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Structural Chromatin Alterations in Peripheral Blood Leukocytes of Alcohol-dependent Individuals During Detoxification Therapy

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Abstract. *Background/Aim: The aim of this study was to investigate the state of chromatin condensation in peripheral blood leukocytes of alcoholics, during the early detoxification period, in order to highlight structural modifications, indicating epigenetic mechanisms regulated by alcohol. Materials and Methods: Blood samples were obtained from alcoholic patients, who were admitted for detoxification on an inpatient basis, and from healthy controls. The level of condensed heterochromatin and de-condensed euchromatin were detected through the ratio of lysine to arginine residues, by the application of the ammoniacal silver reaction (ASR) staining on leukocyte pellets, and through immunohistochemical localization of histone H1 on peripheral blood smears. Results: Lymphocytes and neutrophils with relaxed de-condensed chromatin were found, indicating a more reactive genome in alcoholics, even at the stage of detoxification. Conclusion: The results underline the importance of chromatin structure of leukocytes as a sensitive, peripheral, biological marker for epigenetic studies in living chronic alcoholics.*

Alcohol is one of the most widely used addictive substances, and continued use and abuse can lead to the development of tolerance and dependence (1-3). Alcohol dependence is a common, chronic, relapsing disorder (4), and chronic alcohol abuse exerts a major social and medical toll worldwide (5). Numerous studies have shown that both genetic and environmental risk factors play a role in the development of alcoholism (3, 6-8). Alcohol intake alters gene expression patterns, thereby producing long-lasting cellular and molecular adaptations that might explain the development and maintenance of alcohol dependence. The heterogeneous nature of alcohol dependence indicates a complex etiology involving mechanisms related to motivational behavior, reward and learning, and adaptation in signaling pathways owing to interactions between alcohol and target molecules (4). Although whole-genome expression profiling has highlighted the importance of several genes that may contribute to alcohol abuse disorders (9, 10), more recent findings have added yet another layer of complexity to the overall molecular mechanisms involved in a predisposition to alcoholism and addiction by demonstrating that processes related to genetic factors that do not manifest as DNA sequence changes *i.e.* epigenetic processes also play a role (11, 12). Epigenetic alterations include the direct addition of methyl groups to DNA and chemical modifications (acetylation, methylation, phosphorylation) of the histone proteins around which the DNA is wrapped to form the chromosomes. Both of these mechanisms work in concert to remodel the structure of the protein–DNA complex (*i.e.* the chromatin) and regulate gene expression (3, 13-15).

The chromatin fiber is a dynamic, malleable structure that is targeted by numerous regulatory factors that modify histones and DNA, and remodel the structure of the nucleosomes. The dynamics of the chromatin fiber reflect the combined action of numerous chromatin modifiers, including architectural proteins such as histone H1 (16). The interaction of histone H1 with nucleosomes stabilizes the higher-order compact chromatin structure (17), thereby restricting the ability of regulatory factors, nucleosome remodeling complexes, and histone modifiers to access their chromatin binding sites (18-20). The biology of the H1 and

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Table I. *Clinical characteristics of the alcoholic patients, participants in the leukocyte study.*

Code no.	Age (years)/Gender	Alcohol dependence (years)	Alcohol consumption (g/day)	Abstinence from alcohol (days)
H497	30/F	9	265.44	19
H471	34/M	13	341.3	106
H476	38/M	7	240.16	11
H496	38/M	20	404.48	40
H467	39/M	17	290.7	110
H468	51/M	31	176.96	20
H498	52/M	30	227.52	42
H495	56/M	24	214.88	42
H466	56/M	42	88.5	142

M: Male; F: female.

related linker histones is complex, as they are evolutionarily variable. It exists in multiple isoforms and undergoes a large variety of post-translational modifications in its long, unstructured NH_2- and COOH-terminal tails (21). Contrary to core histones, H1 comprises three domains, one structured ('globular') domain and two unstructured, which specifically are lysine-rich *N*- and *C*-termini domains. In particular, the C-terminal domain accounts for more than half the histone H1 sequence, with ~40% composed of lysine residues (22).

There is an increasing body of evidence pointing to a close integration between the central nervous system and immunological functions, with lymphocytes playing a central role (23). Many investigations have shown that there is a close association between the state of the immune system, regarding leukocytes in particular, and major psychiatric disorders such as mania (24), depression (25), stress and anxiety (26) and schizophrenia (27). As early as 1975, Issidorides and co-workers (28) noted a difference in the nucleo-histone staining patterns of peripheral blood neutrophils in patients with schizophrenia compared to healthy controls, going so far as to suggest an increased dissociation of the lysine-rich H1 linker histone in schizophrenia resulting in a de-condensed pattern of heterochromatin. More recently, similar findings were confirmed by the measurement of the extent of chromatin de-condensation, in blood lymphocytes of first episode, drug-free, patients with schizophrenia, as measured by the level of exposed arginine residues in the core histones (29). This investigation reported a higher ratio of arginine/lysine residues, indicating a more 'reactive' genome in patients with new onset of schizophrenia.

Experimental and clinical data support the conclusion that alcohol is a potent immunomodulatory agent. Although impaired immunity in patients with chronic alcohol use has long been described in the medical literature (30), data of the immune status of chronic alcoholic patients show discrepancies. Chronic alcoholics are more prone to infections with a variety of pathogens, have less ability to fight against infections and have an increased risk of developing cancer (31). While malnutrition, vitamin deficiency, and advanced liver cirrhosis can contribute to some of the immune abnormalities in chronic alcoholics, alcohol itself is a potent modulator of the immune system and it can affect it at the level of innate and acquired immune responses (32).

Considering all the above, the present study was designed to investigate the state of chromatin condensation in the peripheral blood leukocytes of alcoholic patients, applying histochemical and immunohistochemical techniques.

Materials and Methods

Participants. The study cohort included nine alcoholics (Table I), selected out of a group of 25 randomly registered individuals over a 1-year period, who had successively contacted the Drug and Alcohol Addiction Clinic of the Athens University Psychiatric Clinic at the Eginition Hospital. All patients voluntarily sought treatment and fulfilled the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) diagnostic criteria for alcohol abuse/dependence, 'primary alcoholism' (33), and were admitted at this specialized clinic for alcohol detoxification on an inpatient basis. Detailed information on the objectives of the study was provided to all participants, who all provided their informed consent. Ethical permission for the study was obtained from the Special Scientific Committee of the hospital (Approval Number 181/31.5.2007) and the procedures followed were in accordance with the Declaration of Helsinki (1964), as amended in the 59th World Medical Association General Assembly in Seoul (2008) (34).

The nine participants registered in the present investigation were selected from the above in-patient group, according to the following criteria: (i) age 30-56 years; (ii) good nutritional state and absence of serious physical illness (as assessed through physical examination and routine laboratory screening); (iii) absence of another pre- or co-existing major psychiatric disorder on the DSM-IV axis I, and (iv) absence of other drug/substance abuse. The mere presence of affective symptoms of anxiety was not considered to be an exclusion criterion. Alcohol abusers who fulfilled a DSM-IV diagnosis of

depressive disorder were excluded from the study if a major depressive episode had been recorded before the onset of alcoholism.

The experimental group had abstained from alcohol for a mean±SD of 59.11±49.39 days prior to blood collection. They used to consume a mean of 250.00±91.75 g alcohol per day and they had a mean of 21.44±11.46 years of alcohol dependence (Table I). Upon admission, alcohol detoxification was initiated and completed over one week (7-10 days). Detoxification comprised of vitamin replacement (vitamin C, vitamin E, vitamins of B complex) and oral administration of diazepam (10-40 mg in divided doses), with gradual taper off over one week. Thereafter, all patients were given mirtazapine (30-60 mg in 1-2 divided doses per day) in addition to the standard treatment with a short-term psychotherapy of cognitive-behavioral orientation.

Nine healthy volunteers, aged 29 to 55 years (Table II), were also included in the study as a control group, and they also gave their written informed consent.

Table II. *Control participants in the leukocyte study.*

Code no.	Age (years)/Gender
H427	29/F
H512	33/F
H509	33/M
H425	34/M
H428	37/F
H433	37/M
H418	46/M
H472	48/M
H474	55/M

M: Male; F: female.

Preparation of blood cells. Peripheral blood, obtained by venipuncture from both controls and alcoholics, was collected in EDTA-K3-salt-containing tubes and processed for the application of the Ammoniacal Silver Reaction (ASR). Blood smears were also prepared for histone H1 immunohistochemistry.

ASR of Black and Ansley (35), modified for en bloc staining. The ammoniacal silver reaction (ASR) method was applied to blood leukocyte pellets. Briefly, 3 ml of peripheral venous blood from alcoholic patients or controls collected in tubes containing 3 mg EDTA-K_3-salt were left undisturbed for two hours at 4˚C and for a further hour at room temperature (RT). After sedimentation of the erythrocytes, the leukocyte layer with the supernatant plasma was removed, transferred to a centrifuge glass tube and centrifuged at 330× *g* for 10 min at RT in a swing-out rotor. The supernatant was discarded and the leukocyte pellet was then fixed with 10% formalin, and neutralized with 2% sodium acetate for 24 h at RT. After dissecting the fixed pellet into small pieces and washing them in distilled water, they were stained with ammoniacal silver reagent carefully prepared as described by Black and Ansley (35), for 8 min, at RT. Then, they were placed for 2 min in 3% formalin solution, and washed. Subsequently, they were dehydrated, embedded in epoxy resins, and polymerized at 60˚C for 24 h. Semi-thin sections (1 μm) from 10 randomly selected epoxy resin capsules per participant were cut with an ultramicrotome Leica Ultracut R (Leica Mikrosysteme GmbH, Vienna, Austria), mounted on microscope glass slides and examined under a Leitz Aristoplan light microscope (Leica Microsystems CMS, GmbH, Wetzlar, Germany), equipped with a CCD color camera. Image analysis was then performed using the Image-Pro Plus software by Media Cybernetics (Silver Spring, MD, USA). Further thin sections (160-180 nm) from the capsules were cut, mounted on copper grids, viewed without further staining in a Philips EM 201C electron microscope (Amsterdam, The Netherlands) and photographed with Agfa Copex HDP13 microfilm (Belgium).

Histone H1 immunocytochemistry. Blood smears were prepared on pre-cleaned SuperFrost Plus microscope slides. The preparations were fixed in a buffered formalin-acetone solution (10% formaldehyde, 45% acetone, pH 6.6) for 30 s at 4˚C, which is one of the best fixatives for preservation of nuclear morphology and for immunocytochemical applications (36, 37). After air-drying, they were rehydrated in Tris-buffered saline (TBS) and pre-treated in avidin-biotin blocking kit (HK102-5K; Biogenex, San Ramon, CA, USA), for the elimination of non-specific background staining. The preparations were incubated with primary monoclonal antibody to H1 (clone 1415-1; MS-628-P; LabVision/Neomarkers, Fremont, CA, USA) at a concentration 3 μg/ml in TBS, for 30 min, at RT, and then washed in TBS. In order to visualize the end-product of the immunocytochemical reaction, blood smears were incubated consecutively with a multilink biotinylated secondary antibody, an alkaline phosphatase-conjugated streptavidin solution, and naphthol phosphate/fast red chromogen (Super Sensitive Detection Kit QA200-OX; Biogenex, San Ramon, CA, USA). The preparations were finally counterstained with Gill's hematoxylin, mounted in an aqueous mounting medium and examined under a ×100 oil immersion objective in a Leitz Aristoplan microscope. Photomicrographs were taken with a Leitz Orthomat E automatic camera (Leica Microsystems CMS, GmbH, Wetzlar, Germany), using Kodak Ektachrome 64T color reversal film (Kodak Limited, England).

Image Analysis – Morphometry of ASR-stained Epon semi-thin sections. Image analysis was carried out using the Image-Pro Plus, version 3.0, software for Windows NT (Media Cybernetics, Inc. Rockville, MD, USA). The purpose was to measure the yellow nuclear areas representing lysine and the brown-black nuclear areas representing arginine in about 300 nuclei of leukocytes per individual, automatically using a number of commands from the software, as previously described (29). Briefly, the yellow areas were defined and measured in μm^2 in every nucleus, while the brown-black areas were calculated automatically by the subtraction of the yellow area from the whole defined nuclear area. The relative availability of lysine and arginine was expressed in percentages and all the measurements were automatically recorded and statistically assessed.

Statistical analysis. All the statistical tests were performed with the STATISTICA 6.1 (StatSoft, Inc. Tulsa, OK, USA) software package. In order to compare the lysine and arginine areas between the polymorphonuclear and mononuclear cells of the two groups, a standard factorial MANOVA approach was employed (38). The two groups and the two types of cells, with the two dependent variables, lysine and arginine, constitute a 2×2 design.

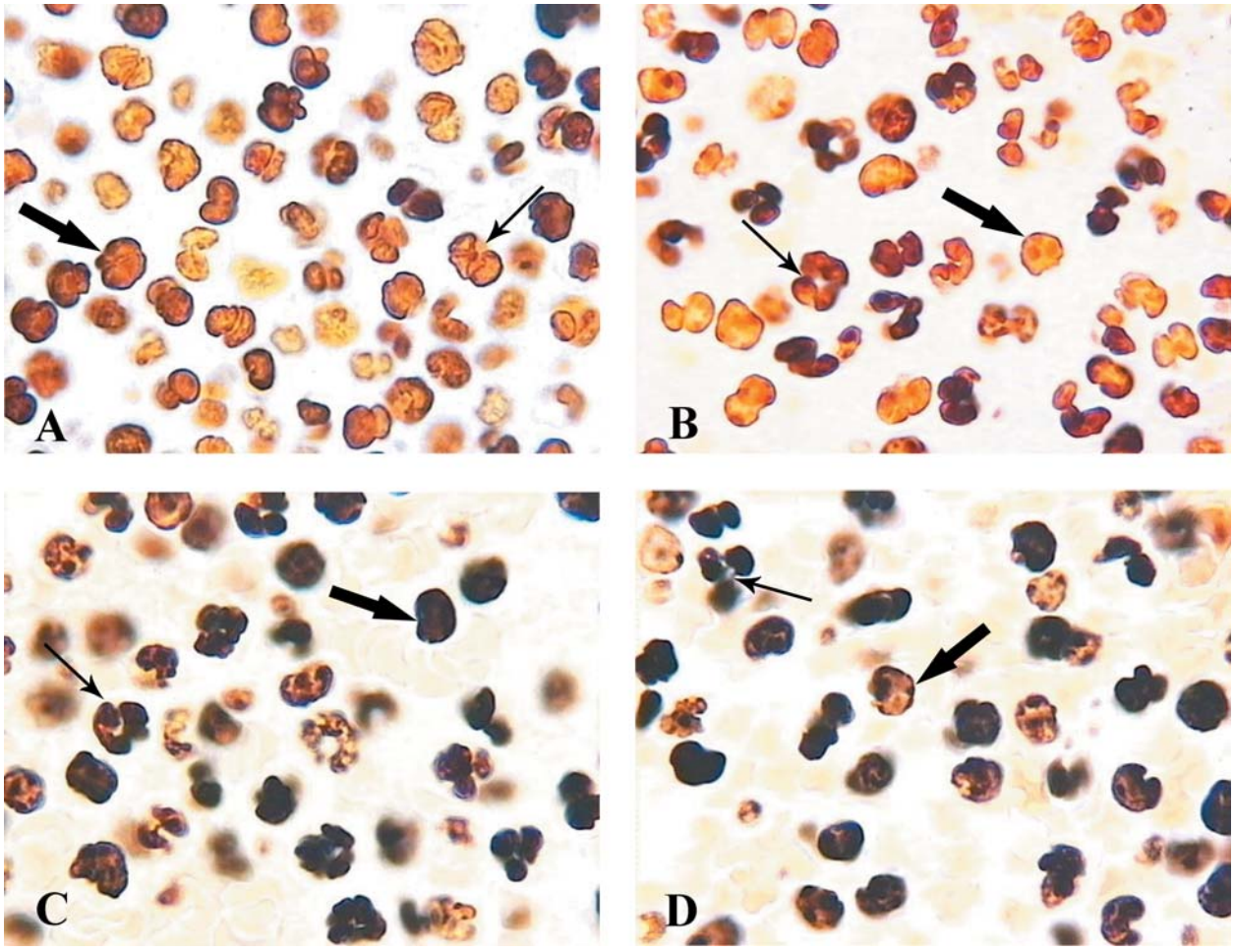

Figure 1. *Photomicrographs of representative fields of the Epon semi-thin sections of the leukocyte nuclei from controls as stained with ASR (A, B). Prevalence of yellow nuclear ASR staining, which represents lysine-rich histones, in the majority of mononuclear (thick arrows) and polymorphonuclear cells (thin arrows) is evident. In contrast, leukocyte nuclei from the alcoholic patients (C, D) show the prevalence of an increased amount of nuclear brown-black ASR staining, which represents arginine-rich histones, in the majority of mononuclear (thick arrows) and polymorphonuclear cells (thin arrows). Original magnification, ×2,500.*

Results

ASR staining. Light microscopy: Under light microscopy, a survey of all the ammoniacal-stained Epon semi-thin sections of the leukocyte pellets from the controls revealed an almost common staining pattern of all nuclei: the characteristic colors of the ASR, ranging from yellowish brown to blackish, with a notable prevalence of yellowish brown staining, which represents lysine-rich histones, in the majority of both mononuclear and polymorphonuclear nuclei. Representative fields of these sections, from two different controls, are shown in Figure 1A and B. In contrast, the ASR staining of both mononuclear and polymorphonuclear cells from the alcoholic patients revealed the prevalence of brown-black nuclear staining, which represents arginine-rich histones. Representative fields of these sections, from two different alcoholic patients, are shown in Figure 1C and D.

After image analysis of the semi-thin sections, the comparison of the percentages of lysine and arginine areas showed a statistically significant predominance ($p \leq 0.05$) of lysine *versus* arginine in the nuclei of both mononuclear (lysine/arginine: 0.80/0.20±0.20) and polymorphonuclear (lysine/arginine: 0.66/0.34±0.24) cells of controls. On the contrary, the same comparisons in the alcoholic patients showed a predominance of arginine over lysine in the nuclei of both mononuclear (arginine/lysine: 0.57/0.43±0.25) and polymorphonuclear (arginine/lysine: 0.68/0.32±0.21) cells,

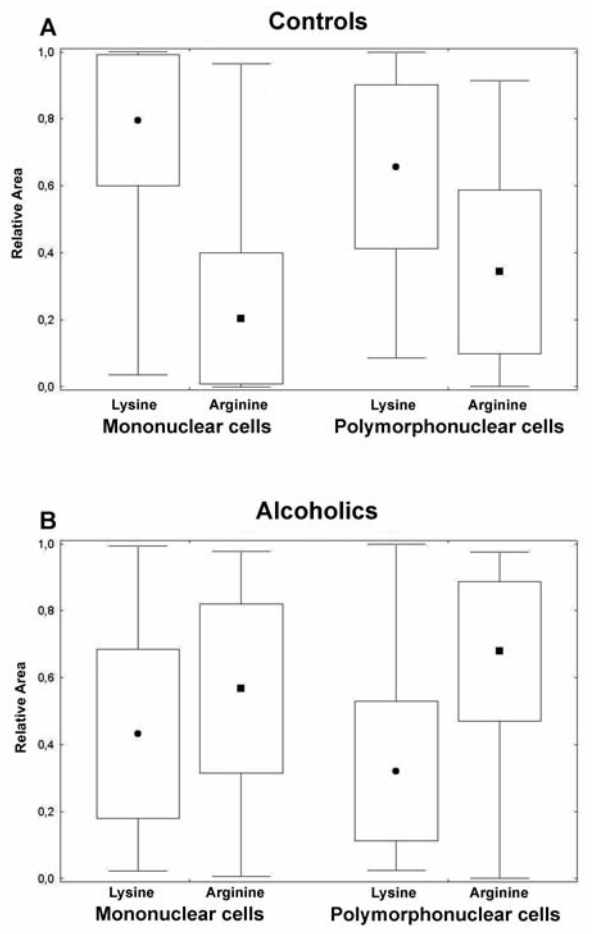

Figure 2. *Box and whisker plots showing the distribution of the percentages of lysine and arginine areas in the nuclei of mononuclear and polymorphonuclear lymphocytes of the controls (A) and alcoholic patients (B). They illustrate the predominance of lysine in controls and of arginine in alcoholics. The box represents the interquartile range, and the whiskers represent the minimum and maximum of the range, according to image analysis measurements reported in the text. The Y axis values are reduced to the unit.*

which was statistically significant only in the latter ($p \leq 0.05$). These results are further illustrated in Figure 2 using box and whisker plots.

In order to investigate putative correlations between certain parameters in the study (age, years of alcohol dependence, alcohol consumption, abstinence from alcohol) and lysine and arginine concentrations, Spearman's correlation coefficient analysis was used as being insensitive to the distribution of the variables involved. However, the analysis revealed no statistically significant correlations between the concentrations of lysine and arginine and these parameters in the mononuclear or polymorphonuclear cells ($p > 0.05$).

Electron microscopy: Viewed under electron microscopy, discrete electron-opaque particles were distributed in the area of heterochromatin of leukocyte nuclei from the controls (Figure 3A and B) and the alcoholic patients (Figure 3C and D). In the nuclei of mononuclear and polymorphonuclear lymphocytes from the alcoholic patients, there was an increase in deposits observed as heavy grains associated with heterochromatin, as opposed to the lymphocyte nuclei of the controls, where the deposits observed as light grains were associated with heterochromatin. By comparing the ASR results from light and electron microscopy, it was found that the coarse deposits corresponded to the prevalence of the blackish stain seen under light microscopy in the majority of mononuclear and polymorphonuclear lymphocytes of alcoholics. In contrast, the yellow nuclear stain, seen under light microscopy in the majority of mononuclear and polymorphonuclear lymphocytes of controls, corresponded only to the light grains of ASR in the area of heterochromatin, as it was seen under electron microscopy.

Immunocytochemistry. The results from the study of the nine controls showed that H1 immunoreactivity was distributed over the whole nucleus in the majority of mononuclear leukocytes and over the entire area of the nuclear lobes of polymorphonuclear leukocytes (Figure 4A and B). The reaction appeared more intense along the periphery of the lobes and staining accumulated adjacent to the nuclear envelope, where heterochromatin is located. Diffuse immunoreaction was also observed throughout the nuclear lobes, while the cytoplasm was completely unstained. Contrary to the above, in the alcoholic patients, H1 immunoreactivity was found to be highly reduced, (Figure 4C and D). Specifically, the immunoreaction of polymorphonuclear and mononuclear lymphocytes was shown to be irregular by distributed, incomplete, vacuolated, patchy or completely absent. These patterns were distinctive for each alcoholic patient.

Discussion

The present study was designed to investigate the state of chromatin condensation in the peripheral blood leukocytes of alcoholic patients, during the early detoxification period, as compared to age- and sex-matched healthy individuals. For this reason, we used the histochemical ASR staining, as well as the immunohistochemical method for the detection of histone H1.

The staining pattern under light microscopy in the majority of lymphocyte nuclei of the alcoholic patients, when compared to controls, was characterized by a change in the color of the ASR staining from yellowish to brown-black, which is associated with an increase in the ratio of arginine-to-lysine residues, and was especially apparent in

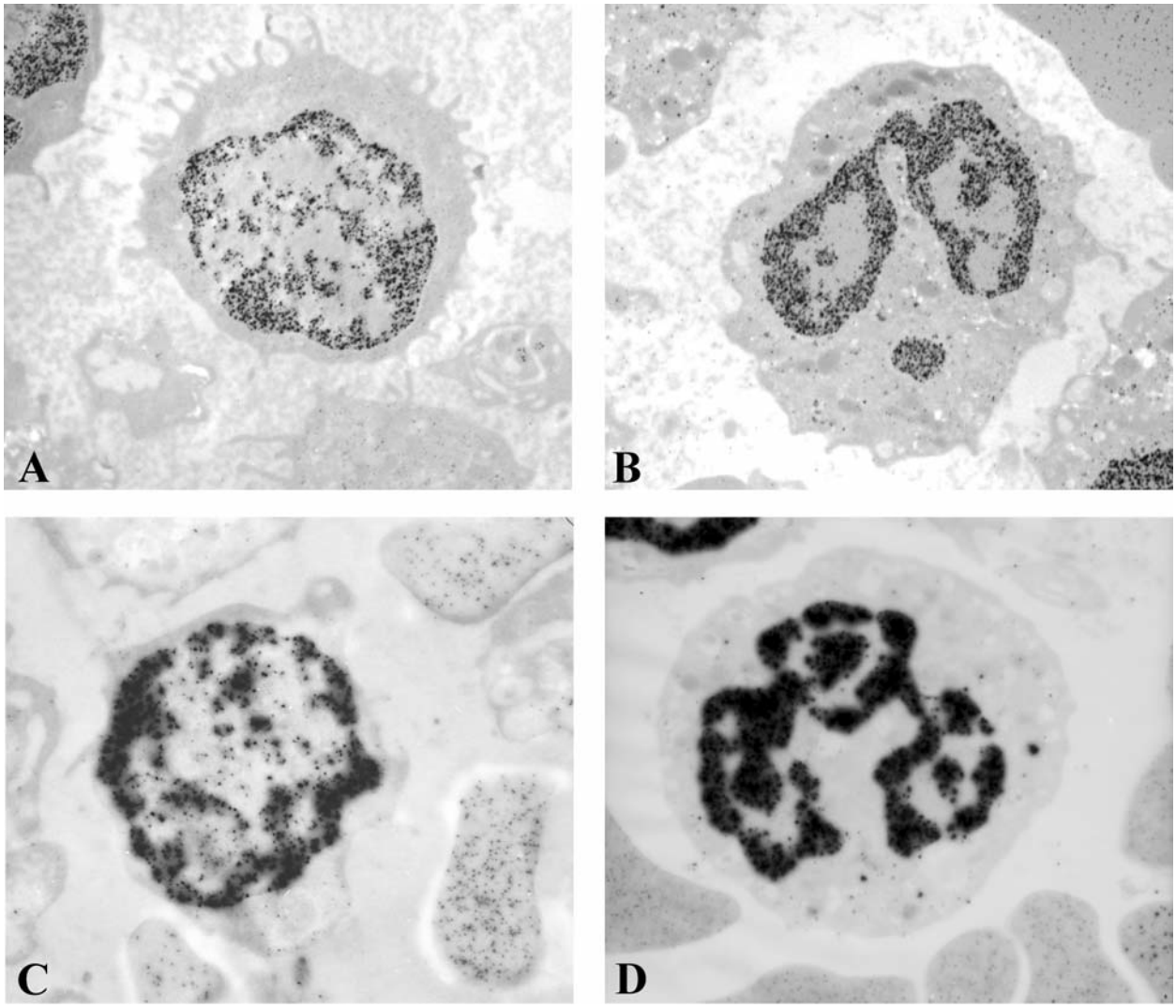

Figure 3. *Electron micrographs of leukocytes stained with ASR, from controls and alcoholic patients. The discrete, electron-opaque ASR deposits, associated with heterochromatin, are shown in mononuclear (A) and polymorphonuclear (B) lymphocytes of controls. On the contrary, in the corresponding cells of alcoholics, the ASR deposits heavily cover the nuclear areas of mononuclear and polymorphonuclear lymphocytes (C, D). Original magnification, ×9,600.*

the polymorphonuclear leukocytes ($p \leq 0.05$). These observations were confirmed under electron microscopy, where fine particles by ASR were seen covering the heterochromatin areas of nuclei of mononuclear and polymorphonuclear leukocytes of controls, as opposed to coarse particles covering the corresponding area on the leukocytes of alcoholics. These findings are consistent with earlier experiments in developing erythroid cells of chick bone marrow (39) where the ASR coarse, discrete electron-opaque particles (190-200 Å in diameter), which observed in EM, appeared to correspond to the brown–black stain seen with the LM. The authors argued that this was due to some specific interaction of silver with reactive centers in arginine and was detectable in either newly-synthesized proteins with a high content of arginine, or in some structural rearrangement or reorientation of the histones, which made previously masked reactive sites available to the ASR.

From these results, it becomes clear that staining by ASR reflects conformation changes in histone molecules and thus, in chromatin structure. As early as 1968, Tokuyasu and co-workers demonstrated that the first visible sign of lymphocyte activation under electron microscopy was the de-condensation of the compact chromatin structure of resting nuclei (40). Later, another study argued that the decondense

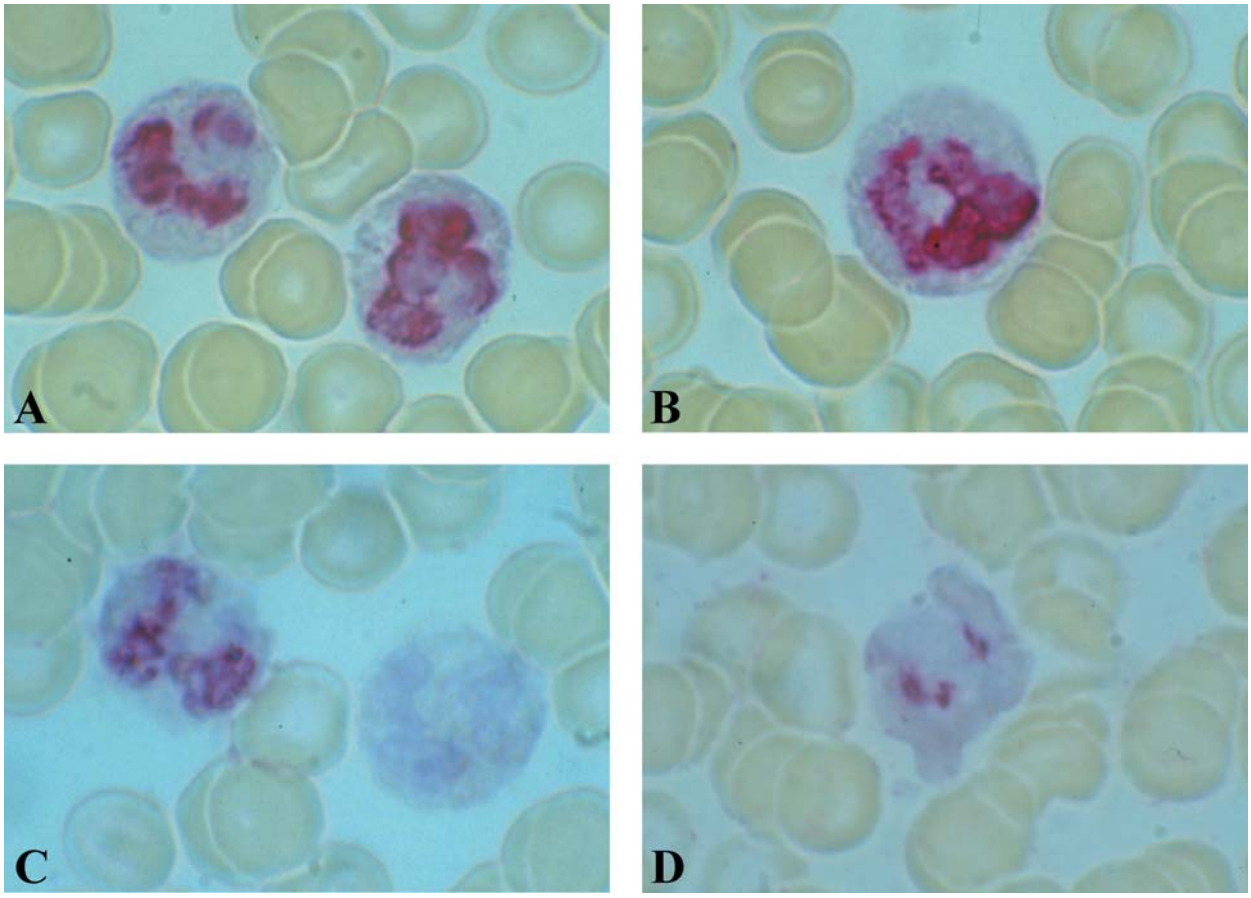

Figure 4. *Photomicrographs of representative fields of blood smears, demonstrating patterns of distribution of histone H1immunoreactivity (red color), in the nuclei of polymorphonuclear leukocytes. Intense immunoreaction covers the majority of the nuclear area of polymorphonuclear taken from the controls (A, B), while reduced and irregular immunoreaction (incomplete vacuolated, patchy or completely absent) is shown in the nuclear lobes of polymorphonuclear leukocytes from the alcoholic patients (C, D). Immunostaining with antibody to histone H1, Fast Red chromogen system method. Original magnification, ×1,300.*

state of the chromatin is due to dissociation of the lysine-rich histone H1 from the nucleosomes, exposing the arginine residues of the core histones H3 and H4 (41). Thus, the notable prevalence of the yellow stain of lysine-rich histones in the majority of both mononuclear and polymorphonuclear lymphocytes of controls in our study indicates a state of chromatin condensation and compact condition of the genome. These results compare favorably with those obtained from the immunohistochemistry, where H1 immunoreactivity was distributed over the entire area of nuclei of the majority of leukocytes, revealing the equivalence of the condensed state of the chromatin. All these findings were expected, since the controls selected for the study were physically and psychologically healthy, infection-free, with normal complete blood count, and thus, their mononuclear and polymorphonuclear lymphocytes were in a 'resting' phase (42, 43). On the contrary, in alcoholic patients, the decrease in the yellow-staining fraction, representative of the increased arginine-to-lysine ratio, indicates an aberrant de-condensed, relaxed state of the chromatin, allowing expression of the genome. Chromatin de-condensation was also confirmed by the reduction of the H1 immunoreactivity, particularly in the lobes of nuclei of polymorphonuclear cells.

All of the above are in agreement with previous studies showing that transcriptionally active chromatin contains less histone H1 than transcriptionally inactive regions (44). As pointed out earlier, histone H1 is an essential player in modulating and maintaining chromatin architecture (45). The globular domain of histone H1 interacts with both the nucleosome dyad and two short (10 bp) sequences at the very beginning of each of the two linker DNAs. The C-terminus of H1 binds to the remaining linker DNA, bringing together the two linkers and forming the stem-like structure,

and thus leads to chromatin compaction (46). Harshman and co-workers indicated that the length, charge and number of posttranslational modification sites of the C-terminal tails vary between histone H1 isoforms (47), suggesting that individual H1 variants may play distinct roles in the regulation of higher-order chromatin structure. The compaction of chromatin by the linker histone H1 in general has a global and repressive impact on transcription. Binding of H1 to DNA at the termini of nucleosomes inhibits spontaneous wrapping and unwrapping of DNA and hence would prevent the binding of transcription factors. Another possibility in which H1 could affect transcription is by occupying the binding sequences of those transcription factors whose binding sites are located in the linker region. This suggests that transcription factors will have to compete with H1 to bind to their cognate sites. Several studies have provided evidence that in certain cases linker histone can be directly displaced by transcription factors (48, 49).

In reviewing the literature, an interesting example of the involvement of histone H1 in alcohol use is the family of transcription factors NF-κB/REL (50), which are sequence-specific DNA-binding proteins that initiate transcription from a variety of genes that are involved in immune response and inflammatory processes (51). Chronic alcohol use sensitizes peripheral blood leukocytes of the innate immune system (macrophages, dendritic cells, mast cells, neutrophils and other leukocytes) to lipopolysaccharide (LPS)-mediated proinflammatory cytokines through, amongst others, activation of NF-κB (52). LPS, a Gram-negative bacterial endotoxin, is normally localized to the gut. Ethanol jeopardizes the tight junctions of the intestinal mucosa, allowing LPS to enter the systemic circulation. Once in the bloodstream, LPS is thought to activate peripheral toll-like receptor signaling cascades that initiate the release of pro-inflammatory cytokines and other immune mediators, via activation of the NF-κB (53). These cytokines then initiate immune responses in liver, blood and other tissues, including the brain (54, 55). Once activated, NF-κB dimers translocate to the nucleus and bind to their cognate sites in target genes, initiating their transcription. Apart from cytoplasmic regulation, NF-κB-dependent genes are also regulated inside the nucleus by the local chromatin structure (56). In agreement with other studies, Lone and co-workers found that the NF-κB binding completely displaces histone H1 from the nucleosomes and they propose that histone H1 eviction is needed for NF-κB to bind specifically to its recognition sequence embedded in the nucleosomes (53).

A quite interesting finding in our study is the demonstration of the activated polymorphonuclear lymphocytes of alcoholic patients, through the statistically significant predominance of the presence of arginine compared to lysine in the nuclei of polymorphonuclear cells ($p \leq 0.05$). According to the literature, a widespread but incorrect view of the neutrophil portrays it as a short-lived, terminally-differentiated cell with a highly condensed nucleus and, hence, unable to express genes (57). However, several studies have expanded the scope of neutrophil gene expression by showing a wide range of mRNAs present in unstimulated neutrophils (58, 59). The study of Zhang and co-workers indicates that a combinatorial transcriptional regulation, including alterations of chromatin structure, may play a role in the rapid changes in gene expression that occur in these cells, which constitute one of the main cell groups of the innate immune system (60).

The influence of innate immunity on the etiology and progression of alcohol abuse is a rapidly expanding area of interest in alcohol research. There is strong evidence for the neuroimmune role of addiction, with the innate immune system being linked to brain changes associated with acute and chronic alcohol exposure (55). Changes in expression of neuroimmune genes and microglial transcripts were first identified in postmortem brains from alcoholics (61). In addition, alcoholics showed altered levels of microRNAs that are known to regulate immune function (62). Furthermore, chronic alcohol treatment in mice induced pro-inflammatory gene expression that persisted for at least one week of abstinence (63), while LPS-induced neuroimmune activation persisted for months (64). These recent data make sense in an earlier interesting study, where the effect was analyzed of ethanol withdrawal on the immune system of chronic alcoholic patients without liver disease and without malnutrition through investigation of the distribution of peripheral blood lymphoid subsets (65). The authors showed that ethanol effects persisted or even became more evident after suppression of ethanol intake for a 9-month period, and suggested that in patients suffering from chronic alcoholism, once ethanol intake has been discontinued, a persistent immunological stimulus may contribute to the persistent of immunological changes. Given the fact that the alcoholics included in the present study were free from liver disease or other serious physical illness and had been abstinent from alcohol for an average of 58.5 days, our results show that immunological imbalances persist in spite of the absence of alcohol intake, through morphological chromatin alterations of mononuclear and polymorphonuclear lymphocytes. This imbalance is also supported by a recent clinical study according to which serum levels of interleukin-6, a multi-functional cytokine mainly produced by lymphocytes and which characterizes alcoholics without hepatic disease, decreased significantly in alcohol-dependent individuals during the detoxification process (66); however, its alteration during and after detoxification therapy is not yet clearly understood.

Reviewing several microarray studies in human and animal models concerning the identification of individual genes as candidates for addiction phenotypes (67), it becomes clear

that an integrated view of molecular and cellular changes underlying alcohol addiction is lacking. However, more recent studies focus on and identify critical epigenetic components in gene co-expression and propose a central role for epigenetic regulation in alcohol-induced changes in global gene expression (68). This important study applied a novel systems approach to transcriptome profiling in postmortem human brains and generated a systemic view of brain alterations associated with alcohol abuse. The study identified previously unrecognized epigenetic determinants of gene co-expression relationships and discovered novel markers of chromatin modifications in brains of alcoholic patients, such as histone and DNA methylation. Another example constitutes findings which illustrate that changing the level of chromatin condensation by affecting DNA methylation or histone acetylation controls the level of excessive alcohol drinking and seeking behaviors in pre-clinical rodent models (69). This study demonstrates that modifications in chromatin remodeling by alcohol may be a focal point in neuroadaptations resulting in continuous excessive alcohol seeking and intake. If so, modifiers of chromatin structure can be used for the treatment of alcohol use disorders. In this respect, lack of a specific receptor target for alcohol in the brain makes a potential treatment strategy that targets chromatin structure particularly promising.

In conclusion, our findings demonstrate the importance of chromatin structure and provide additional arguments for using blood chromatin of nucleated blood cells as a sensitive tool for epigenetic studies in living chronic alcoholic patients (70). Even though many questions still remain, it is clear that the post-translational modification of histone lysine and arginine residues is a critical signal-transduction pathway reminiscent of other well-established cascades (71). While the small number of cases studied, as well as lack of sequential blood sampling of our alcoholic patients during the detoxification period, do not allow us to make statistical comparisons with respect to the time of abstinence, our results revealed conformational changes in chromatin structure through the ASR method and histone H1 immunolocalization. These histochemical and immunohistochemical approaches applied to samples of peripheral blood leukocytes of alcoholics, coupled with modern molecular procedures, could be used to monitor epigenetic modifications that correlate to the harmful effects of alcohol. Indeed, these approaches could lead to methods of screening problem drinkers who engage in harmful excessive alcohol use, as well as alcohol-dependent individuals during the detoxification therapy.

Declaration of Interest

The Authors report no conflicts of interest and they alone are responsible for the content and writing of this article.

Acknowledgements

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