Review

The role and diagnostic value of cell-free DNA in systemic lupus erythematosus

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ABSTRACT

Cell-free DNA (cfDNA) represents a small fraction of total DNA pool that circulates freely in the blood both in normal and pathological conditions. Data indicate that cfDNA plays an important role in the pathogenesis of systemic lupus erythematosus (SLE) and hypomethylation may be crucial for its immunogenic properties. Although differences in quantification methodology hinder the comparison of results between the studies, it appears that levels of cfDNA are abnormally elevated in SLE patients and correlate with various antibody titers, but not with disease activity. Increased cfDNA concentration, however, may be associated with active lupus nephritis. Most of the studies confirmed apoptosis as the major cfDNA release mechanism in various conditions, but formation of neutrophil extracellular traps may significantly contribute to the cfDNA generation in SLE patients. In this review, we summarise current knowledge about the role and possible origin of cfDNA in SLE patients, and discuss why cfDNA testing for diagnostic and prognosis of SLE remains questionable.

Introduction

Most of the human DNA is located within a cell – in the nucleus and mitochondria. Cell-free DNA (cfDNA) represents a small fraction of total DNA pool that circulates in the bloodstream of healthy individuals. Its cellular origin and release mechanisms are still not fully understood. Interestingly, changes in cfDNA concentration have been associated with many disorders including various types of cancers (1-6), myocardial infarction (7-8), stroke (9), pregnancy pathologies (10-12), and autoimmune diseases (13-15). Circulating cfDNA appears to play a central role in the pathogenesis of systemic lupus erythematosus (SLE), therefore, it was extensively studied in lupus patients.

SLE is a chronic autoimmune disease with a wide spectrum of clinical presentations. It can affect almost every organ of the body and its severity ranges from mild to potentially life-threatening. The course of disease is unpredictable, with periods of flares and remissions. Common clinical manifestations include mucocutaneous features, arthritis, Raynaud's phenomenon, nephritis, vasculitis, gastrointestinal tract and nervous system involvement, lymphadenopathy, pleuritis, and pericarditis (16). Lupus pathogenesis is based on genetic and environmental factors, but precise pathomechanism still remains elusive (17). The disease may occur in genetically susceptible individuals and is initiated by certain environmental factors. Triggering stimuli, that may be relevant to the pathogenesis of SLE, include ultraviolet light, drugs, chemical factors, and possibly infectious agents (18-23). Lupus is characterised by several immunological abnormalities, both in the adaptive and innate immunity. Defective immunoregulation leads to the production of autoantibodies, lymphocytes hyperactivation, chronic inflammation, and immune complexes deposition, which causes tissue and organ damage (24).

Cell-free DNA levels

Ranges and mean cfDNA concentration, both in healthy and ill subjects, vary between the studies. Moreover, differences in sample processing and lack of standardisation of cfDNA extraction and quantification do not permit a direct comparison of the results (25). Most of the investigators confirmed that plasma cfDNA concentration in healthy individuals ranges from 0 ng/ml to about 89.8 ng/ml (26-28). Cell-free DNA levels in SLE patients were

significantly increased comparing with healthy controls in all studies (29-38). In the analysis performed by Tug *et al*. in 59 SLE patients mean concentration of total cfDNA was 44.7±53.5 ng/ml, with concentration ranging from 2.05 ng/ml to 223.2 ng/ml (35). Cell-free DNA levels observed by Hendy *et al*. in comparable group of patients (n=52) were lower, with mean concentration of 16.31±2.58 ng/ml (36). Using different quantification method, Zhang *et al*. reported higher cfDNA ranges in both SLE patients (mean 236.66±40.09 ng/ml) and healthy controls (mean 187.96±40.55 ng/ml) (37). Therefore, in order to compare the results between the studies and to determine whether analysis of cfDNA levels might be useful for discriminating between healthy individuals and SLE patients, a standardisation of cfDNA measurements is necessary (Table I).

Release mechanisms

Multiple sizing experiments reveled significant differences between cfDNA fragments length in SLE and in healthy controls. Suzuki *et al*. found that in healthy population plasma cfDNA mostly consists of 176 bp fragments, with no units larger than 2000 bp (40). Further sequencing studies confirmed the size distribution of plasma cfDNA molecules, showing the major peak around 166-169 bp, with the number of smaller peaks at intervals of approximately 10 bp (41-42). This periodicity was observed only in autosomal fragments of cfDNA, whereas mitochondrial cfDNA fraction exhibited no major peaks and a wider range of sizes (42). Circulating cfDNA in SLE patients was characterised by an abnormal pattern of cfDNA fragmentation. Gel electrophoresis studies revealed that plasma cfDNA from SLE patients consists of 150-200 bp, 400 bp, 600 bp, and 800 bp fragments (43). Additionally, Galeazzi *et al*. observed a high molecular weight DNA band of approximately 33kb length in all SLE samples, but not in healthy individuals and systemic sclerosis (SSc) patients (44). Sequencing studies revealed that cfDNA in SLE patients contained fewer 166-bp fragments and more DNA molecules of different length, particularly fragments shorter than 115 bp, comparing with healthy controls (45). Those changes were more pronounced in SLE patients classified as the active group, with SLEDAI over 6. Interestingly, the percentage of cfDNA fragments shorter than 115 bp correlated positively with anti-dsDNA antibody level and SLEDAI score. Those short cfDNA fragments were also enriched in the IgG-bound fraction.

Cell death

Differences in cfDNA size distribution between healthy subjects and SLE patients imply that, at least to a certain extent, the process of cfDNA generation proceeds differently in SLE. Three sources of circulating cfDNA have been postulated in the literature: apoptosis, necrosis, and active secretion from viable cells (46, 47). During apoptosis DNA is first cleaved into 50–200 kb fragments and subsequently into much smaller 180 bp nucleosomal units, while necrotic cells produce much larger fragments, even longer than 10000 bp (48, 49). Presumably, the major 166 bp peak observed in sizing experiments in both healthy population and SLE patients represents nucleosomal DNA, while the peaks at 10 bp periodicity are the result of enzymatic cleavage of DNA wrapped around the nucleosome histone core. It also appears that cfDNA associated with one full nucleosomal subunit creates a stable structure that is partially protected from further degradation (42). Therefore, most of the investigators agree that majority of cfDNA molecules in healthy individuals and in SLE enter the bloodstream fol-

Table I. Comparison of methods used for cfDNA analysis in SLE.

Author, year	Patients	Concentration of cfDNA	Isolation method	Quantification method	Material
Raptis *et al*., 1980 (29)	n=7	293±57 ng/ml (inactive SLE) 3231±1122 ng/ml (active SLE)	Phenol-based extraction	DNA labelling by nick translation	Plasma
McCoubrey-Hoyer *et al*., 1984 (30)	n=31	66.90±83.68 ng/ml	Phenol-based extraction	Counterimmunoelectrophoresis and precipitin line comparison	Plasma
Chen *et al*., 2007 (31)	n=12	78.1 ng/ml	Silica-based extraction	PicoGreen fluorometric assay	Serum
Atamaniuk *et al*., 2011 (33)	n=13	90 (64–103) pg/µl*	Not specified	Vistra Green fluorometric assay	Plasma
Bartoloni *et al*., 2011 (34)	n=44	35.0±9.0 ng/ml	Silica-based extraction	Real-time PCR (hTERT gene)	Plasma
Cepika *et al*., 2012 (32)	n=15	Not specified	Silica-based extraction	Real-time PCR (Alu-Ya5 gene)	Serum
Tug *et al*., 2014 (35)	n=59	44.7 ±53.5 ng/ml ($L1PA2_{90}$) 28.9±42.8 ng/ml ($L1PA2_{222}$)	Analysis of unpurified plasma	Real-time PCR ($L1PA2_{90}$, $L1PA2_{222}$ genes)	Plasma
Zhang *et al*., 2014 (37)	n=54	236.66±40.09 ng/ml	Analysis of unpurified plasma	PicoGreen fluorometric assay	Plasma
Fragoulis *et al*., 2015 (38)	n=5	60.0 (9.8–88.0) ng/ml*	Silica-based extraction	Real-time PCR (β-globin)	Serum
Hendy *et al*., 2016 (36)	n=52	16.31±2.58 ng/ml	Silica-based extraction	Real-time PCR (GAPDH gene)	Plasma

Data are presented as mean ± SD, unless stated otherwise. *median (quartiles).
cfDNA: cell-free DNA; SLE: systemic lupus erythematosus; PCR: polymerase chain reaction; hTERT: human telomerase reverse transcriptase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

lowing programmed cell death. However, the precise mechanisms by which excessive amounts of highly fragmented cfDNA enter the circulation in SLE remain unclear. Accumulation of short cfDNA fragments might be caused by an abnormal DNA cleavage during cell death, since it was found that SLE patients show decreased ability to degrade chromatin released from both necrotic and viable cells (50). Moreover, based on the observation that short cfDNA fragments were enriched in the IgG-bound fraction, Chan *et al*. proposed a preferential binding of anti-DNA antibodies to those fragments, which protects them from enzymatic degradation and clearance mechanisms (45). It is also possible that observed differences in cfDNA size distribution are caused by the increase of DNA secretion from living cells, possibly in the form of neutrophil extracellular traps (NETs).

Neutrophil extracellular traps

NETs formation is the unique antimicrobial mechanism, in which neutrophils secrete netlike structures to trap and kill microbes (51). This process, termed NETosis, is distinct from apoptosis and necrosis and does not require DNA fragmentation (52). Neutrophil traps are composed of DNA, histones, and various antimicrobial proteins (53). In healthy individuals, NETs are secreted at the site of inflammatory response and are subsequently degraded by endonuclease DNase I (54). Interestingly, it was shown that the excessive production and impaired clearance of NETs might be associated with increased levels of cfDNA in SLE. Patients with SLE have decreased ability to degrade NETs, which is mainly caused by the presence of DNase I inhibitors and anti-NETs antibodies that protect neutrophil traps from enzymatic cleavage (54, 55). Interestingly, NETs-degrading ability changes with the course of disease and could be partially restored by the addition of exogenous DNase I (55). Inefficient NETs degradation was also associated with SLEDAI, kidney involvement and pleuritis (54-56). NETs-degrading status was also strongly correlated with anti-NETs and anti-dsDNA antibody titers (54). Additionally, low density granulocytes (LDGs), an abnormal neutrophil subset with the enhanced capacity to form NETs, secrete excessive amounts of NETs in SLE patients (57). All NETs expose dsDNA, hence it was postulated that NETs formation could be an additional source of circulating cfDNA. Zhang *et al*. found that the percentage of LDGs was significantly higher in SLE patients compared with healthy controls, and plasma cfDNA levels correlated positively with LDG and neutrophil levels (37). Moreover, cfDNA concentration was higher in SLE patients with elevated LDG level. Those correlations were not observed in healthy controls. The authors postulated that in SLE patients about 41.2% of the cfDNA level elevation is caused by the excessive release of NETs by LDGs and neutrophils. It also appears that the contribution of NETosis to the plasma cfDNA generation in healthy subjects is negligible. Interestingly, despite the fact that DNase I activity in SLE patients was significantly lower compared with healthy individuals, no significant correlation was observed between cfDNA level and DNaseI activity (37). The authors proposed that lack of correlation might have been caused by the interference of other factors that prevented DNase I from degrading NETs, for example anti-NETs antibodies (Fig. 1).

Genomic distribution

Circulating cfDNA in SLE exhibits significant changes in genomic distribution comparing with healthy controls. High-throughput sequencing of serum cfDNA in healthy subjects revealed that 87% of sequences were attributable to known database sequences, of which 97% were genomic, and 3% were xenogeneic. There were no difference in genes, pseudogenes, transcribed regions, and protein-coding DNA sequences between cfDNA and genomic DNA, but circulating DNA tended to have higher proportion of *Alu* repetitive elements and lower proportion of long interspersed nuclear elements L1 and L2 (41, 58-59). Representation of serum cfDNA sequences correlated with chromosome size, with the exception for chromosome 19 which showed only 81% of the expected expression (41). Interestingly, chromosome 19 has the highest gene density, amount of *Alu* elements, and GC content of all chromosomes (60). Further analysis did not reveal any significant correlations between chromosomal representation in the serum and chromosome gene density or GC content (41). Differently, plasma cfDNA profiles in SLE patients showed significant differences in genomic distribution (45). Multiple under- and over-representations called aberrant measured genomic representations (MGRs) were found in cfDNA pool of SLE patients, but not in healthy individuals. The median percentage of genome segments with aberrant MGRs was 8.1% in SLE patients with active disease (range 1.1–52%), and 6.5% in patients with inactive disease (range 0.5–32.1%). Interestingly, the extent of aberrant MGRs correlated with anti-dsDNA antibody level, but not with the disease activity measured by SLEDAI. Also, more IgG binding occurred at genomic locations with increased MGRs.

Data obtained from sequencing studies suggest that non-random degradation or clearance of cfDNA occurs both in healthy individuals and in SLE patients (42). Beck *et al*. postulated that cfDNA generated as a result of apoptotic or necrotic unspecific nuclear DNA cleavage should be distributed uniformly in the entire genome. Only some highly histone protected regions would be overrepresented in cfDNA pool (41). Such a distribution was observed neither in healthy population nor in SLE patients. Moreover, uneven genomic representation was more pronounced in SLE patients. This could be explained by the significant disturbances in the process of non-random DNA degradation. However, based on the observation that IgG antibody binding of plasma cfDNA may be related to the presence of aberrant MGRs, Chan *et al*. proposed a different hypothesis. Preferential binding of anti-DNA antibody to particular cfDNA sequences and formation of antibody-cfDNA immune complexes could protect certain cfDNA molecules from enzymatic degradation and clearance mechanisms.

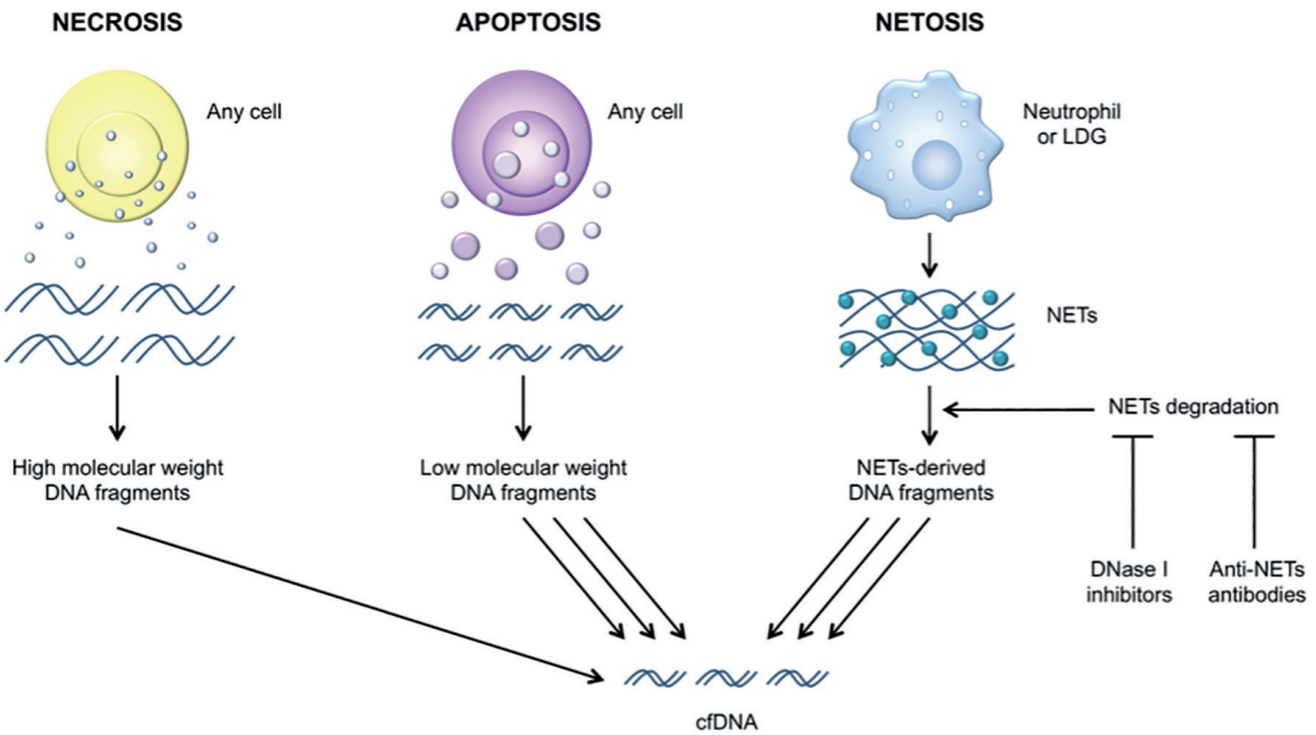

Fig. 1. Potential mechanisms of cell-free DNA (cfDNA) formation in systemic lupus erythematosus (SLE): apoptosis, NETosis and necrosis. Necrotic cells produce high molecular weight DNA fragments, whereas apoptosis results in short DNA fragments, mostly 166 bp nucleosomal units. Neutrophils and low density granulocytes (LDGs) excessively release neutrophil extracellular traps (NETs), which may comprise an important cfDNA source in SLE. This process is additionally reinforced by hindered NETs degradation that is mediated mainly by DNase I inhibitors and NETs antibodies.

Retention of antibody-bound cfDNA would increase the genomic representation in some regions, whereas regions with less preferential antibody binding would be underrepresented. These differences might have been detected as increased or decreased MGRs (45). Why preferential binding and retention of certain antibody-cfDNA complexes would be observed only in SLE patients remains unclear. Further studies are necessary to determine what is the significance of aberrant MGRs in cfDNA pool of SLE patients.

Methylation status and immunogenicity

Circulating cfDNA in patients with SLE may be immunogenic. It was found that plasma DNA obtained from patients with active SLE induces polyclonal and monoclonal production of anti-DNA antibodies after injection into experimental animals (61). Interestingly, hypomethylation may be crucial for cfDNA to exhibit its immunogenic properties. Administration of syngeneic apoptotic DNA triggered SLE-like disease with proteinuria and anti-DNA antibodies production in non-susceptible mice, but only when DNA was hypomethylated (62). The relevance of DNA demethylation in the pathogenesis of SLE was further confirmed in several studies (63-66). Both genomic DNA in T cells (67) and plasma cfDNA is hypomethylated in patients with SLE (45). Interestingly, the extent of cfDNA hypomethylation correlated with the anti-dsDNA antibody level and SLEDAI. Also, in patients with active disease cfDNA was more hypomethylated compared with both the inactive SLE group and with healthy individuals. Further analysis showed that especially cfDNA fragments of ≤115 bp showed decreased methylation density (45). These findings suggest that cfDNA hypomethylation may be associated with the pathogenesis of SLE. Based on the observation that plasma cfDNA exhibits immunogenic properties and its methylation status is associated with SLE activity, it seems probable that changes in cfDNA methylation influence the course of the disease, possibly by causing excessive anti-dsDNA production and lupus flares.

Clinical significance

SLE activity

The association between cfDNA level and SLE activity was confirmed only partially so far. Although it was shown that after therapy with cyclophosphamide, azathioprine, mycophenolate mofetil, or chloroquine cfDNA levels were significantly reduced (31, 36), investigators did not find any correlations between cfDNA levels and SLE Disease Activity Index (SLEDAI) (32-36). Despite no differences in cfDNA concentrations between SLE patients with certain clinical manifestations such as nephritis, arthralgia, myalgia, and cutaneous features, Tug *et al*. observed a significant correlation between the medical evaluation of SLE patients and cfDNA level fluctuations (35). Changes in disease activity from stagnation/remission to deterioration were especially represented by cfDNA levels. Additionally, Zhang *et al*. reported that cfDNA level in patients with lupus nephritis (LN) was significantly higher compared with patients without kidney involvement. Moreover, the level of cfDNA in patients with active LN was signifi-

Table II. Studies evaluating association between plasma cfDNA levels and disease activity parameters in SLE patients.

Author, year	Patients	SLEDAI	ANA	Anti-dsDNA antibodies	Anti-nucleosome antibodies	Anti-histone antibodies	C3	C4	CRP
Atamaniuk *et al*., 2011 (33)	n=13	4 (3-8)*		N	N	P			
Bartoloni *et al*., 2011 (34)	n=44	2 (0–16)*		N			N	N	
Tug *et al*., 2014 (35)	n=59	5.74±4.5	I	I			P	P	N
Hendy *et al*., 2016 (36)	n=52	7.31±5.26	I	I	P		I	N	P

Data are presented as mean ± SD, unless stated otherwise. *median (quartiles).
cfDNA: cell-free DNA; SLE: systemic lupus erythematosus; SLEDAI: SLE Disease Activity Index; ANA: antinuclear antibodies; dsDNA: double-stranded DNA; C3: complement factor C3; C4: complement factor C4; CRP: C-reactive protein; P: positive correlation with cfDNA level; I: inverse correlation with cfDNA level; N: no correlation with cfDNA level.

cantly higher than in those with inactive LN (37). The authors also found significant correlations between cfDNA concentration and parameters closely related to the activity and severity of LN. Concentration of cfDNA correlated positively with the degree of proteinuria, and negatively with the albumin concentration and creatinine clearance rate, suggesting that elevated cfDNA level might be associated with the LN activity. Further investigations are necessary to determine whether analysis of cfDNA concentration could be useful for monitoring of the LN activity, but at the moment most of the studies excluded measurement of cfDNA level as a potential tool to predict flares or assess disease activity in SLE patients. Interestingly, cfDNA may serve as a promising biomarker of disease activity in SSc and primary Sjögren's syndrome (SS). In the study performed by Mosca *et al*. in 122 SSc patients cfDNA level did not differ from healthy controls, but individuals with active disease had significantly higher cfDNA levels than those with inactive disease (14). In SS patients cfDNA level was higher with respect to healthy controls and correlated with disease activity index (34, 38). Similar to SSc, SS subjects with active disease displayed significantly increased cfDNA levels comparing with inactive patients. Unfortunately, only few studies of cfDNA in both disorders are available, therefore cfDNA utility as a marker for SSc and SS activity monitoring requires further confirmation.

Autoantibodies

Systemic lupus erythematosus is characterised by the presence of numerous autoantibodies directed against double stranded DNA (dsDNA), histones, and other nuclear components (39). Multiple investigators confirmed significant correlations between cfDNA levels and autoantibodies titers in SLE patients (Table II). Concentration of cfDNA correlated positively with anti-nucleosome (36) and anti-histone antibodies (33), and negatively with antinuclear (ANA) and anti-dsDNA antibody titers (35, 36). Additionally, patients negative for anti-dsDNA antibodies had higher cfDNA levels compared to those with positive anti-dsDNA-antibodies (35). To explain these results, the authors suggested that analysis of cfDNA may be hindered in patients with high autoantibody titers, possibly by the formation of antibody-DNA immune complexes that directly interfere with cfDNA detection or accelerate the clearance of antibody-bound DNA from the circulation.

Inflammatory response

Contradictory results were reported on the associations between cfDNA levels and complement proteins C3, C4, and C-reactive protein (CRP) levels. Tug *et al*. found a positive correlation between cfDNA concentration and levels of C3 and C4 in SLE patients and in healthy controls (35). Hendy *et al*. *obtained* an inverse correlation between cfDNA level and C3 level in SLE group (36), whereas Bartoloni *et al*. did not confirm any correlations between cfDNA and complement levels (34). Hendy *et al*. found a positive correlation between cfDNA level and CRP (36). In contrast, Tug *et al*. did not find any relation between cfDNA concentration and CRP levels in SLE patients (35). These conflicting results might have been caused by the differences in methodology and clinical diversity of patients' populations. Levels of C3, C4, and CRP change over time, thereby results may vary depending on SLE activity at the point of examination. Nonetheless, findings suggest the possible involvement of cfDNA in the inflammatory response. Tug *et al*. postulated that cfDNA in SLE may act as an acute-phase reactant and the increased cfDNA concentration reflects inflammatory conditions (35). This notion seems to be further supported by the positive correlation between cfDNA and CRP levels observed by Hendy *et al*. in SLE patients (36).

Summary

Available data indicate that cfDNA is involved in the pathogenesis of SLE. Multiple disturbances in the immunoregulatory mechanisms lead to the excessive cfDNA release into the bloodstream. Apoptosis and NETosis are the two most significant mechanisms in this process. Abnormally elevated cfDNA level correlate with various autoantibodies titers and is associated with active lupus nephritis, which indicates the future prospects for cfDNA utility as a novel biomarker for SLE diagnostics. Unfortunately, considerable differences in sample processing and quantification methodology, as well as the heterogeneity of patients' population, do not permit a direct comparison of the results between the studies. Therefore, standardisation of cfDNA measurement is necessary to accurately examine its potential applications in clinical practice. Also, further studies are necessary to truly understand the interplay between cfDNA and various autoantibodies in SLE, and to determine the role of cfDNA-antibody complexes in lupus nephritis development.

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