Effect of supraphysiologic levels of C1-inhibitor on the classical, lectin and alternative pathways of complement

Erik Waage Nielsen\textsuperscript{a,c,*}, Christian Waage\textsuperscript{a}, Hilde Fure\textsuperscript{b}, Ole L. Brekke\textsuperscript{b,c}, Georgia Sfyroera\textsuperscript{e}, John D. Lambris\textsuperscript{e}, Tom E. Mollnes\textsuperscript{b,c,d}

\textsuperscript{a} Department of Anesthesiology, Nordland Hospital, N-8092 Bodø, Norway
\textsuperscript{b} Department of Laboratory Medicine, Nordland Hospital, N-8092 Bodø, Norway
\textsuperscript{c} University of Tromsø, Norway
\textsuperscript{d} Institute of Immunology, Rikshospitalet University Hospital, University of Oslo, Norway
\textsuperscript{e} Department of Pathology and Laboratory Medicine, University of Pennsylvania, PA, USA

Received 5 June 2006; received in revised form 3 October 2006; accepted 13 October 2006
Available online 13 November 2006

Abstract

C1-inhibitor (C1-INH) was named according to its ability to inhibit the C1 subcomponents C1r and C1s in the classical pathway and is the only known physiologic inhibitor of these proteases (Pensky et al., 1961). Early studies of patients suffering from hereditary angioedema (HAE) shed further light on C1-INH’s role in the classical pathway (Landerman et al., 1962; Donaldson and Evans, 1963). These patients’ heterozygous C1-INH deficiency leads to an increased spontaneous activation of C4 and C2 (Donaldson and Rosen, 1964). As this breakdown takes place in the fluid phase, and not on a solid surface like, e.g. bacteria, C4b is rapidly inactivated by factor I. This has been the main explanation as to why the classical C3 convertase C4b2a does not assemble in HAE patients (Klein, 1990). A higher ratio of C4b-binding protein to C4 may also hinder activation beyond C4 and C2 (Gronski et al., 1988). Later, sensitive assays showed a very modest activation of C3 in HAE patients (Nielsen et al., 1995). During attacks of HAE even a minor increase in the terminal complement complex (TCC) was revealed (Nielsen et al., 1996).

In the lectin pathway, discovered in the late 1980’s, mannose-binding lectin (MBL) or ficolins bind structures containing sugar

1. Introduction

C1-inhibitor (C1-INH) was named according to its ability to inhibit the C1 subcomponents C1r and C1s in the classical pathway and is the only known physiologic inhibitor of these proteases (Pensky et al., 1961). Early studies of patients suffering from hereditary angioedema (HAE) shed further light on C1-INH’s role in the classical pathway (Landerman et al., 1962; Donaldson and Evans, 1963). These patients’ heterozygous C1-INH deficiency leads to an increased spontaneous activation of C4 and C2 (Donaldson and Rosen, 1964). As this breakdown takes place in the fluid phase, and not on a solid surface like, e.g. bacteria, C4b is rapidly inactivated by factor I. This has been the main explanation as to why the classical C3 convertase C4b2a does not assemble in HAE patients (Klein, 1990). A higher ratio of C4b-binding protein to C4 may also hinder activation beyond C4 and C2 (Gronski et al., 1988). Later, sensitive assays showed a very modest activation of C3 in HAE patients (Nielsen et al., 1995). During attacks of HAE even a minor increase in the terminal complement complex (TCC) was revealed (Nielsen et al., 1996).

In the lectin pathway, discovered in the late 1980’s, mannose-binding lectin (MBL) or ficolins bind structures containing sugar
on the surface of foreign particles and microbes (Kawasaki et al., 1989; Lynch et al., 2004). MBL share similarities with C1q in the classical pathway. Complexes of MBL and a C1s-like enzyme named MBL-associated serine protease (MASP)-2 become activated when bound to the target. The resulting sequential cleavage of C4 and C2 creates a C4bC2a complex indistinguishable from the C3 convertase of the classical pathway (Matsushita and Fujita, 1992; Thié et al., 1997). MASP-2 is inhibited by C1-INH and alpha-2 macroglobulin (Matsushita et al., 2000; Terai et al., 1995). Furthermore, a role for C1-INH in regulating the activation of C3 has been extensively described previously (Sahu et al., 1996). C1-INH (Berinert-P®) from Aventis Behring GmbH, Marburg, Germany Berinert®-P, is separated from cryo-depleted human plasma by adsorption and precipitation steps. According to information from the company the purity is 99%. Subsequently, the purified material is pasteurized by heat treatment in sterile filtrated and lyophilized in the absence of preservatives (De Serres et al., 2003).

2.2. Effect of C1-INH on solid-phase complement activation in serum

The effect of C1-INH on the function of classical, lectin and alternative pathways was examined in the Wieslab TM Complement System Screen (Euro-Diagnostica, Malmö, Sweden) (Seelen et al., 2005), which is an enzyme immunoassay (EIA) for specific detection of the three pathways with deposition of C5b-9 as a common read-out. Serum from healthy blood donors was collected and distributed into three different normal human serum pools (n > 10 for each). NHS was then added into tubes containing PBS, C1-INH, albumin or ethylene-diaminetetraacetic acid (EDTA) whereby NHS was diluted 5/7. Final concentrations of exogenously added C1-INH were 0.5, 2.5 and 5.0 mg/ml. These concentrations of added C1-INH corresponded to 2.8 times (2.8×), 14 times (14×) and 28 times (28×) physiologic values. The ratio of C1-INH to, e.g. that of the activating component C4 is approximately the same. Corresponding final concentrations of added albumin were 0.5, 2.5 and 5.0 mg/ml. Final concentration of EDTA was minimum 20 mM. Identical experiments were done with the same serum pools, including IgG as a supplement. Concentrations of IgG were equivalent to the concentrations of C1-INH and Albumin. Further dilutions were done according to the instructions, i.e. 1:101 for the classical and lectin pathway and 1:18 for the alternative pathway.

2.3. Assessment of classical and lectin pathways at low serum dilutions by EIA

Since the Wieslab Complement System screen experiments are based on high dilution of serum for the classical and lectin pathway, we made additional experiments using close to physiologic serum concentration in newly described assays for the classical (Harboe et al., 2004) and lectin pathways (Harboe et al., 2006). Microtiter wells were coated with IgM 10 μg/ml in PBS or mannann 5 μg/ml in carbonate buffer, pH 9.6, overnight at room temperature. After washing, blocking was performed with PBS containing 1% bovine serum albumin and 0.1% Tween 20. NHS was first diluted 5/7 with PBS, C1-INH or albumin. The final concentration of exogenously added C1-INH and albumin was 0×, 2.8×, 14× and 28× physiologic values. NHS was further diluted 1/2 with veronal buffer pH 7.5 containing 0.5 mM MgCl2, 2 mM CaCl2, 0.05% Tween 20 and 0.1% gelatin, 50 μl of each dilution was added to the wells and incubated at 37°C for 30 min (mannann coated wells) or 60 min (wells coated with IgM). The amount of C4 or TCC deposited on the surface of the washed wells was measured.

2.4. Effect of C1-INH on LP measured in a MBL/MASP functional assay

C4 deposition as a function of MBL/MASP activity was measured by EIA (Petersen et al., 2001). Microtiter wells were coated
with 10 μg/ml mannan in carbonate buffer, pH 9.6, overnight at room temperature. After washing, blocking was performed with 1 mg/ml bovine serum albumin in 10 mM Tris–HCl, 140 mM NaCl, pH 7.4. A serum pool from 20 healthy donors was used as standard, diluted 1/10 and then a twofold dilution series with a buffer containing 1 M NaCl. Three different serum pools were diluted 1/50. Serum from an MBL-deficient donor was used as a control. Standards, samples and controls were added in triplicates to the wells and incubated overnight at +4 °C. A dilution series of C1-INH in concentrations of 0.25, 0.75, 2.75 or 5.25 mg/ml was made. This corresponds to 1 ×, 3 ×, 11 × or 21 × physiological concentration. Albumin was diluted correspondingly. After a further 1/50 dilution of either the C1-INH- or the albumin solution, corresponding to the 1/50 serum dilution in the first step, 100 μl was added to the washed wells in a cocktail with 5 μg/ml C4 (Quidel, San Diego, CA). A baseline sample with only PBS and C4 in similar concentration was also added. After incubation at 37 °C for 1.5 h, deposited C4 was detected by the addition of HRP-conjugated anti-human C4 (04-032, Immun-system, Uppsala, Sweden) diluted 1/1000, and incubated at room temperature for 1.5 h. The substrate solution was 0.3 mM ABTS (Sigma, St. Louis, MO) in 0.15 M acetate buffer, pH 4.0, and 0.024% hydrogen peroxide. Optical density reading at 405 nm.

2.5. Effects of C1-INH on fluid-phase complement activation in a human whole blood model

The model has previously been described in detail (Mollnes et al., 2002). All incubations were performed at 37 °C. All equipment (tubes, tips, etc.) and solutions used in the model were endotoxin-free according to information from the manufacturers. Polypropylene tubes were used to obtain low background activation of complement. Whole blood anti-coagulated with lepirudin 50 μg/ml, was collected and distributed immediately into tubes containing PBS, or exogenous C1-INH in final concentrations of 2.8 ×, 14 × or 28 × physiologic value, or albumin in equivalent (mg/ml) concentrations. Compstatin, 50 μM final concentration or EDTA, 20 mM were included as controls. The samples were preincubated for 4 min until PBS (baseline samples), HAIGG (0.1 mg/ml) or CVF (0.2 U/ml) were added. After activation for 2 h or 30 min (CVF-incubations), EDTA in 10 mM final concentration was added and the tubes were centrifuged for 15 min at 4000 × g at 4 °C. The plasma was stored at −70 °C until analyzed for the terminal sC5b-9 complex (TCC) (Mollnes, 1997). As a supplement, blood from three additional donors were activated with CVF according to the guidelines above, including IgG in equivalent concentrations to C1-INH and albumin.

2.6. Detection of factor H in the C1-INH concentrate

The possible presence of factor H in the C1-INH sample was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and a sandwich ELISA. The latter was performed by coating microtiter plates with 50 μl of 2 μg/ml monoclonal anti-factor H antibody MH22 in PBS for 2 h at room temperature. Then wells were blocked with 200 μl of 1% BSA/PBS for 1 h. Purified factor H or the C1-INH preparation were serial diluted using 1% BSA/PBS and bound factor H was detected using a 1/1000 dilution of polyclonal anti-factor H antibody.

2.7. Statistics

Effects of increasing doses of C1-INH compared to albumin were analyzed with two-ways repeated measurements analysis of variance in Prizm (Graphad Software, San Diego, CA). Bonferroni post-test were used to adjust for repeated measurements. A p-value < 0.05 was considered significant.
2.8. Ethics

The study was approved by the Regional Ethical Committee.

3. Results

3.1. Effect of C1-INH on classical-, lectin- and alternative pathway examined in Wielisa

3.1.1. Solid-phase classical pathway (Fig. 1, upper panel)

C1-INH reduced classical pathway mediated C5b-9 deposition dose-dependently \((p = 0.0003)\). The inhibition was, with Bonferroni post-tests in parentheses, 2% (n.s.), 27% \((p < 0.05)\) and 40% \((p < 0.001)\) using 2.8×, 14× or 28× physiologic concentration of C1-INH.

3.1.2. Solid-phase lectin pathway (Fig. 1, middle panel)

C1-INH reduced lectin pathway mediated C5b-9 deposition dose-dependently \((p < 0.0001)\). The inhibition was 33% \((p < 0.001)\), 77% \((p < 0.001)\) and 86% \((p < 0.001)\) using 2.8×, 14× and 28× physiologic concentration of exogenous C1-INH.

3.1.3. Solid-phase alternative pathway (Fig. 1, lower panel)

C1-INH in up to 28× physiologic concentration had no effect on alternative pathway mediated C5b-9 deposition.

For all the above analyses albumin in equivalent concentrations (w/v) as C1-INH had no significant effect, while EDTA reduced C5b-9 deposition by 93% (Fig. 1, all panels). IgG in equivalent concentrations (w/v) reduced C5b-9 deposition in solid phase lectin pathway by 12% without a dose–response and had no significant effects in the classical or alternative pathways.

3.2. Effect of C1-INH on classical and lectin pathway at low serum dilution

Since the first sets of experiments (Wielisa) were done with serum dilution 1:101 for the classical and lectin pathway we examined the effect of C1-INH in these two pathways under low serum dilution, closely resembling physiologic conditions as recently described (Harboe et al., 2006).

3.2.1. Solid-phase classical pathway at low serum dilutions (Fig. 2, upper panel)

C1-INH reduced classical pathway mediated C5b-9 deposition dose-dependently \((p < 0.0001)\). The inhibition was 23\% \((p > 0.05)\), 70\% \((p < 0.001)\) and 85\% \((p < 0.001)\) using 2.8×, 14× and 28× physiologic concentration of exogenous C1-INH. Albumin in equivalent concentrations had no significant effect, while EDTA reduced C5b-9 deposition by 89%.

3.2.2. Solid-phase lectin pathway at low serum dilutions (Fig. 2, lower panel)

C1-INH reduced lectin pathway mediated C5b-9 deposition dose-dependently \((p < 0.0001)\). The inhibition was 62\% \((p < 0.001)\), 82\% \((p < 0.001)\) and 86\% \((p < 0.001)\) using 2.8×, 14× and 28× physiologic concentration of exogenous C1-INH.

Fig. 2. Effect of C1-INH on solid-phase activation at low serum dilution. Increasing doses of C1-INH, indicated as times the physiologic concentration in serum \((x\text{-axis})\), was added to normal human serum pools using albumin in equivalent microgram amounts as control. The samples were examined using enzyme-immunoassays for the classical (CP; upper panel) and lectin (LP; lower panel) pathway designed for using low serum dilutions (see Section 2). Readout was deposition of C5b-9, a normal serum pool defining 100\% \((y\text{-axis})\). Data are mean and S.E.M. of \(n = 3\) experiments.

3.3. Functional MBL/MASP assay

C1-INH reduced MBL/MASP-mediated C4 deposition dose-dependently \((p < 0.0005)\) (Fig. 3). The inhibition was 77\% \((p < 0.001)\), 83\% \((p < 0.001)\), 86\% \((p < 0.001)\) and 86\% \((p < 0.001)\) using 1×, 3×, 11× or 21× physiologic concentration of C1-INH. C4 deposition in serum from a MBL-deficient (81 ng/ml) donor was reduced by 82\% compared to normal human serum.

3.4. Effect of C1-INH on fluid-phase complement activation

3.4.1. Fluid-phase classical pathway (Fig. 4, upper panel)

The addition of HAIGG to serum led to a marked increase in soluble terminal C5b-9 complement complexes (TCC). C1-INH reduced HAIGG-mediated TCC generation dose-dependently \((p < 0.0001)\). The inhibition was −12\% \((p > 0.05)\), 41\% \((p < 0.001)\) and 77\% \((p < 0.001)\) using 2.8×, 14× and
Fig. 3. Effect of C1-INH on functional lectin pathway activation. Standards, normal human serum pools and serum from an MBL-deficient donor (81 ng/ml) were incubated on mannan-coated microtiter wells in high salt concentration to enable binding of MBL/MASPs, but to avoid further activation. After washing, increasing doses of C1-INH, indicated as times the physiologic concentration in the pre-incubated serum (x-axis), were mixed with purified C4 and added to the wells, using albumin in equivalent microgram amounts as control. Readout was deposition of C4, a normal serum pool defining 100% (y-axis). Data are mean and S.E.M. of 3 × 3 experiments.

28 × physiologic concentration of C1-INH. Albumin in equivalent concentrations had no significant effect, while compstatin reduced TCC generation by 96%.

3.4.2. Fluid-phase alternative pathway (Fig. 4, lower panel)

The addition of cobra venom factor to serum led to a marked increase in TCC-generation. C1-INH reduced cobra venom factor-mediated TCC generation dose-dependently (p < 0.0001). The inhibition was 7% (p > 0.05), 59% (p < 0.001) and 87% (p < 0.001) using 2.8 ×, 14 × and 28 × physiologic concentration of C1-INH. Albumin and IgG in equivalent microgram amounts had no significant effect, while EDTA reduced TCC-generation by 99%.

3.5. Analysis of factor H in the C1-INH concentrate

The purity of the C1-INH concentrate, including a possible contamination by factor H, was assessed by SDS-PAGE and ELISA (Fig. 5). The C1-INH preparation was highly pure, containing a major protein band corresponding to the molecular weight of the active protein (105 kDa) and a weak smaller protein band corresponding to inactive C1-INH (90 kDa) (Fig. 5A). No other bands were observed; in particular no bands with molecular weight similar to factor H. Similarly, when the C1-INH preparation was tested in a sandwich ELISA for the detection of factor H, no activity was present (Fig. 5B).

4. Discussion

C1-INH’s effects on the three initiating pathways of complement varied considerably. The classical pathway solid-phase was only modestly inhibited by C1-INH when high serum dilution was used, but at low serum dilutions which are more close to the physiological conditions the effect was more pronounced. Notably, however, low C1-INH concentration only slightly reduced the activation and doses of 14–28 times physiologic concentration was needed to abolish the activation. This is somewhat surprisingly as C1-INH is the only known regulator of C1. The early works by Ziccardi (1982), however, described C1-INH as a poor inhibitor once C1 was activated by immune complexes. The speed by which immune complexes could activate C1 was believed to overwhelm C1-INH. On the other hand,
was non-immune activation of C1 efficiently controlled, and interpreted as C1-INH’s primary role. The IgM coating in the wells, used in both classical pathway solid-phase assays in this paper, is a very potent and rapid activator of C1. HAIGG added to undiluted whole blood may activate C1 even more forcefully as the highest dose of C1-INH was needed to get a substantial inhibition of fluid-phase classical pathway activation. Our results fit partly in with the findings of Doekes et al. (1983) A C1-INH/C1 ratio of 8, as in normal serum, nearly abolished soluble IgG-induced C4 consumption. The effect of increasing the C1-INH/C1 ratio from 10 to 100, nearly corresponding to our dose of 14 times physiologic concentration, was marginal. The effect of increasing C1-INH/C1 ratio from zero to normal values was, on the other hand, formidable in their study. This lead Doekes et al. (1983) to suggest that C1-INH in normal serum was needed to get a substantial inhibition of fluid-phase classical pathway activation. Our results fit partly in with the findings of Doekes et al. (1983)

Fig. 5. Purity of the C1-INH concentrate. (A) The C1-INH inhibitor preparation in two concentrations (lane 2: 5 μg; lane 3: 10 μg) and purified factor H (lane 4: 5 μg) were subjected to SDS-PAGE. C1-INH showed one major band corresponding to the active protein and one minor band (arrows) corresponding to inactivated C1-INH. No other bands were observed, in particular no corresponding to the molecular weight of factor H. lane 1: Molecular weight markers (37, 54, 98, 115 and 206 kDa). (B) The C1-INH preparation was examined in a sandwich ELISA for detection of factor H, using purified factor H as positive control. No factor H (lower detection limit below 0.1 μg/ml in this assay) could be found in the C1-INH concentrate using concentrations up to 50 μg/ml.

was non-immune activation of C1 efficiently controlled, and interpreted as C1-INH’s primary role. The IgM coating in the wells, used in both classical pathway solid-phase assays in this paper, is a very potent and rapid activator of C1. HAIGG added to undiluted whole blood may activate C1 even more forcefully as the highest dose of C1-INH was needed to get a substantial inhibition of fluid-phase classical pathway activation. Our results fit partly in with the findings of Doekes et al. (1983) A C1-INH/C1 ratio of 8, as in normal serum, nearly abolished soluble IgG-induced C4 consumption. The effect of increasing the C1-INH/C1 ratio from 10 to 100, nearly corresponding to our dose of 14 times physiologic concentration, was marginal. The effect of increasing C1-INH/C1 ratio from zero to normal values was, on the other hand, formidable in their study. This lead Doekes et al. (1983) to suggest that C1-INH in normal serum was needed to get a substantial inhibition of fluid-phase classical pathway activation. Our results fit partly in with the findings of Doekes et al. (1983)

Fig. 5. Purity of the C1-INH concentrate. (A) The C1-INH inhibitor preparation in two concentrations (lane 2: 5 μg; lane 3: 10 μg) and purified factor H (lane 4: 5 μg) were subjected to SDS-PAGE. C1-INH showed one major band corresponding to the active protein and one minor band (arrows) corresponding to inactivated C1-INH. No other bands were observed, in particular no corresponding to the molecular weight of factor H. lane 1: Molecular weight markers (37, 54, 98, 115 and 206 kDa). (B) The C1-INH preparation was examined in a sandwich ELISA for detection of factor H, using purified factor H as positive control. No factor H (lower detection limit below 0.1 μg/ml in this assay) could be found in the C1-INH concentrate using concentrations up to 50 μg/ml.

was non-immune activation of C1 efficiently controlled, and interpreted as C1-INH’s primary role. The IgM coating in the wells, used in both classical pathway solid-phase assays in this paper, is a very potent and rapid activator of C1. HAIGG added to undiluted whole blood may activate C1 even more forcefully as the highest dose of C1-INH was needed to get a substantial inhibition of fluid-phase classical pathway activation. Our results fit partly in with the findings of Doekes et al. (1983) A C1-INH/C1 ratio of 8, as in normal serum, nearly abolished soluble IgG-induced C4 consumption. The effect of increasing the C1-INH/C1 ratio from 10 to 100, nearly corresponding to our dose of 14 times physiologic concentration, was marginal. The effect of increasing C1-INH/C1 ratio from zero to normal values was, on the other hand, formidable in their study. This lead Doekes et al. (1983) to suggest that C1-INH in normal serum was needed to get a substantial inhibition of fluid-phase classical pathway activation. Our results fit partly in with the findings of Doekes et al. (1983)
A major part of C1-INH’s therapeutic success has been in animal and human ischemia-reperfusion injuries (IRI) (Bergamaschini and Cicardi, 2003). The effect we observed on the classical pathway could have been involved in IgM-dependent and probably other classical pathway-mediated mechanisms of IRI (Weiser et al., 1996; Williams et al., 1999; Zhang et al., 2004). Recently, C1-INH’s beneficial effect on cerebral IRI in mice also were found to be partly independent of C1q (De Simoni et al., 2004). The possibility of regulating the lectin pathway by C1-INH is attractive as this pathway was found to be central in, e.g. murine myocardial IRI (Jordan et al., 2001), murine renal IRI (de Vries et al., 2004) and IRI after human thoraco-abdominal aneurism repair (Fiane et al., 2003). The latter paper also described how the lectin pathway, once activated, was amplified by the alternative pathway. The role of the alternative pathway as amplification loop was recently documented also for the classical pathway, showing that more than 80% of terminal pathway activation induced by the classical pathway was indeed mediated through alternative amplification (Harboe et al., 2004). Thus, in vivo, the initial activation mechanism through classical and/or lectin pathway activation in IRI may be greatly enhanced through the alternative pathway. Furthermore, a recent study suggested an important and primary role for the alternative complement pathway in local and remote tissue injury after gastrointestinal IRI in mice (Stahl et al., 2003). Our data indicate that C1-INH in high physiologic concentration may have a selective impact on all three complement activation pathways.

Many earlier and even current demonstrations of C1-INH’s efficiency in various pathophysiological and clinical conditions were often and intuitively ascribed to inhibition of the classical pathway. Our findings, suggesting a potent inhibition by C1-INH of the lectin pathway, emphasize this pathway to be considered, in particular since C4 is activated both through the classical and lectin pathway and should not be considered specific for the classical. Furthermore, our findings of inhibition of the alternative pathway also have to be taken into account when interpreting C1-INH’s effects. Additionally, C1-INH may regulate processes in which proteases are not involved (Liu et al., 2005; Davis et al., 2004). Finally, complement is but one of several important biological cascades where C1-INH is an important regulator. Notably, coagulation, fibrinolysis and the kallikrein–kinin system are also involved in inflammation and IRI (Schoenmakers et al., 2005; Souza et al., 2003). In several diseases a tailored therapy of the immune system is wanted. In human diseases in which cascade system activation is undesired, C1-INH in obtainable doses could be useful.

5. Conclusions

The present data indicate that C1-INH in supraphysiologic doses in human serum inhibits solid-phase classical- and lectin pathway activation in a similar manner, but with a more pronounced effect on the lectin pathway at low doses. C1-INH also inhibited alternative pathway activation, but this was limited to fluid-phase cobra venom factor mediated activation. To our knowledge this is the first study documenting and comparing the effect of C1-INH on all three complement pathways under similar conditions. The data have implications for the interpretation of previous studies using C1-INH to modulate the inflammatory reaction and for C1-INH as a therapeutic agent in the future.

Acknowledgements

Financial support was provided by Sigvald Bergesen D.Y. and wife Nanki’s Foundation, The Family Blix Foundation, The Norwegian Foundation for Health and Rehabilitation, The Norwegian Council on Cardiovascular Disease and NIH Grant R01-EF-003968-01.

References


