

Neutropenia Through Enhanced Neutrophil Apoptosis and Secondary Necrosis in Wolfram Syndrome 1

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Wolfram syndrome (WFS)(MIM #222300), a rare autosomal recessive disorder resulting from wolframin deficiency, is clinically characterized by diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (DIDMOAD) in its classical form (1). Wolframin, crucial for regulating endoplasmic reticulum (ER) stress and calcium homeostasis, is highly expressed in cells with mitochondria that have significant protein folding demands, such as pancreatic β -cells and neurons, where its deficiency can induce ER stress-mediated apoptosis (2,3). By suppressing ATF6 α in the unfolded protein response (UPR) (4,5), wolframin plays a vital role in cell viability. While its expression has been identified in various cells (6), other affected cell types whose roles in the disease are yet to be determined likely exist. Given neutrophils' reliance on proper protein folding for antimicrobial functions and their possession of both ER and mitochondria, they may be susceptible to ER stress-induced dysfunction. Indeed, there are certain diseases caused by ER stress-induced apoptosis in neutrophils, and furthermore, there are conditions where neutrophil apoptosis leads to neutropenia (7-9). This study investigated the impact of the WFS gene mutation on neutrophil survival and apoptosis in a patient with WFS1 who presented with unexplained chronic neutropenia. Complete blood counts (CBCs) were performed utilizing a Beckman Coulter DCH900 automated hematology analyzer to quantify blood cell populations and corroborate the observed neutropenia. Cell viability was assessed via Trypan Blue exclusion assay. Flow cytometric analysis was employed to quantify cellular apoptosis and necrosis, utilizing Annexin V/propidium iodide (PI) staining. Furthermore, the expression levels of apoptotic markers, specifically active caspase-3 and Bcl-2, were determined through flow cytometry.

A 24-year-old Turkish male, born to healthy, unrelated parents, was diagnosed with isolated neutropenia at age nine. This diagnosis was made during an evaluation for the insertion of tympanostomy tubes due to serous otitis media that developed following a middle ear infection. During follow-up, the isolated neutropenia persisted (stable neutrophil count of 150–700/mm³). Initial genetic and bone marrow evaluations were unremarkable: Karyotyping and the Chromosomal Breakage (DEB) assay were negative, as was Schwachman-Diamond syndrome (SDS) gene sequencing. Bone marrow examination showed no evidence of pathology in the myeloid compartment. During follow-up, he developed poor growth and diabetes mellitus (DM). Hyperglycemia was first observed in the patient at 16 years of age, progressing to insulin-dependent DM by age 24. Insulin therapy has been initiated recently.

On admission, his anthropometric measurements were 70 kg for weight (3rd–10th percentile), 170 cm for height (10th percentile), and 55 cm for head circumference (25th percentile). Laboratory results showed persistent neutropenia (absolute neutrophil count [ANC] 300/mm³) with a normal red blood cell picture and platelet count. Biochemical analysis revealed hyperglycemia (fasting glucose 293 mg/dL) and elevated HbA1c (10.4%); other biochemical tests were normal. Anti-neutrophil antibodies were negative. The patient declined a repeat bone marrow examination. Whole-exome sequencing subsequently identified a homozygous pathogenic variant in the WFS1 gene (c.1672C>T, p.Arg558Cys) according to the recommendations of ACGS and ClinGen guidelines (Figure 1). In silico analyses determined that the variant impairs the protein's structure and function. No other causative variants in any gene, including known cytopenia-associated genes, were detected. A family-based genetic analysis was not possible due to financial constraints.

The patient's chronic neutropenia, which began in childhood, was not attributable to any underlying chronic disease, medication use, or infection. Bone marrow evaluation revealed no impairment in neutrophil production,

nor were there any antibodies present that would lead to neutrophil destruction. The remaining plausible explanation for the observed neutropenia was **cell death**. Given that **WFS is characterized by apoptosis-related cell loss**, we investigated apoptosis in the patient's neutrophils to elucidate the cause of neutropenia. For this purpose, neutrophils derived from the patient were subjected to comprehensive analysis. CBC was performed utilizing a Beckman Coulter DCH900 automated hematology analyzer to quantify blood cell populations and corroborate the observed neutropenia. Cell viability was assessed via Trypan Blue exclusion assay as described in the literature (10). Flow cytometric analysis was employed to quantify cellular apoptosis and necrosis, utilizing Annexin V/propidium iodide (PI) staining (11). Furthermore, the expression levels of apoptotic markers, specifically active caspase-3 and Bcl-2, were determined through flow cytometry. Apoptotic levels of neutrophils were determined using the Sony FITC Annexin V Apoptosis Detection Kit with Propidium Iodide (PI). Cells were stained with FITC Annexin V (to detect early apoptosis) and PI (Propidium Iodide). The samples were then analyzed by flow cytometry to differentiate between viable, early apoptotic, and late apoptotic/necrotic cells. The comparison was conducted with a healthy control matched for age and gender. Before enrichment, the patient had a WBC count of 2100/ μ l (5% neutrophils), while the healthy control had 7000/ μ l (60.2% neutrophils). After enrichment, the patient showed a WBC count of 200/ μ l (45.8% neutrophils) and the control 2400/ μ l (96.7% neutrophils). Flow cytometry of isolated neutrophils revealed a lower neutrophil percentage in the patient (70.29%) compared to the control (81.92%). The Trypan Blue test indicated significantly reduced viability in the patient sample (53%) compared to the control (92%). Annexin V/PI staining demonstrated significantly higher rates of apoptosis (30.04%), necroptosis (1.73%), and necrosis (40.73%) in the patient compared to the control (apoptosis 0.04%, necroptosis 0.00%, necrosis 6.09%) (Figure 2). Active caspase-3 levels were approximately three times higher in the patient (30.53%) than in the control (10.85%). Analysis of the anti-apoptotic protein Bcl-2 showed comparable expression levels between the patient (91.86%) and the control (85.88%).

Mutations in the *WFS1* gene account for more than 90% of WFS cases (12). The specific mutation identified in our patient holds a unique significance among the known *WFS1* mutations. A comprehensive review examining the clinical, laboratory, and genetic characteristics of eight patients homozygous for this mutation revealed a mean age of diagnosis of 30 years (with one patient diagnosed at 62 years) (13). The mean ages of onset were 19 years for DM (range: 15-21 years) and 29 years for optic atrophy (OA) (range: 15-48 years). DM was observed in seven of the eight patients, while OA was present only in six. Neither diabetes insipidus (DI) nor deafness was reported in any of the patients. Protein modeling and normal mode analysis (NMA) simulations of the variant's effect on wolframin protein thermodynamics demonstrated that the p.R558C variant is associated with a milder, later-onset WFS phenotype (13). In our patient, insulin-dependent DM developed at a later stage, as expected with this mutation. While DI and other characteristic features have not yet emerged, the patient is under close clinical observation for these conditions. The notable finding in our patient is the prominence of neutropenia. Although WFS is primarily recognized for its endocrine manifestations, its hematological aspects are less known. To our knowledge, a WFS patient presenting with a prominent hematological finding has not been reported to date. However, a careful review of various genetic databases collecting WFS information clearly indicates that neutropenia is a documented finding in WFS (14-16). Our patient also had DM as a metabolic disorder, but his neutropenia was present before DM diagnosis.

In general terms, neutropenia may arise from decreased marrow production, increased tissue sequestration, or increased peripheral destruction (17). In our *WFS1* patient with neutropenia, the absence of bone marrow production defects or increased antibody mediated neutrophil destruction suggested cell death as the underlying mechanism, encompassing apoptosis, autophagy, necrosis, and entosis. Among these, apoptosis, a rapid, caspase-mediated type I cell death, involves intricate molecular events through extrinsic and intrinsic pathways converging on caspase-3 activation, leading to cell dismantling and phagocytosis (18).

Annexin V staining confirmed apoptosis in the patient's neutrophils, supported by caspase-3 activation and a significantly elevated apoptosis rate (30.04%) compared to normal physiological levels. While the specific apoptotic pathway remains undetermined, the wolframin-related nature of the disease suggests the intrinsic pathway, particularly UPR and ER stress-induced apoptosis, potentially evidenced by the observed necroptosis, which can occur in this context. Severe, prolonged ER stress primarily induces apoptosis but can progress to secondary necrosis if apoptotic cells are not adequately cleared, potentially explaining the high necrosis rate (40.73%) observed, consistent with findings by Silva et al (19).

Interestingly, despite high apoptosis and necrosis ($p < 0.001$), Bcl-2 expression was similar between the patient and control (91.86% vs 85.88%). This seemingly paradoxical result aligns with Gong et al.'s work demonstrating that *WFS1* mutations increase ER stress and apoptosis independently of Bcl-2 levels, where mutant *WFS1* failed to rescue cell viability despite anti-apoptotic mechanisms (20). The elevated Bcl-2 in our patient may represent an insufficient compensatory response to chronic ER stress triggered by *WFS1* deficiency.

Persistent ER stress due to the *WFS1* mutation may impair neutrophil production and survival. Increased apoptosis and subsequent secondary necrosis could contribute to neutropenia, consistent with the patient's low neutrophil count and reduced viability in assays. Even with extensive research on WFS, a common

hematological manifestation like neutropenia remains understudied. This is primarily because WFS is so well-known for its endocrine and neurological symptoms that a hematological abnormality is often overlooked. While this may seem like a new discovery, information from genetic databases suggests that neutropenia is indeed a documented finding in WFS (14-16). However, these data have yet to be effectively translated into clinical awareness. The lack of reported cases where neutropenia is a primary feature highlights a gap in our clinical understanding and may be due to a lack of screening for hematological features in WFS patients.

Given that apoptotic cell loss is a key pathophysiological mechanism in WFS, the susceptibility of neutrophils to ER-stress-induced apoptosis becomes particularly relevant. Furthermore, the documented instances in which neutrophil apoptosis leads to neutropenia underscore the necessity for focused attention on neutropenia within the context of WFS. Among blood cells in WFS, wolframin expression has only been examined in lymphocytes to date. Wolframin expression in neutrophils has not been previously investigated. Considering the potential risks and persistence of neutropenia in WFS patients, we propose a retrospective evaluation of reported cases and recommend routine monitoring for this underappreciated aspect, even though this patient did not experience neutropenic fever requiring hospitalization or persistent infections. The variable impact of *WFS* mutations could explain the earlier and more severe effect on neutrophils in our patient compared to β -cells and neurons (21). It has been shown that even with the same mutation in the same individual, different cell types can be affected to varying degrees (6). This may account for the fact that the neutrophils were the first and most severely affected cells in our patient.

Although our flow cytometric analysis unequivocally demonstrated elevated apoptosis and caspase-3 activation in WFS neutrophils, corroboration through Western blot analysis of wolframin protein expression and ER stress markers would substantially reinforce these observations. These complementary methodologies could provide direct evidence of wolframin deficiency within neutrophils and validate the hypothesized ER stress-mediated apoptotic pathway. Subsequent investigations, incorporating these advanced techniques and expanded patient cohorts, will be crucial for a comprehensive elucidation of the molecular mechanisms contributing to neutropenia in Wolfram syndrome.

In conclusion, our study provides evidence of neutrophil apoptosis in a neutropenic patient with WFS. These findings highlight the need for further research to fully understand the role of apoptosis in WFS-related neutropenia and to identify potential therapeutic targets. The results also suggest that treatment strategies for neutropenia in WFS should focus on improving ER stress management and enhancing apoptotic cell clearance, considering both apoptosis and secondary necrosis as potential mechanisms.

Key words: Wolfram syndrome, neutrophil, apoptosis, ER stress

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Abbreviations:

WFS	Wolfram syndrome
DIDMOAD	Diabetes insipidus, diabetes mellitus, optic atrophy, and deafness
ER	Endoplasmic reticulum
UPR	Unfolded protein response

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Figure Legends

Figure 1. Integrative Genomics Viewer (IGV) screenshot demonstrating the homozygous WFS1 variant NM_004006.2:c.1672C>T. Sequencing reads are visualized in both forward (blue) and reverse (red) orientations, with the variant position highlighted in red at nucleotide position 1672. The uniform presence of the C>T substitution across all aligned reads confirms the homozygous state of the variant.

WFS1(NM_004006.2):c.[1672C>T];[1672C>T]

