned skin diseases which occurred in animals on a massive scale, affecting economic results of the farm. Serious or less serious ulcerations, hair loss as well as poorly healing wounds occurred in about 50% of animals. Hides with evident damages were worthless for the tanning industry. Very serious problem for the above-mentioned farm included miscarriages and deaths of calves during the first days of their lives. They occurred in 22% of all pregnancies in 1999 and deteriorated every year (26% in 2000). Another serious problem was blindness which occurred in 14% of born alive calves, causing nervousness in young animals and affecting breeding efficiency, as animals running around lose much energy and so gain body weight much slower. Veterinary services failed to find treatment for the disease and were unable to solve this problem. Taking all this into consideration, it appeared very interesting to compare DNA changes in animals from the two groups living in different conditions, with different levels of environmental contamination.

The groups of animals under study represented beef type cattle of different upgrade percentage of the Limousin breed blood. The animals were characterised by early sexual maturation and low milk yield. The size of animals was considered to be moderate. Their growth increment, understood as the daily body weight gain, was very fast. The obtained slaughter material was characterised by low fat content and high productivity (dressing percentage). The animals from the experimental group failed to reach daily body weight gains which were expected on the basis of the genetic material of the parents. Limousin breed bulls used to mate heifers from the Skidniów farm in order to constitute an elite breeding material. However, even though the animals were ensured optimal living conditions and veterinary care, and the most recent breeding methods were used, the effect in terms of the rate of daily body weight gains was far from sat-

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isfactory. The knowledge from the area of rearing beef type animals did not help to obtain good results, either. Roughages produced in the Skidniów farm derived from plants which grew on fertile soils from the wheat-beet complex which constitutes over 75% of the farm area. Full coverage of the daily requirements of animals was ensured thanks to the supplementation of the daily diets by concentrates manufactured by the farm own feed mixing plant. Study was performed on female progeny (heifers) obtained from commercial crossings which were purchased at various ages by the owner of the Skidniów farm. It was established on the basis of information obtained from the veterinary services of the farm that mean body weight gains were as follows: 820 g in the 6–9 months age group, 910 g for 10–15 months old animals, and 980 g in the group of animals older than 15 months. Both the owner's expectations and data from other breeders of the beef type animals indicated that the potentials were over 20% higher. This decreased breeding effectiveness of the farm by increasing the costs of daily body weight gains and discouraged the owner from continuing this type of animal breeding.

The animals from the control group in Sława, which were bred in similar climatic conditions, achieved the following results: 970 g mean body weight gain in the 6–9 months age group, 1090 g in 10–15 months old animals, and 1120 g in the group of animals older than 15 months. It should be emphasised that the recorded daily body weight gains were higher in comparison with Skidniów farm, even if Sława is much poorer, with regard to the environmental conditions, region in which over 50% of arable land is taken up by the rye-potato complex. The level and quality of the obtained crops in Sława farm depended on high farmyard manure doses applied on all fields. Satisfaction from the selected direction of cattle breeding entirely coincided with economic effects measured as the costs of rearing young cattle and the price obtained for the sold meat type heifers. Slaughter houses from the area of Sława recorded slaughter yield exceeding 59% for animals purchased from the farm in Sława. In addition, the results recorded from the five prime cuts were by 4% better. Cattle carcasses contained less fat and the meat percentage content was even by 5% higher in comparison with the mean values obtained from other animals.

Differences observed between the experimental and control animals, especially blindness developing during pregnancy in the experimental animals may have been caused by the contamination of lands where the animals were kept. Accumulation of heavy metals in soil was important here and inappropriate agricultural exploitation of the lands only exacerbated this influence because heavy metals which were liberated as a result of deep ploughing and improper soil pH were incorporated into the food chain. It should be mentioned here again that this farm is situated very close to the source of emissions: the Głogów Copper Smelters. Arable lands in this area had been exposed to direct dust and gas emissions, and problems of soil contamination were getting worse for years and it was only recently that the trend was stopped and reversed. The content of heavy metals in soils is influenced directly by the distance of the contaminants' emitter (Pilc et al., 1999; Rosada, 2005; Rosada and Przewocka, 2015) but it is also necessary to take into consideration prevailing winds which can extend the zone with increased

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heavy metal content even to several kilometres. Historically achieved soil contamination will pose a serious threat for plant and animals for years to come.

The two groups of animals analysed in this study were kept in extremely different conditions with regard to heavy metal content in soils. The experimental group lived in the neighbourhood of the Głogów Copper Smelters and the control group was kept in almost total isolation from industrial contamination. The observed differences in the development of animals from the experimental group can be associated with the accumulation of microelements in soil, in amounts many times exceeding the levels found in soils free from industrial contamination (Baluk et al., 1993; Grzebisz et al., 1997). Moreover, the deterioration of soil structure may have also affected the obtained results.

Very few data exist on the influence of copper smelter on human and animal health (De Olivera et al., 2012). Occupational exposure in copper smelting industry is associated with an increased risk of cancer among workers. It has been shown by comet assay that DNA damage levels in peripheral blood leukocytes of copper smelter workers are significantly increased, suggesting a potential health risk. The proton-induced X-ray emission (PIXE) analysis used to determine the metal content of leukocytes showed the presence of copper, iron, and other metals but no correlation between DNA damage or metal concentration and mean age mean or time of exposure was found (De Olivera et al., 2012). Analysis of breast cancer incidence showed increased occurrence in more developed countries as a result of pollution of soil, surface water and food by heavy metal salts. Level of DNA methylation of cancer tissues was correlated with increased level of iron, copper, zinc, lead, chromium and nickel (Romaniuk et al., 2017). In humans, exposure to copper dust results in respiratory irritation, mainly associated with exposure to arsenic, additionally nervous system and renal function were also affected and increased frequency of chromosome aberrations were observed. There is no data indicating that copper may cause birth defects in humans but such problems were observed in cattle living in the vicinity of KGHM Polish Copper Głogów Smelters. Contact with higher amounts of heavy metals like iron and copper may lead to the generation of reactive oxygen species (ROS) with the consequent enhancement in membrane lipid peroxidation, DNA damage, and protein oxidation (De Olivera et al., 2012). Reactive oxygen species generated by this process could attack DNA, leading to base damage and DNA strand excision. The real strand breaks and/or the strand breaks formed as an intermediate step in the excision repair of altered bases could explain the increased DNA damage shown by the comet assay. Another study on workers from three copper smelters in south-western Poland showed a significant increase in micronuclei frequency in peripheral blood leukocytes and in buccal epithelial cells but no correlation between arsenic content in urine or toenail samples and the genotoxic effect was found (Lewińska et al., 2007). Because cancer risk is mainly associated with exposure to arsenic, only the presence of this element in organisms of workers was analysed but it is obvious that a role of other agents, like heavy metals (Pb, Zn, Cd, Cu) or polycyclic aromatic hydrocarbons, in inducing the observed genotoxic effects can-

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not be excluded. Such studies were not performed for my groups of animals but we can hypothesise that such changes could have also occurred in cattle organisms.

Very interesting data were obtained in the Russian Federation for one of the most polluted cities in the world, Karabash which is located in the Chelyabinsk region and whose pollution is associated with the presence of a copper smelter (Skalny et al., 2016). It was indicated that the levels of Fe, Cu, Zn, Cd, Pb, and As in water and snow in Karabash by far exceed the existing standards and cause health problems like impaired nervous and hematopoietic systems functioning in children, high number of pregnancy complications, premature births and neonatal morbidity. Similar changes were observed in cattle from my experimental group living in the vicinity of Głogów Copper Smelters. Toxic metals may enter the organism by inhalation of metal particles from the atmosphere, transdermal absorption, and ingestion of polluted water and local foodstuffs. Contamination is also present in plants and mushrooms growing in the polluted areas may also reach the livestock (cattle) through the food chain. Skalny and colleagues (Skalny et al., 2016) emphasised the pro-inflammatory and pro-oxidant role of heavy metals and their co-exposure due to their synergistic effect on cellular mechanisms, increasing health risk. It was shown that children living in a polluted area near a copper smelter have significantly increased blood concentrations of As and Pb being associated with elevated As, Cd, Ni, and Pb levels. On the other hand, the exposed children had significantly lower levels of Ca and Mg, whereas blood concentration of phosphorus (P) was increased. The authors suggest that adverse health effects in persons living near a copper smelter may be associated not only with toxic metal over-exposure but also with altered mineral homeostasis (Skalny et al., 2016).

The influence of the environment on living organisms may be observed by the occurrence of epigenetic changes at the DNA level, including DNA methylation and oxidation. This concept can be further verified by studies using two groups of animals of a similar genetic background and bred using similar methods. In our experimental group of cattle's we observed decrease of global methylation of DNA which may lead to the first stages of carcinogenesis. Almost thirty years have passed since it was demonstrated that methylation levels in tumour cells are lower than in normal cells (Feinberg et al., 1988). A decrease in methylation levels occur at very early stages of carcinogenesis, even before the actual tumour development (Zukiel et al., 2004). Experimental data from rodent studies showed that the decrease in dietary methyl group donors led to liver carcinogenesis and DNA hypomethylation (Wainfan and Poirier, 1992). It is assumed that epigenetic silencing of tumour suppressor genes by promoter methylation associated with lowered global DNA methylation level leads to the development of tumours (Dobrovic and Kristensen, 2009; Esteller, 2008). There are also reports concerning hypermethylation of specific genes involved in control of carcinogenesis. For example, hypermethylation of CpG islands of the retinoblastoma susceptibility gene promoter was shown to be associated with reduced expression of the gene in 10% of patients with retinoblastoma (Greger et al., 1994). Promoter hypermethylation of tumour suppressor Wilms' tumour gene 1 (WMT1) and the calcitonin gene was also re-

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ported in colon cancer cases, and the *APC* gene promoter showed hypermethylation in over 50% of these patients. In experiments on mice with hundreds of polyps in the intestine, carrying a mutation in the *APC* gene, the methyltransferase inhibition resulted in a huge reduction in polyp numbers (Laird et al., 1995).

It is well documented that stress caused by diet disturbances in the balance of methionine, choline, folate and selenium as well as excessive alcohol consumption can lead to DNA hypomethylation. This was observed in rats which were fed diets poor in choline and methionine, which resulted in global DNA hypomethylation and liver cancer. Symptoms reminded the effects of exogenous carcinogens (Li et al., 2003; Edwards and Myers, 2007). It was also demonstrated that the environment as well as the diet of parents may influence the development of the progeny as well as possibly contribute to the occurrence of tumours and other developmental disorders by changes in DNA methylation (Li et al., 2003; Edwards and Myers, 2007). Chemically induced changes in gene regulation are associated with complex human diseases, including cancer, diabetes, infertility, diseases of the respiratory system, allergies as well as neurodegenerative diseases, and they are heritable (Edwards and Myers, 2007). Similar problems connected with a reduction in the levels of DNA methylation may occur in animals living in the vicinity of the Głogów Copper Smelter as a consequence of living in a polluted environment (Monkiewicz, 1988; Eeva et al., 2006).

The investigations presented in this monograph revealed reduced methylation levels in animals from the experimental group as compared to those in the control group. Although single animals with reduced levels of DNA methylation were found also among controls, the recorded differences between the two groups were statistically significant. It is possible that methylation was not the only parameter of DNA from these animals which underwent changes due to environmental contamination. Further investigations could focus on determining the levels of DNA adducts. As it was impossible to determine which of the elements was directly responsible for the observed phenotypic changes, it cannot be ruled out that the methylation changes reported in this study could have also resulted from other changes, e.g. those associated with enzymes taking part in the methylation process. It would also be interesting to perform analyses on the cytogenetic level, in particular of chromosomal instability following cell incubation with bleomycin, 5-azacytidine or 5-bromodeoxyuracil. It was impossible to perform lymphocyte culturing of the peripheral blood for such a large group of animals in the course of collection of the experimental material because animals are no longer viable. But analysis performed on DNA level showed changes caused by living in neighbourhood of Głogów Copper Smelters and continuation of such experiments is possible because DNA preparations of all animals have been stored in our laboratory DNA bank. The collected biological material is particularly valuable because the animal groups comprised half-sisters, the daughters of Jaśmin bull, which reduced the population genetic heterogeneity.

Another change observed at DNA level as one of the results of the exposure of living organisms to environmental pollution is DNA oxidation which plays an important role

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in the development of different diseases, including cancer (Meyer et al., 2000). Many studies focus on the analysis of 8-hydroxydeoxyguanosine (8-oxo2dG), a product of the hydroxylation of the 8-deoxyguanosine by a hydroxide radical, which is a typical oxidised nucleoside serving as an indicator of DNA oxidative damages (Kasai et al., 1987; Shigenaga et al., 1989; Umemura et al., 1990; Ichinose et al., 1997; Lloyd and Phillips, 1999; Sato and Aoki, 2002; Aoki et al., 2001; Sato et al., 2001).

Chemicals, radiation and biological agents (e.g. viruses, bacteria and parasites) can have direct effects on genome integrity, causing the formation specific DNA lesions (like 8-oxo-7,8-dihydroguanine (8-oxoG), cyclobutane pyrimidine dimers, bulky adducts), inducing gene mutations and chromosome aberrations. Environmental exposure can also cause genome instability by indirect mechanisms involving single-nucleotide polymorphisms (e.g. SNPs in genes whose protein products are involved in chemical metabolism, DNA repair, cell cycle), changes in the DNA repair enzyme activity, changes of nuclear and mitochondrial DNA copy number (mtDNAcn) or microsatellite instability (MSI), changes in epigenetic regulation of gene expression (e.g. differential promoter region methylation) or inducing alterations in levels of specific proteins (Langie et al., 2015). Heavy metals may cause DNA breaks through the generation of reactive oxygen species and inhibition of DNA repair proteins or other enzymes caused by the displacement of Mg and Zn from cellular proteins (Kale et al., 2006; Langie et al., 2015).

Blood collected from the animals kept in the contaminated environment provides interesting material for investigations on the influence of pollution on DNA. A comparison of global oxidation levels between the experimental group and controls showed a statistically significant difference (P-value = 0.0000), with higher oxidation levels in animals living in the vicinity of copper smelters.

A comparison of global methylation levels between the experimental and control group showed a statistically significant difference ($Z = 5.76$; P-value = 0.0000). Mean global DNA methylation was lower in animals from the experimental group, which could have caused health problems associated with abnormal regulation of gene expression in young animals. A similar situation was observed for DNA oxidation, with global oxidation levels being significantly higher in experimental animals ($Z = -5.07$; P-value = 0.0000).

The mean age in the control group was 382 days (the youngest animal was 154 days old and the oldest 659 days), while in the experimental group it was 556 days (the youngest individual was 262 days old, the oldest 2423 days). The experimental group was significantly older than the control group (P-value = 0.0007). The level of oxidative DNA damage may depend on age (de la Asuncion et al., 1996; Roszkowski, 2002; Judge and Leeuwenburgh, 2007; Reddy Thavanati et al., 2008). On the basis of the obtained results of investigations of the oxidation level expressed by the 8-oxo2dG/dG ratio, I conducted an analysis of dependence correlation between the oxidation level and age of the examined animals. The oxidation levels are expected to increase with age of animals and such a correlation was found when all animals were analysed together

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(*P*-value = 0.0135). The same significant positive correlation, of an average strength was found when analysing controls alone (*R* = 0.44, *P*-value = 0.0014). However, such a correlation was not observed for the experimental group. In experimental animals, the environmental pollution induced changes in the oxidation profile and a negative moderate strength correlation between age and global oxidation levels was observed. On the other hand methylation level should decrease with age of animals and such a negative correlation, very week, was observed for all animals (*P*-value = 0.0201) but not for experimental or control animals analysed separately.

The overall analysis of results obtained for each group confirms the reports of other researchers indicating the existence of a correlation between the age of organisms and the level of oxidative damage (Roszkowski, 2002; Humphreys et al., 2007). The low correlation probability calculated for the control and experimental groups could be attributed to their small size. I have not observed correlation between decrease of global methylation and increase of global oxidation levels with age of animals.

It is assumed that global methylation level is decreasing with age and the content in DNA of oxidatively modified nucleotides increased with the age of the examined animals according to accumulation of DNA changes. Similarly to all groups, Jaśmin progeny did not show a correlation of global methylation with global oxidation, even if such a tendency was observed (*P*-value = 0.0815).

The influence of the environment on DNA of analysed animals could be observed more precisely due to a very similar genetic background of the two groups, both including the progeny of Jaśmin bull. Global methylation levels for the 35 daughters of Jaśmin bull did not significantly differ from those in other animals (*P*-value = 0.5583), and the same situation was observed also for global oxidation (*P*-value = 0.1672).

On the other hand, stability of the epigenetic changes at DNA level of animal organism and repeatability of the observed influence of environment on global methylation or global oxidation levels could be confirmed by an analysis of the 16 daughters of another bull called Jasny, included in the experimental group. This group of animals had lower global methylation levels in comparison to those in other animals (*P*-value = = 0.0691) and significantly higher levels of global oxidation (*P*-value = 0.0002). This pattern is probably a natural characteristic of the Jasny progeny. This conclusion is in accordance with the observation that there were no differences in global methylation and global oxidation patterns between the Jaśmin progeny and those in other animals between the experimental and control group.

Analysis of the Jaśmin progeny gave interesting results concerning the influence of the environment on global methylation levels which were significantly lower for animals in the experimental group than in control group (*P*-value = 0.0008). As it was expected, global oxidation levels were significantly higher in these animals (*P*-value = = 0.0001), which may have resulted from the exposure of animals from the experimental group to contaminants present in the environment. Increased levels of oxidative damage were reported by other researchers in animals following their exposure to different types of chemical substances. The reported levels of oxidative damage ex-

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pressed as the ratio of 8-oxo2dG to dG ranged from about 0.00002 to 0.00004 at an approximately ten-fold lower initial exposure level (Umemura et al., 1990). Other researchers reported about five-fold increase in the oxidation level following exposure to contamination (Yamaguchi et al., 1996). The experimental group analysed in my study had about two-fold higher oxidation levels compared to the control group. The smaller difference than those reported by other researchers may be contributed to the fact that the animals exposed to increased contamination levels were kept in laboratory conditions, whereas the results obtained in this study refer to animals which were exposed to contamination in a polluted environment.

Heavy metals and other pollutants in the vicinity of a copper smelter may also impair the ability to repair DNA changes, and the *Apex1* and *Nudt1* gene promoters may be two of many possible targets. To verify this hypothesis, I determined the methylation status of the promoter region of the *Apex1* gene which is a multifunction DNA repair gene. The APEX1 nuclease is an apurinic/apyrimidinic endodeoxyribonuclease 1 in *Bos taurus* and is conserved in the human, chimpanzee, *Rhesus* monkey, dog, mouse, rat, chicken, zebrafish, mosquito, *Arabidopsis thaliana*, rice, and frog. This enzyme is involved in identifying and repairing apurinic/apyrimidinic (AP) sites which occur frequently in DNA molecules by spontaneous hydrolysis, induced by DNA damaging agents or by DNA glycosylases that remove particular abnormal bases. The resulting abasic sites can block the progress of the DNA replication machinery and cause mutations. These sites must be repaired to restore genetic integrity. According to the available databases, the APEX1 enzyme is linked to a number of processes including DNA demethylation, DNA recombination, DNA repair, base-excision repair, nucleic acid phosphodiester bond hydrolysis, oxidation-reduction process, regulation of mRNA stability, regulation of transcription and DNA-templated transcription. The second gene which I analysed in detail was *Nudt1* encoding nudix hydrolase 1 (7,8-dihydro-8-oxoguanine triphosphatase) which is involved in removing oxidised bases. Oxygen radicals, which can be produced through normal cellular metabolism, are thought to play an important role in mutagenesis and tumorigenesis. Among various types of oxidative DNA damage, 8-oxo-7,8-dihydroguanine (8-oxoG) is the most important because of its abundance and mutagenicity. It is repaired by the NUDT1 enzyme which hydrolyses oxidised purine nucleoside triphosphates, such as 8-oxo-dGTP, 8-oxo--dATP, 2-hydroxy-dATP, and 2-hydroxy rATP, to monophosphates, thereby preventing misincorporation. The encoded protein is localised mainly in the cytoplasm, and to a lesser extent in the mitochondria, suggesting that it is involved in the sanitizing nucleotide pools both for nuclear and mitochondrial genomes. NUDT1 is connected with processes encompassing DNA protection, DNA repair, aging, dATP and dGTP catabolic process, male gonad development, nucleobase-containing small molecule catabolic process, purine nucleotide catabolic process, response to cadmium ion and response to oxidative stress.

Methylation analyses of specific DNA fragments presented in this dissertation were carried out on the same groups of cattle living in different conditions of environment.

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All DNA samples were subjected to conversion by sodium bisulphite followed by two parallel PCR reactions using primers specific for sequences in which at least two CpG islands occurred. For the first PCR reaction, the primers were designed to match unchanged cytosine within CpG islands (amplification of fragments in which cytosine was methylated within islands), whereas for the second one, the primers matched sequences in which all cytosine residues were replaced by thymine (amplification of fragments in which cytosine was unmethylated within islands).

The analyses did not reveal a statistical significant association between the methylation of the *Apex1* and *Nudt1* gene promoters and the impact of the environment. The methylation of the *Apex1* promoter was more frequent among controls (*P*-value = = 0.2544). This observation is consistent with higher global methylation levels in animals from the control group. For the methylation of the *Nudt1* gene promoter, no correlation with the environmental exposure was found. The methylation status of the *Apex1* and *Nudt1* gene promoters in Jaśmin progeny did not differ from that of other animals (*P*-value = 0.4304 and *P*-value = 0.2915 respectively).

Interestingly, I found that methylation levels of the *Apex1* gene promoter in Jasny bull progeny were decreased as compared to other animals (*P*-value = 0.0028), whereas no changes in the methylation status of the *Nudt1* gene promoter were observed. This may suggest that higher global oxidation levels in Jasny progeny were not directly connected with the promoter methylation of the *Nudt1* gene which is involved in removing oxidased bases. Higher oxidation levels in animals from the experimental group correlated with unmethylated promoter of the *Apex1* gene (*P*-value = 0.0436). Methylation status of the *Nudt1* gene promoter did not influenced the global oxidation status in all groups of animals. Also, there was no correlation between the methylation status of the *Apex1* gene promoter and *Nudt1* promoter, and global methylation levels were not associated with the methylation status of the *Apex1* and *Nudt1* gene promoters.

Animals from the experimental group with methylation of the *Apex1* gene promoter were older than the cattle with unmethylated promoter of the *Apex1* gene (*P*-value = = 0.0350). The analysis of the *Nudt1* gene promoter methylation and age showed no statistically significant correlation. The obtained results suggest a connection between the methylation of the *Apex1* gene promoter with the influence of the environment. This could seem logical because this gene encodes a protein involved in DNA repair. This relationship could also emerge accidentally as no such relationship was observed for the *Nudt1* gene. Because cytosine hypermethylation in the CpG in promoter segments may result in an inhibition of a given protein synthesis (Shen et al., 2007), and the results of my analyses indicate privileged methylation of the analysed fragment in all individuals, it can be presumed that the analysed fragment probably does not take part in the methylation-mediated regulation of the *Apex1* gene expression or that the regulation process of this gene through methylation differs from the one described in literature for a majority of studied genes.

An important part of my studies involved the analysis of *Alu* sequences using *Alu*-PCR. This method can be employed when large-sized groups of animals are involved

and electrophoregrams containing about 30 DNA bands are obtained. The choice to analyse *Alu* sequences was made on the basis of literature reports that qualitative changes in these sequences (occurrence of additional peaks or shifts) can provide a rapid and inexpensive technique of evaluation of genomic instability (Krajinovic et al., 1996). Such investigations were conducted, among others, by Debniak and colleagues (Debniak et al., 2001) who carried out experiments on colon cancer patients without polyposis (HNPCC) and found quantitative and qualitative changes of *Alu* sequences. Part of these fragments may have contain microsatellite sequences whose length changes occurred in the course of the colon tumour development. The occurrence of additional or reallocated peaks of fluorescence did not always correlate with the phenotype. In the study presented in this monography, in a certain number of cases I observed additional or shifted fluorescence peaks, and the appearance of two or more changes in *Alu* profiles in the experimental group of animals was highly significant (*P*-value = 0.0001). Analysis of changes in *Alu* profile observed in half-sisters in both herds of cattle may suggest the influence of environment on *Alu* profile. The occurrence of changes in the *Alu*-PCR, in cases where peak relocation occurred, may be caused by a change in the length of amplified fragments as a result of exposition to heavy metals and other pollutants connected with copper smelter industry and may be connected with a microsatellite instability in mononucleotide sequences (Debniak et al., 2001). The presence of heavy metals in the environment of studied animals may have had a great impact on their organisms and mobile element activation must be considered as one of the mechanisms responsible for genomic damage/instability in response to environmental agents (Kale et al., 2005; El-Sawy et al., 2005). Water-insoluble forms of several heavy metals, such as nickel oxide, cadmium sulphide and mercury sulphide, present a higher carcinogenic potential than its soluble counterparts, and stimulate human L1 mobile element activity leading to genomic instability. Genome instability is an enhanced tendency for the genome to acquire mutations, ranging from changes in the nucleotide sequence to chromosomal amplifications, rearrangements or loss (Kale et al., 2006). The following substances may indirectly contribute to genomic instability: heavy metals (influencing DNA repair, epigenetic modification, DNA damage signalling and telomere length), acrylamide (influencing DNA repair, chromosome segregation), bisphenol A (influencing epigenetic modification, DNA damage signalling, mitochondrial function, chromosome segregation), benomyl (chromosome segregation), quinones (epigenetic modification), and nano-sized particles (epigenetic pathways, mitochondrial function, chromosome segregation and telomere length) (Langie et al., 2015).

The exposure to chemical contamination can also contribute to the loss of genetic diversity called genetic erosion. Such changes are observed in wild animal populations and may even lead to species extinction. Genetic erosion is the loss of genetic variation by loss of alleles determining the value of a specific trait or set of traits influencing fitness, environmental plasticity, co-tolerance mechanisms, trade-off mechanisms and tolerance to pathogens in amphibian populations (Fasola et al., 2015). Similar changes, even though probably on a smaller scale, may occur in farm animals e.g. analysed cattle

groups and humans due to diversification of food and feed sources, and lack of dependence of the aquatic habitat. Studies concerning genetic diversity in two small insectivorous passerines: the great tit (*Parus major*) and the pied flycatcher (*Ficedula hypoleuca*), leaving near two copper smelters or nuclear material reprocessing plant showed an increase of heavy metal concentrations (mainly arsenic) and significantly higher nucleotide diversity in mitochondrial DNA in the *P. major* population living near a smelter and increased nucleotide diversity in *F. hypoleuca* population living near the source of radioactivity. The authors suggested that the differences between the two species may result from their different ability to metabolise pollutants (Eeva et al., 2006).

Similar results on the effect of a long-term exposure to elevated concentrations of metals (Cd, Pb, Zn) on genetic diversity in rove beetle (*Staphylinus erythropterus*) populations living along a pollution gradient were reported in Poland. A genome-wide analysis of 615 nuclear RADseq loci and mtDNA fragment in 96 individuals showed weak differentiation between populations. The highest genetic diversity was found in a population living in the most polluted site, probably due to increased mutation rates associated with elevated oxidative stress levels, but there no correlation between the genetic diversity and metal pollution or other soil properties was observed (Giska et al., 2015).

Srikanta and colleagues (2009) suggest a role of DNA double-strand break repair and its association with intra-chromosomal translocations, *in vitro* RNA recombination, and synthesis-dependent strand annealing, which may explain my results. Genomic instability and changing pattern of *Alu* fragments were determined by analysis of the sequences flanking the deletion breakpoints and revealed an enrichment of *Alu* repeat elements (de Smith et al., 2008). The mechanism of the formation of some deletions remain to be elucidated but some models for these mechanisms in humans have been proposed (Kim et al., 2016).

Alu elements are frequently involved in genomic rearrangements in the human genome due to their abundance and sequence identity between them. The genomic rearrangements caused by Alu elements may lead to genetic disorders, such as blood disorders and neurological disorders. It has been suggested that *Alu* elements are associated with approximately 0.1% of human genetic disorders (Kim et al., 2016). Such changes may be connected with health problems observed in the experiment group of cattle living in the neighbourhood of the Głogów Copper Smelter, the major cause of environmental pollution in the area.

The diagnostic value of the *Alu*-PCR method in comparison to other techniques is limited by its low specificity (Krajinovic et al., 1996; Debniak et al., 2001). Moreover, *Alu*-PCR is not complementary with screening methods of mutation detection and it is believed that substituting the microsatellite sequence analysis by the *Alu*-PCR is not always fully justified. In this study, this technique was employed exclusively with the aim to identify DNA differences between the experimental and control animals. However, it would be wrong to assume that it is possible to draw reliable conclusions concerning correlations between the phenotype of the examined animals and changes

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observed in the *Alu*-PCR profiles. Among factors substantially limiting full utilisation and interpretation of the results obtained with *Alu*-PCR is the fact that in cattle very complex images are obtained, comprising on average 30 peaks in the analysed range of magnitude. Interestingly, in most somatic tissues highly methylated *Alu* elements accumulate in gene-rich regions which harbour up to 33% of the total number of CpG sites in the genome. A high content of CpG dinucleotides in *Alu* elements may be connected with a high frequency of point mutation (Kim et al., 2016). *Alu* and LINE-1 elements are usually heavily methylated in normal cells, thus maintaining transcriptional inactivation and inhibiting retrotransposition. Hypomethylation of these elements is hypothesised to facilitate genomic instability by resulting in retrotransposition of transposable elements, dysregulation of DNA repair genes and altered expression of important genes (Udomsinprasert et al., 2016). Demethylation of *Alu* elements occurs during aging and cancer processes and has been associated with gene reactivation and genomic instability (Rodriguez et al., 2008). Genotoxic agents may induce methylation of retrotransposons in addition to oxidative DNA damage in the form of 8-hydroxy-2'-deoxyguanosine (8-OHdG). Methylation of retrotransposons induced by these agents may contribute to different physiological conditions and health problems (Udomsinprasert et al., 2016).

The repeat-specific DNA demethylation may cause structural alterations of adjacent chromosomal regions and it seems most likely that hypomethylation precedes rearrangements (Cadieux et al., 2006). Active transposons are demethylated in mammalian genomes and our data (not shown) confirm a decrease of global methylation levels in the experimental group compared to the control animals. Similar studies suggested that, independently of risk factors, hypomethylation of retrotransposable DNA elements in peripheral blood leukocytes is associated with shorter telomeres, elevated oxidative DNA damage and a higher risk of biliary atresia in humans (Udomsinprasert et al., 2016). In my study, I did not analyse *Alu* fragments methylation but the existence of a correlation of a higher retrotransposition of *Alu* elements in the experimental group with the observed decrease of global methylation level may suggest a similar mechanism.

As a continuation of my studies, it would be necessary to determine the sequences of the fragments for which changes were observed and this would be possible after cloning the *Alu*-PCR products. This would considerably exceed the scope of this study and, therefore, the performed *Alu*-PCR analyses can only be described here as experiments which confirmed the occurrence of changes in the DNA of animals living in a region with a considerable content of heavy metals in the soil. *Alu*-PCR electrophoretic images for animals from the control group were almost identical, whereas in the experimental group, differences were observed. However, it is not possible to determine for sure if these differences resulted from the appearance of new or disappearance of the existing *Alu* fragments, or else from changes in their length.

It is now assumed that the exposure to low doses of different chemicals present in the environment could be associated with the emergence of accumulating health prob-

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lems and development of cancer by indirectly affecting genome stability. As it was earlier mentioned, heavy metals may interfere with DNA damage signalling, DNA repair, epigenetics, telomere integrity and as nano-sized particles on mitochondrial functions (Langie et al., 2015). The impact may be worsened by the effect of combined exposure, and exposure to a mixture of genotoxins might be more likely to induce cancer than single chemicals (e.g. quaternary mixture lead, arsenic, cadmium and chromium). In the environment, mixtures of numerous metals (arsenic, mercury, lead, cadmium, chromium, copper, vanadium) with benzo(a)pyrene in the form of airborne particulates are frequently found. Metals may decrease DNA damage caused by oxidation of benzo(a)pyrene particles by changing the activity of CYP cytochrome families (Langie et al., 2015). Therefore, it is necessary to take into account the cumulative effect of environmental pollution on the health of the analysed cattle and a very complicated mechanism by which DNA changes leading to genomic instability and health problems develop.

Increased differences between methylation levels in animals of different ages observed in the experimental group can indicate the influence of DNA damage caused by the environmental contamination on the level of methylation of CpG islands present in DNA. The analysis of all animals for which the gene methylation status was determined allowed to find correlations between oxidation and methylation levels.

It has been suggested that chronic exposure to a mutagen (pollution by copper smelter industry) more favours the development of disease or even cancer than an acute exposure to a similar total dose of mutagen. These observations and the principle that carcinogenesis includes a cascade of specific mutations occurring over a period of time in an organism confirm the hypothesis that chronic exposures to mutagens might be more mutagenic than acute exposures (Langie et al., 2015). DNA mutations may also be inherited from parents, and any exposure or disruption of biological functions that leads to increased mutation rates in either of the parents may influence the susceptibility of the child to cancer. A dysregulated epigenome in offspring originating from the paternal or maternal genome through reproduction will potentially influence genetic instability and susceptibility to cancer in the offspring. There are indications that parental lifestyle can be associated with an increased risk of childhood cancer, and the significance of the father's exposure may be particularly important (Langie et al., 2015).

Maternal environmental exposures to pollutants may contribute to adverse outcomes during pregnancy and later in life as suggested by the developmental origins of health and disease hypothesis (Green and Marsit, 2015). These effects are connected with the incorrect regulation of DNA methylation in offspring tissues, specifically placental tissue, which plays a critical role in foetal development. DNA methylation may be influenced by cigarette smoke, endocrine disruptors, heavy metals and maternal diet. It has been suggested that DNA methylation plays an important role in mediating the effects of the intrauterine environment on children health and that there is a need for additional research to better clarify the role of this epigenetic mechanism (Green and Marsit, 2015).

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In utero exposure to chemicals may have a direct effect on the genome integrity (Perera et al., 2002; Laubenthal et al., 2012). In this context, epigenetic changes were shown as the mechanism of environmental interactions with organisms function (Hou et al., 2012; Vanhees et al., 2014). About value of observation of influence of foetus environment on methylation of animals many studies were performed. Also BM1308 COST Action Advances on Large Animal Models: bridging the gap between biomedical research and clinical translation is involved in study of programming effects in preimplantation development and during final conference in September 2017, few reports concerning methylation and foetus development were presented. Influence of environmental pollution on development in utero may explain the changes observed in young animals in Skidniów farm. This is in accordance with the tests carried out on zebrafish larvae exposed to heat and copper stresses leading to increased mortality rates and delayed hatching and upregulation of the expression of *de novo* DNA methyltransferase genes (Dorts et al., 2016).

Epigenetics may thus provide a new tool for understanding mechanisms underlying well-recognised gene-environment interactions and their role in carcinogenesis. Environmental epigenetics may help in identification of: 1) early life exposures causing persistent changes to the epigenome changing disease susceptibility; 2) epigenetic biomarkers of past exposures; 3) reversible epigenetic modifications to target therapeutically or nutritionally (Goodrich et al., 2015). For proper evaluation of correlation between exposures and DNA methylation at key genes it is important to apply stringent quality control measures in the laboratory and careful statistical analysis. Influence of various levels of air pollution and radiation, especially in prenatal development may lead to adaptation to such stressor by epigenetic mechanism (Rossnerova et al., 2017). Linking methylation with gene expression profiles and health outcome data will improve the understanding of the biological implications of observed changes (Goodrich et al., 2015; Rossnerova et al., 2017).

Significant amounts of heavy metals can accumulate in fish tissues (Fatima et al., 2015), modifying reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CATA) activities in brachial, neural, renal and hepatic tissues. Eating fish from polluted rivers may probably influence human health (Fatima et al., 2015). In the same way humans may be exposed to heavy metals by eating polluted fishes and similar situation occurs for cattle's eating polluted grass. Studies performed on tilapia (*Oreochromis mossambicus*) showed accumulation of copper(II) oxide nanoparticles (CuO-NPs) leading to oxidative stress and induction of antioxidant defence which confirmed genotoxicity of heavy metals (Shahzad et al., 2018). Toxic metals are considered as important environmental hazards due to their wide distribution and long persistence leading to accumulation in biological systems. Mercury, lead, nickel, cobalt cadmium and arsenic have been reported to inhibit DNA repair by interfering with the nucleotide and base excision repair (BER) at low, non-cytotoxic concentrations (Asmuss et al., 2000; Hartwig et al., 2002). Various steps of the repair process can be affected by alteration of the binding activity of zinc finger proteins through displacement of

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Zn(II) by those heavy metals. Toxic metals have been shown to affect DNA methylation and DNMTs and therefore influence gene transcription in animal and human studies. DNA repair is also inhibited by the production of ROS (Galaris and Evangelou, 2002; Zawia et al., 2009). Prenatal arsenic exposure is related with 5mC alterations in human cord blood (Rojas et al., 2015). Exposure to heavy metals is connected with induction of post-translational histone modifications by affecting the enzymes involved in their modifications (Langie et al., 2015) and changes in miRNA expression levels were also observed.

Environmental pollution may be caused by the presence of heavy metals as nanoparticles. Nanoparticles affect mitochondrial respiration and can induce genome instability but the mechanism of this phenomenon may depend on the type of pollutants, their size and chemical composition, and the surrounding substance (Langie et al., 2015; Boyles et al., 2016; Murugadas et al., 2016; Wongrakpanich et al., 2016).

The area where the animals from the experimental group were kept can be described as particularly suitable for carrying out investigations concerning influence of environmental factors on animal organism. The blood collected from animals from the experimental group can also be used for other experiments and provide unique model material for studies. The uniqueness of the collected material will allow to carry out further research, possibly including retrospective studies after broadening the knowledge about the impact of environmental contamination on genetic material.

In my work, I demonstrated that contamination of the environment with heavy metals has an enormous impact on animal breeding. The results of the studies were also interesting, because it was possible to obtain material for testing from cattle groups living in extremely different environmental conditions. It may seem that we are currently witnessing the limitation of emissions of harmful substances due to legal regulations and more effective protection of the environment. However, the facts that have happened in recent months have contradicted activities limiting the release of harmful substances into the environment. The innovative technology of the flash furnace, implemented last year in one of the branches of the Głogów Copper Smelter, brings problems instead of benefits due to occurrence of severe failures in the steelworks. The innovative solution should increase the production efficiency of the smelter and reduce its negative impact on the environment. Meanwhile, the level of arsenic in Głogów's air still exceeds the permitted standards

Additonally, in our country, there were massive fires of landfills, which clearly indicated that environmental pollution in Poland still occurrs on an extremely large scale. Dramatically high concentrations of hazardous substances, including arsenic, lead and cadmium, were detected in soil samples collected by the Greenpeace organization in the site of fires of waste storage facilities in Zgierz and Trzebinia. The organization submits notifications to the Regional Directorates of Environmental Protection in Łódź and Kraków about the occurrence of damage to the environment. It also announces referral to the prosecutor's office. This is one of the ways to fight the burning waste problem. The scale of fires and the results of the analyses are alarming. Magdalena

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Figura from Greenpeace Polska believes that the Minister of the Environment should strengthen the institutions responsible for controlling waste storage, so that they have real tools to protect the environment and human health. Prof. dr hab. eng. Adam Grochowalski from the Institute of Inorganic Chemistry and Technology of the Cracow University of Technology also believes that hazardous substances, including heavy metals, which can damage the nervous, circulatory and respiratory systems have been released into the environment. High concentrations of polycyclic aromatic hydrocarbons have also been found in the samples, the toxicity of which is extremely high. Specifically trained Greenpeace activists collected soil samples for testing, three samples from different locations of the same fire site, as well as one background sample taken outside the landfill. Tests carried out by the accredited laboratory Wessling Polska sp. z o.o. showed the presence of high levels of arsenic, lead, cadmium, zinc and copper in all samples. Their concentrations in most samples from the investigated fire sites were several times higher than the permissible contents for industrial sites, defined in the Regulation of the Minister of the Environment of 1st September 2016 on the way of assessing the pollution of the earth's surface (Figura and Pazderski, 2018).

In small amounts, zinc and copper are essential elements for the good functioning of the human body, however, in excess they can lead to kidney and liver damage. A common feature of heavy metals is that they accumulate in the body, and their excretion can be very slow. For example, half of once absorbed lead by a man is excreted only after 50 years, and cadmium after 15 years. Lead and cadmium also accumulate in the placenta of pregnant women and penetrate into the fetal bloodstream, causing severe damage to newborns. Arsenic in turn accumulates in an unnoticeable way for a long time, and symptoms of severe intoxication sometimes appear after a few years.

In the samples tested, there were also high concentrations of polycyclic aromatic hydrocarbons (PAHs), i.e., substances formed during the incomplete combustion of various organic substances. PAHs are classified as persistent organic pollutants characterized by a high tendency to bioaccumulation. They can cause cancer, impair fertility and harm the unborn child. Most PAHs, including benzo(a)pyrene, is classified as toxic at very low doses. The International Agency for Research on Cancer (IARC) included this compound and its derivatives to the substances with the strongest carcinogenic effect for humans already in 1987.

Landfill fires occur frequently in many countries (Cogut, 2016; Gwenzi et al., 2016). In the USA, there are around 8,300 fires per year, and in the UK around 300 (Foss-Smith, 2010). With the potential loss of life and environmental consequences, the need to understand the often mysterious nature of landfill fires is greater than ever. Municipal solid waste has been found to contain a significant quantity of heavy metals, such as Cd, Zn, Pb, and Cu, all of which may eventually end up in the soil and leach down the profile. This qualifies municipal solid wastes among the principal sources of heavy metals in the environment (Alves et al., 2017).

Other identifiable sources include atmospheric deposition, manure and fertilizers, pesticides and industrial discharge. The concern about these heavy metals is that they

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are not biodegradable and may therefore accumulate in the environment. Thus, one of the development challenges facing this decade is how to achieve cost-effective and environmentally-sound strategies to deal with the global waste crisis faced by both developed and developing countries. The crisis has threatened the assimilative and carrying capacity of the earth, which is our life support system.

Heavy metal in a landfill is a chronic environmental problem (Xaypanya et al., 2018). Pollution not only remains around its vicinity during its period of operation, but may linger on for a long time after the landfill or dumpsite ceased operations. To overcome the problem, the management aspect should be systematic and efficient not only at the early phase of operation, but also during and after the landfill is closed from active operation. The regime of actions required to manage issues related to heavy metal pollution requires a substantial reduction of waste sources through the application of integrated management of waste by reducing the generation of wastes at their respective source, recycling, compositing and thermal burning that could reduce the concentration of heavy metals in the wastes.

The situation in Poland is different in this way that fires were probably set deliberately, as the cheapest way to dispose of harmful substances. The lack of any records of stored materials supports such reasoning and indicates that for the sake of profit, considerations about environmental purity are irrelevant. The direct causes of fires are still being investigated. Nevertheless, it can be assumed that arsons could have been started to get rid of waste at least in the illegal dumpsites (Zgierz, Trzebinia and Wszedzień). Only within one week, wastes were burned in Warsaw, Olsztyn, Zgierz, Trzebinia, Jelenia Góra and Wszedzień.

The State Fire Service confirms the alarming increase in statistics concerning fires in these types of facilities, in 2010, there were 59 such interventions, in 2013 and 2014 – around 100, while in 2016 and 2017, there were 117 and 132 interventions, respectively; in 2018, it was already over 80 fires (data from Ministry of Environment webpage with modification, access on 29.05.2018).

In conclusion, the release of harmful substances into the environment by industry is constantly being reduced, however, the import of waste to Poland and its combustion creates a new type of threat and new challenges for the assessment of the impact of such activities on plants, animals and humans.

6. Conclusions

1. The experimental and control groups studied in this dissertation are unique groups of animals for testing the influence of the environment on their genotype and phenotype because they have been bred in dramatically different environmental conditions. The presence of related individuals in both groups further increases the value of the collected biological material. Thus, an unique model was created to study the influence of industrial pollution on living organisms, never reported in the literature.

2. The results confirmed the usefulness of global methylation and global oxidation analyses as markers of epigenetic changes indicating the influence of the environment on animal organisms.

3. It was not possible to determine which of the elements, like heavy metals or other pollutants, coming from the nearby Copper Smelters Głogów I and II were directly responsible for the observed phenotypic, genetic and epigenetic changes but the data from other studies indicated a similar influence of pollutants on living organisms.

4. The studies on two animal groups with a similar genetic background (Jaśmin progeny) living in different environmental conditions may be considered as a unique cattle model for such studies. These animals are no longer available but it is still possible to continue valuable studies on the remaining biological material from these animals as it has been collected in relatively large quantities. Similar studies may also be conducted using new available models.

5. The epigenetic changes and genome instability observed in this study are consistent with the hypothesis that methylation changes occur by environmental pollution in the vicinity of copper industry facilities, due to the action of reactive oxygen species.

6. Statistically significant lower global methylation level observed in experimental animals as compared to control animals ($Z = 5.76$; P-value = 0.0000) confirm the influence of environment pollution near copper smelters on cattle's DNA epigenetic modifications, leading to a decrease in global methylation in the experimental group. This result was confirmed by an analysis of global methylation in Jaśmin progeny in both groups, which eliminated the role of the genetic background ($U = 49.5$; P-value = = 0.0008).

7. The age distribution in the experimental and control group significantly differed ($Z = -3.38$; P-value = 0.0007), with the experimental group being significantly older than the control group. A similar situation was observed for Jaśmin bull progeny which was significantly older than other animals in both groups. This observation validates Jaśmin progeny as a valuable indicator of the environmental pollution.

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8. A decrease in global methylation and an increase in global oxidation levels with age observed for all animals and for controls alone was expected as a result of naturally occurring processes. However, the influence of the environmental pollution has led to unexpected accumulation of oxidative DNA changes in younger animals in the experimental group ($R = -0.35$; P-value = 0.0189).

9. The content of 8-oxo2dG in DNA of animals from the experimental group was significantly higher than among controls ($Z = -5.07$; P-value = 0.0000), which may have resulted from the exposure of the experimental group to pollutants present in their breeding environment. The observed differences in oxidative DNA damage levels between the experimental group and the control group confirm the observations of other investigators. The hypothesised environmental impact on global DNA oxidation levels was confirmed by excluding the role of genetic factors as shown by higher global oxidation level in Jaśmin progeny in the experimental group versus those in the control group ($U = 22$; P-value = 0.0001). Additionally, higher global oxidation levels found in the 16 daughters of Jasny bull in the experimental group ($U = 253.5$; P-value = 0.0002) confirm the stability of oxidation pattern in Jasny progeny among experimental animals, suggesting the influence of other factors.

10. Only weak correlation between global methylation and oxidation levels in Jaśmin progeny ($R = -0.32$; P-value = 0.0815) is not sufficient to confirm the inverse relationship between the level of global methylation and the global oxidation of DNA.

11. Living in a polluted environment is significantly associated with the appearance of one or more changes in the *Alu* profiles as observed in the experimental group as compared to controls (P-value = 0.0001). This observation is consistent with a well-known increase of DNA genetic instability due to environmental pollution.

12. The analysis of alterations in genes involved in DNA damage repair enabled evaluation of the influence of environmental pollution on the global methylation and oxidation of DNA. However, due to the complexity of epigenetic changes in regulatory mechanisms, a direct association of the observed changes in the global methylation and oxidation patterns with the methylation status of selected fragments of the *Apex1* and *Nudt1* gene promoters was not possible to indicate. This may suggest that the analysed fragments were not involved in regulation of gene transcription by methylation of the CpG islands. However, the percentage of animals with the methylated *Apex1* promoter was slightly higher among controls and a significantly lower prevalence of the *Apex1* promoter methylation was observed in the experimental group (P-value = = 0.0028) among Jasny bull progeny. Furthermore, higher levels of global oxidation were observed in those animals from the experimental group who did not have changes in methylation of the *Apex1* promoter ($U = 47.5$, P-value = 0.0436). This suggests that methylation of the *Apex1* promoter is crucial for regulating DNA oxidation level.

13. The observed significant correlation of the *Apex1* gene promoter methylation with age in the experimental group ($U = 52$; P-value = 0.0350) may be associated with higher DNA oxidation levels leading to hypermethylation of specific gene regions and

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affecting the efficiency of oxidative DNA damage repair. Such a correlation was not observed for the methylation of the Nudt1 promoter. However, it should be remembered that in both cases only fragments of promoter regions were analysed.

14. It is of utmost importance to realize that the alterations observed in animals may also occur in humans, especially that despite a significant reduction of pollutant emissions from copper smelters, heavy metal contamination is still present in soil, water and plants. The release from the contaminated soil of heavy metals could affect the health of next generations even after many years. Therefore, it is important to design a durable environment monitoring system in order to prevent the "release" of soil pollutants into human and animal habitats.

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Methylation and oxidative changes in DNA samples of cattle living in environmental conditions contaminated with heavy metals

Abstract

Background. A continuous exposure to pollutants weakens the organism and may lead to permanent changes in its cell structures. It also contributes to an increased incidence of a variety of specific health problems, notably in farm animals bred in polluted areas. The studies described in this dissertation were initiated due to diseases observed in farm animals kept in the Skidniów farm situated in the neighbourhood of Głogów Copper Smelters. The most severe problems were premature pregnancy terminations and deaths of calves during the first days of life, accounting for 22% pregnancy complications altogether. Phenotypic changes in mature animals were difficult to detect. One of the most troublesome problems was the occurrence of skin diseases which affected economic results of the farm. However, although these problems were observed continually and have been confirmed by scientific experiments, the specific influence of contaminants present in the environment on living organisms has not yet been elucidated and is a topic of many studies all over the world.

Objectives. The objective of my studies was to compare methylation levels and oxidative changes in DNA extracted from leukocytes of cattle living in the environment polluted with heavy metals as compared to a control group bred in a pollution-free region.

Methods. The animals under study constituted a representation of the meat type cattle which was upgraded with different proportions of the Limousin breed blood. The progeny of bull Jaśmin was present in both groups (20 half-sisters in the experimental group and 15 among controls) to ensure a similar genetic background between the two groups. Additionally, in the experimental group were also present 16 daughters of other bull – Jasny. Animals from both groups were fed similar diets. Therefore, it can be assumed that the only factor discriminating the two groups was living in extremely different environmental conditions, with the experimental group bred in the environment polluted with heavy metals. In order to determine a possible increase of genetic instability due to environmental pollution, fingerprinting of *Alu* sequences was carried out. The *Alu* profiles were analysed by an electrophoretic analysis of PCR products generated using an *Alu*-specific primer. Moreover, methylation levels of 5-methylcytosine were measured by thin layer chromatography and direct sequencing of promoters of the *Apex1* and *Nudt1* genes. The levels of 8-hydroxydeoxyguanosine as ratio of 8-hydroxydeoxyguanosine (8-oxo2dG) to non-oxidased form of 2-deoxyguanosine (2dG) were determined using an HPLC system with a Gynkotek autosampler and the Hypersil

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BDSTMC18 column with a pre-column (Thermo Hypersil). 8-oxo2dG and 2dG were separated on a 4.6 × 150 mm column.

Results. The average age of animals was 382.22 days in the control group and 556.38 days in the experimental group, and this difference was statistically significant. Similarly, Jaśmin progeny was significantly older than that of other animals.

The methylation status of the *Apex1* gene promoter was analysed for 68 animals (38 controls and 30 from the experimental group) and that of the *Nudt1* gene for 42 animals (26 controls and 16 from the experimental group), both genes have been analysed for 33 of these individuals (21 from the control group and 12 from the study group). I found that changes of Alu fragment patterns in experimental animals occurred more frequent than in the control group, and this difference was highly significant (P-value = 0.0001).

A comparison of global methylation levels between the experimental and control groups showed a statistically significant difference ($Z = 5.76$; P-value = 0.0000). The control group had higher levels of global DNA methylation, while the experimental group showed higher values of global DNA oxidation. Then, a comparison of promoter methylation of the *Apex1* and *Nudt1* genes in both groups was performed. Methylation of the *Apex1* gene promoter was slightly higher in the experimental group than among controls, however this difference was not statistically significant (P-value = 0.2544). Similarly, for the frequency of the *Nudt1* gene promoter methylation, no significant differences between the experimental and control group were observed (P-value = 0.8186).

Age was negatively correlated with global methylation in all animals ($R = -0.23$; P-value = 0.0201), while global oxidation levels positively correlated with age, with a similarly low association strength ($R = 0.25$ and P-value = 0.0135). In control animals alone, a similar correlation was observed only for global oxidation, however the strength of the association was slightly higher ($R = 0.44$; P-value = 0.0014). In the experimental group, global oxidation was negatively associated with age of animals ($R = -0.35$; P-value = 0.0189). Global methylation did not correlate with age neither in the control ($R = 0.14$; p = 0.2734), nor in the experimental group ($R = -0.22$; P-value = 0.1237).

Global methylation levels for the 16 daughters of the Jasny bull from the experimental group were lower than for other animals, however this difference was borderline significant (U = 478.5; P-value = 0.0691). The offspring of bull Jasny had higher global oxidation levels than other cattle ($U = 253.5$; P-value = 0.0002).

Among the progeny of bull Jasny, the *Apex1* gene promoter methylation levels were significantly lower than among other animals from the experimental group (P-value = 0.0028). Higher oxidation levels were observed in cattle from the experimental group with unmethylated *Apex1* gene promoter ($U = 47.5$; P-value = 0.0436).

In the experimental group, the cattle with methylated *Apex1* gene promoter turned out to be older than cattle with unmethylated *Apex*1 ($U = 52$, P-value = 0.0350). Global methylation levels in Jaśmin progeny from the experimental group were significantly lower than in those from the control group ($U = 49.5$; P-value = 0.0008). A comparison of global oxidation in Jaśmin progeny between the experimental and control group showed statistically significant differences ($U = 22$; P-value = 0.0001), with higher oxidation levels for the experimental group. There was no correlation between global methylation and global oxidation for Jaśmin progeny but the results were borderline significant ($R = -0.32$; P-value = 0.0815).

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Discussion and conclusions. The experimental and control group studied here consist of unique groups of animals to test the environmental influence on the phenotype and the genotype due to the fact that the animals have lived in dramatically different environmental conditions. The presence of related individuals in both groups further increases the experimental value of the related biological material. I chose to analyse global methylation and global oxidation levels as markers of epigenetic changes resulting from the influence of the environment on animal organisms. It was interesting to analyse two genes involved in DNA repair, however, due to the complexity of the regulatory mechanisms of epigenetic changes, a direct association of the observed changes with the methylation or oxidation status of selected fragments of the *Apex*1 and *Nudt1* gene promoters was not possible.

A decrease of global methylation and increase in global oxidation levels with age observed for all animals was expected as a result of naturally occurring processes. However, the accumulation of oxidative damage in younger animals from the experimental group was atypical and is likely due to environmental pollution. Significantly lower global methylation levels observed in animals from the polluted area as compared to a control group confirm the influence of environment near copper smelters on genetic stability in farm animals.

The 8-oxo2dG content in DNA of animals from the experimental group was significantly higher than in controls, which may be a result of the experimental group's exposure to pollutants present in their environment. The results of a correlation analysis between global methylation and oxidation in Jaśmin progeny may suggest that the regulation of epigenetic changes may involve interactions between DNA methylation and oxidation levels. Living in a polluted environment was significantly associated with the appearance of one or more changes in the *Alu*-PCR profiles, as was observed in the experimental group in contrast to controls. This observation is consistent with an increase of DNA genetic instability due to environmental pollution, which is widely recognised. It was not possible to determine which of the elements (like heavy metals or other pollutants) coming from the nearby Głogów I and II Copper Smelters was directly responsible for phenotypic, genetic and epigenetic changes but it is consistent with data from other studies indicating such association. It is of utmost importance to realise that the genetic and epigenetic alterations observed in animals may also occur in humans, especially that, despite a significant reduction of pollutant emissions from copper smelters, we still observe the presence of heavy metal contamination in soil, water and plants. Attention should also be paid to the problem of environmental pollution caused by heavy metals resulting from their release during increasingly frequent in Poland landfill fires and smog.

Keywords: DNA methylation, DNA oxidation, heavy metals, environmental pollution, *Alu* sequences, cattle, epigenetic

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Zmiany metylacji i oksydacji w próbkach DNA bydła bytującego w środowisku zanieczyszczonym metalami ciężkimi

Abstrakt

Wprowadzenie. Stała ekspozycja na zanieczyszczenia osłabia organizm i może prowadzić do stałych zmian struktury komórkowej. Przyczynia się także do zwiększenia częstości występowania różnych specyficznych problemów zdrowotnych, szczególnie zwierząt gospodarskich hodowanych na terenach zanieczyszczonych. Badania opisane w niniejszej dysertacji zostały zainicjowane zaobserwowaniem występowania chorób u zwierząt gospodarskich utrzymywanych w gospodarstwie w Skidniowie, usytuowanym w sąsiedztwie Hut Miedzi Głogów. Najpoważniejszym problemem były przedwczesne zakończenia ciąży i upadki cieląt w pierwszych dniach życia – dotyczyły łącznie 22% komplikacji ciąży. Zmiany fenotypowe u dojrzałych zwierząt były trudne do uchwycenia. Jednym z najbardziej kłopotliwych problemów było występowanie chorób skóry, co wpływało na wyniki ekonomiczne gospodarstwa. Chociaż wymienione problemy były stale obserwowane i zostały potwierdzone badaniami naukowymi, specyficzny wpływ zanieczyszczeń obecnych w środowisku, w którym żyją organizmy nie został wyjaśniony i jest głównym tematem wielu badań ogólnoświatowych.

Celem badań było porównanie poziomu metylacji i zmian oksydacyjnych w DNA wyizolowanym z leukocytów bydła żyjącego w środowisku zanieczyszczonym metalami ciężkimi w porównaniu z grupą kontrolną hodowaną w regionie wolnym od zanieczyszczeń.

Metody. Badane zwierzęta reprezentują bydło mięsne z różnymi proporcjami dolewu krwi bydła rasy limousine. Potomstwo buhaja Jaśmina występowało w obydwu (20 półsióstr w grupie eksperymentalnej i 15 wśród grupy kontrolnej) dla zapewnienia podobnego tła genetycznego w obydwu grupach. Dodatkowo w grupie eksperymentalnej występowało 16 córek buhaja Jasnego. Zwierzęta z obydwu grup były karmione według podobnych diet. Stąd można przyjąć, że jedynym czynnikiem różnicującym dwie grupy było bytowanie w ekstremalnie różnych warunkach środowiskowych, z grupą eksperymentalną hodowaną w środowisku zanieczyszczonym metalami ciężkimi.

W celu określenia możliwego wzrostu niestabilności genetycznej w związku z zanieczyszczeniem, środowiska przeprowadzono badania odcisku genetycznego sekwencji *Alu*. Profil *Alu* badano przez rozdział elektroforetyczny produktów PCR uzyskanych z zastosowaniem starterów specyficznych do *Alu*.

Ponadto zmierzono poziom metylacji 5-metylocytozyny z zastosowaniem chromatografii cienkowarstwowej i bezpośredniego sekwencjonowania promotorów genów *Apex1* i *Nudt1*.

Poziom 8-hydroksydeoksyguanozyny oznaczono jako stosunek 8-hydroksydeoksyguanozyny (8-oxo2dG) do nieutlenionej formy 2-deoksyguanozyny (2dG) z zastosowaniem systemu HPLC z autosamplerem Gynkotek i kolumną Hypersil BDSTMC18 i prekolumną (Thermo Hypersil). 8-oxo2dG id 2dG były rozdzielane na kolumnie 4,6 × 150 mm.

Wyniki. Średni wiek zwierząt grupy kontrolnej wynosił 382,22 dnia, a grupy eksperymentalnej 556,38 dnia, różnica ta była statystycznie istotna. Podobnie potomstwo buhaja Jaśmina było istotnie starsze od innych zwierząt.

Status metylacji promotora genu *Apex1* badano u 68 zwierząt (38 kontrolnych i 30 z grupy eksperymentalnej) a genu *Nudt1* u 42 zwierząt (26 kontrolnych i 16 z grupy eksperymentalnej), obydwa geny analizowano dla 33 zwierząt (21 z grupy kontrolnej i 12 z grupy eksperymentalnej). Zaobserwowano, że zmiany w profilach fragmentów *Alu* u zwierząt eksperymentalnych występują częściej niż w grupie kontrolnej, a różnica ta była wysoce istotna ($P = 0,0001$).

Porównanie całkowitego poziomu metylacji w grupie eksperymentalnej i grupie kontrolnej wykazało różnice istotne statystycznie ($Z = 5,76$; $P = 0,0000$). Grupa kontrolna ma wyższy poziom całkowitej metylacji DNA, natomiast grupę eksperymentalną charakteryzuje wyższy poziom całkowitej oksydacji DNA. Następnie dla obydwu grup przeprowadzono porównanie metylacji promotorów genów *Apex1* i *Nudt1*. Metylacja promotora genu *Apex1* była nieznacznie wyższa w grupie eksperymentalnej niż wśród zwierząt kontrolnych, jednak różnice nie były statystycznie istotne ($P = 0,2544$). Podobnie w przypadku częstości metylacji promotora genu *Nudt1* nie wykazano istotnych różnic między grupą eksperymentalną a grupą kontrolną ($P = 0,8186$).

Wiek korelował negatywnie z całkowitą metylacją dla wszystkich zwierząt ($R = –0,23$; $P = 0,0201$), a całkowity poziom oksydacji korelował pozytywne wraz z wiekiem, z podobnie małą siłą ($R = 0,25$; $P = 0,0135$). Wyłącznie u zwierząt kontrolnych podobna korelacja była obserwowana tylko do całkowitej oksydacji, ale siła asocjacji była nieznacznie większa ($R = 0,44$; $P = 0,0014$). W grupie eksperymentalnej całkowity poziom oksydacji był zasocjowany negatywnie z wiekiem zwierząt ($R = –0,35$; $P = 0,0189$). Całkowita metylacja nie korelowała z wiekiem ani w grupie kontrolnej ($R = 0,14$; $P = 0,2734$), ani w grupie eksperymentalnej ($R = –0,22$; $P = 0,1237$).

Całkowity poziom metylacji 16 córek buhaja Jasnego z grupy eksperymentalnej był niższy niż u pozostałych zwierząt, chociaż ta różnica była na granicy istotności ($U = 478,5$; $P = 0,0691$). Potomstwo buhaja Jasnego wyróżniała wyższa całkowita oksydacja niż pozostałe zwierzęta ($U = 253,5$; $P = 0,0002$).

Wśród potomstwa buhaja Jasnego metylacja promotora genu *Apex1* była istotnie mniejsza niż u pozostałych zwierząt z grupy eksperymentalnej ($P = 0,0028$). Wyższy poziom oksydacji zaobserwowano u bydła z grupy eksperymentalnej z niemetylowanym promotorem genu *Apex1* ($U = 47,5$; $P = 0,0436$).

W grupie eksperymentalnej bydło z metylowanym promotorem genu *Apex1* okazało się starsze niż bydło z niemetylowanym *Apex1* ($U = 52$; $P = 0,0350$). Całkowity poziom metylacji potomstwa Jaśmina z grupy eksperymentalnej był istotnie niższy, niż potomstwa Jaśmina w grupie kontrolnej ($U = 49,5$; $P = 0,0008$). Porównanie całkowitej oksydacji u potomstwa Jaśmina między grupą eksperymentalną a grupą kontrolną wykazuje statystycznie istotne różnice ($U = 22$; $P = 0,0001$), z wyższym poziomem oksydacji w grupie eksperymentalnej. Nie

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wykazano korelacji między całkowitym poziomem metylacji a całkowitym poziomem oksydacji dla potomstwa Jaśmina, ale wyniki są na granicy istotności ($R = -0,32$; $P = 0,0815$).

Dyskusja i wnioski. Badane zwierzęta z grupy eksperymentalnej i kontrolnej obejmują unikatowe grupy zwierząt umożliwiające testowanie wpływu środowiska na fenotyp i genotyp, ponieważ zwierzęta żyły w dramatycznie różnych warunkach środowiskowych. Obecność spokrewnionych osobników w obydwu grupach zwiększa dodatkowo wartość eksperymentalną zebranego materiału biologicznego. Wybrano badanie całkowitego poziomu metylacji i całkowitego poziomu oksydacji jako markerów zmian epigenetycznych wynikających z wpływu środowiska na organizmy zwierząt. Interesująca była analiza dwóch genów naprawy DNA, ale ze względu na złożoność mechanizmów regulatorowych zmian epigenetycznych nie była możliwa bezpośrednia asocjacja wybranych fragmentów promotorów genów *Apex1* i *Nudt1* z obserwowanymi zmianami statusu metylacji czy oksydacji.

Obserwowane wraz z wiekiem u wszystkich zwierząt zmniejszenie metylacji całkowitej i zwiększenie oksydacji całkowitej było oczekiwane zgodnie z naturalnie występującymi procesami starzenia. Akumulacja uszkodzeń oksydacyjnych u młodszych zwierząt z grupy eksperymentalnej była jednak nietypowa i prawdopodobnie wynikała z zanieczyszczenia środowiska. Istotnie mniejsza metylacja całkowita obserwowana u zwierząt z zanieczyszczonego obszaru, w porównaniu z grupą kontrolną, potwierdza wpływ środowiska w pobliżu hut miedzi na stabilność genetyczną zwierząt gospodarskich.

Zawartość 8-okso2dG w DNA zwierząt z grupy eksperymentalnej była znacznie większa niż w grupie kontrolnej, co może być wynikiem narażenia grupy eksperymentalnej na zanieczyszczenia występujące w ich środowisku. Wyniki analizy korelacji między całkowitą metylacją a oksydacją u potomstwa buhaja Jaśmina mogą sugerować, że regulacja zmian epigenetycznych może obejmować interakcje między metylacją DNA a poziomem utlenienia DNA. Życie w zanieczyszczonym środowisku było związane istotnie z pojawieniem się jednej lub więcej zmian w profilach *Alu*-PCR, jak stwierdzono w grupie eksperymentalnej w przeciwieństwie do kontroli. Ta obserwacja jest zgodna z powszechnie uznanym wpływem zanieczyszczenia środowiska na wzrost niestabilności genetycznej DNA.

Nie można było ustalić, który z czynników (metale ciężkie lub inne zanieczyszczenia) pochodzących z pobliskich Hut Miedzi Głogów I i II był bezpośrednio odpowiedzialny za obserwowane zmiany fenotypowe, genetyczne i epigenetyczne, ale jest to zgodne z danymi z innych badań wskazującymi występowanie takich powiązań. Niezwykle ważna jest świadomość, że obserwowane u zwierząt zmiany genetyczne i epigenetyczne mogą wystąpić również u ludzi, zwłaszcza że pomimo znacznego zmniejszenia emisji zanieczyszczeń z hut miedzi, nadal stwierdzamy obecność metali ciężkich i innych zanieczyszczeń w glebie, wodzie i roślinności. Należy również zwrócić uwagę na problem zanieczyszczeń środowiska metalami ciężkimi wynikający z uwalniania ich podczas coraz częstszych w naszym kraju pożarów wysypisk śmieci, a także zanieczyszczeń wywołanych smogiem.

Słowa kluczowe: metylacja DNA, oksydacja DNA, metale ciężkie, zanieczyszczenie środowiska, sekwencje *Alu*, bydło, epigenetyka