Epitope Specificity and IgG Subclass Distribution of Autoantibodies to Cardiac Troponin

Tanja Savukoski,1* Aleksandra Twarda,1 Sanna Hellberg,1 Noora Ristiniemi,1 Saara Wittfooth,1 Juha Sinisalo,2 and Kim Pettersson1

BACKGROUND: Autoantibodies to cardiac troponins (cTnAAbs) can interfere with the measurement of cardiac troponin I (cTnI) by immunoassays for the diagnosis of myocardial infarction. Therefore, we determined the cTnI binding sites and IgG subclasses of circulating cTnAAbs.

METHODS: We studied epitope specificity with sandwich-type immunoassays by measuring the recovery of troponin complex added to 10 cTnAAb-negative and 10 cTnAAb-positive sera from healthy volunteers. To study the IgG subclasses, we analyzed admission and 3-month follow-up sera from chest pain patients with a reference assay measuring total IgG (14 cTnAAb negative and 14 cTnAAb positive at 3 months) and with 4 subclass-specific assays measuring exclusively IgG subclasses 1–4.

RESULTS: Mean recoveries of troponin complex in cTnAAb-positive samples for single cTnI epitopes ranged from 37% to 211%, being lowest for the cTnI midfragment (aa 30–110). However, the lowest sample-specific recoveries, 4%–92%, showed that none of the studied epitopes completely escaped the cTnAAb-related interference. Eight chest pain patients of the cTnAAb-positive group became positive between sampling points, and according to all 5 cTnAAb assays, specific signals were generally higher at follow-up. IgG4, with the highest prevalence, was detected in 68% of samples in the cTnAAb-positive group.

CONCLUSIONS: IgG subclass studies confirm that cTnAAb formation may be triggered/boosted in acute cardiac events. This new information about the epitope specificity of cTnAAbs should be used to reevaluate existing recommendations regarding use of midfragment epitopes in cTnI assays. To circumvent the negative interference of the highly heterogeneous cTnAAbs, use of 3 or more unconventionally selected epitopes should be considered.

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REFERENCES:
1. Ternary troponin complex, consisting of subunits I, T, and C, is part of the myofibril contractile apparatus in striated muscle cells. Because 2 of these intracellular proteins, troponin I and T, exist as different isoforms in skeletal and cardiac muscle, the measurement of cardiac-specific isoforms (cardiac troponin) in blood serves as the most sensitive means to prove a recent or persistent myocardial injury. This has made cardiac troponins the recommended biomarkers for the diagnosis of acute coronary syndrome (ACS)3.

2. Cardiac troponin–specific autoantibodies (cTnAAbs), however, can interfere with the detection of these clinically important biomarkers by cTnI assays designed according to the IFCC-recommended midfragment approach used in clinical practice (2–4). cTnAAbs have been found in a high proportion (5%–20%) of individuals with or without cardiac diseases (5–10), and cardiac troponin–specific autoimmune response leading to autoantibody formation can be triggered by any release of cardiac proteins following myocardial injury, for example, after inflammation, ischemia, or cardiotoxic treatments. Nevertheless, the mechanisms for their appearance and maintenance are poorly understood.

3. Besides being able to interfere with cardiac troponin detection by immunoassays, cTnAAbs may have clinical significance. Recent studies using a mouse model have associated circulating cTnI-specific autoantibodies with myocardial infarction and impaired cardiac function.

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The results of epitope specificity studies have been presented as a poster at the 10th International Congress on Coronary Artery Disease in Venice, 2011, and the results of IgG subclass distribution studies at the 21st International Congress of Clinical Chemistry and Laboratory Medicine/19th IFCC-EFCC European Congress of Clinical Chemistry and Laboratory Medicine in Berlin, 2011.

Received August 16, 2012; accepted November 27, 2012.
Previously published online at DOI: 10.1373/clinchem.2012.194860

3 Nonstandard abbreviations: ACS, acute coronary syndrome; cTnAAb, cardiac troponin–specific autoantibody; MAb, monoclonal antibody; SA, streptavidin; ITC, cardiac troponin complex; TSA, Tris-buffered saline with azide; BSA, bovine serum albumin; ILL, insulation layer II.
cTnAAbs with inflammation of the heart muscle and the emergence of symptoms indicative of cardiac disease. These studies have shown that the administration of cTnI-specific monoclonal antibodies (MAbs) induces autoimmune dilated cardiomyopathy in wild-type mice (11) and that provocation of an autoimmune response to cTnI, but not cTnT, leads to increased myocardial inflammation and cardiac dysfunction (12, 13). Moreover, the relatively mild cardiac damage and cTnI release in the acute phase of experimental coxsackievirus B3–induced myocarditis gives rise to cTnAAbs formation (14).

The possible clinical consequences of circulating cTnAAbs have also been studied in humans. A recent study (10) demonstrated that the presence of cTnAAbs may lead to a higher and longer cTnI release in ACS patients. Another study on ACS patients (15) indicated that cTnI-specific cTnAAbs may have a negative impact on the recovery of left ventricular function. Nevertheless, some groups have reported that the outcome of cTnAAb-positive patients was not inferior or was even better than that of cTnAAb-negative patients with ischemic cardiomyopathy, dilated cardiomyopathy, heart failure, and/or ACS (6, 16–19).

The role of cTnAAbs in the etiology of cardiac diseases and the reason for their occurrence even in apparently healthy individuals is not known. Actually, cardiac troponin–specific autoimmunity seems to appear only in certain individuals, and the vast majority of cardiac patients do not develop it (18, 19). In addition, the possible usefulness of cTnAAbs as diagnostic and/or prognostic biomarkers is unclear in the absence of adequately sized studies with long-term follow-up. Because of lack of detailed data on the molecular characteristics of human cTnAAbs, our purpose in this study was to explore more precisely the location of cTnI binding sites and the IgG subclass distribution of cTnAAbs.

Materials and Methods

REAGENTS

All MAbs except 8I7 (International Point of Care, www.ipocdx.com) were kindly provided by HyTest (www.hytest.fi). A summary of MAbs used and their antigens is presented in Table 1. This information was obtained from the manufacturers’ package inserts. We purchased streptavidin (SA)-coated microtiter plates from Kaivogen Oy (www.kaivogen.com) and human cardiac troponin complex (ITC) from HyTest. ITC was diluted into Tris-buffered saline with azide (TSA) (50 mmol/L Tris-HCl, pH 7.75, 150 mmol/L NaCl, 0.5 g/L NaN₃) containing 75 g/L bovine serum albumin (BSA) (Millipore/Intergen, www.millipore.com), and the stocks were stored at −20 °C until use.

Table 1. Summary of the MAbs and their antigens.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Antigen</th>
<th>Binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4–14G6</td>
<td>cTnI</td>
<td>aa 1–15</td>
</tr>
<tr>
<td>23C6</td>
<td>cTnI</td>
<td>aa 15–25</td>
</tr>
<tr>
<td>4C2</td>
<td>cTnI</td>
<td>aa 23–29</td>
</tr>
<tr>
<td>M155</td>
<td>cTnI</td>
<td>aa 26–35</td>
</tr>
<tr>
<td>10F4</td>
<td>cTnI</td>
<td>aa 34–37</td>
</tr>
<tr>
<td>19C7</td>
<td>cTnI</td>
<td>aa 41–49</td>
</tr>
<tr>
<td>247</td>
<td>cTnI</td>
<td>aa 65–74</td>
</tr>
<tr>
<td>560</td>
<td>cTnI</td>
<td>aa 83–93</td>
</tr>
<tr>
<td>8E10</td>
<td>cTnI</td>
<td>aa 86–90</td>
</tr>
<tr>
<td>415</td>
<td>cTnI</td>
<td>aa 104–119</td>
</tr>
<tr>
<td>84</td>
<td>cTnI</td>
<td>aa 117–126</td>
</tr>
<tr>
<td>M46</td>
<td>cTnI</td>
<td>aa 130–145</td>
</tr>
<tr>
<td>817</td>
<td>cTnI</td>
<td>aa 137–148</td>
</tr>
<tr>
<td>581</td>
<td>cTnI</td>
<td>aa 143–152</td>
</tr>
<tr>
<td>441</td>
<td>cTnI</td>
<td>aa 148–158</td>
</tr>
<tr>
<td>625</td>
<td>cTnI</td>
<td>aa 169–178</td>
</tr>
<tr>
<td>472*</td>
<td>cTnI</td>
<td>aa 182–191</td>
</tr>
<tr>
<td>MF4</td>
<td>cTnI</td>
<td>aa 190–196</td>
</tr>
<tr>
<td>p45–19</td>
<td>cTnI</td>
<td>aa 195–209</td>
</tr>
<tr>
<td>7B9</td>
<td>TnC</td>
<td></td>
</tr>
<tr>
<td>3D3</td>
<td>Human IgG</td>
<td>Constant region</td>
</tr>
<tr>
<td>2C11</td>
<td>Human IgG1</td>
<td>Constant region</td>
</tr>
<tr>
<td>3C7</td>
<td>Human IgG2</td>
<td>Hinge region</td>
</tr>
<tr>
<td>5G12</td>
<td>Human IgG3</td>
<td>Hinge region</td>
</tr>
<tr>
<td>5C7</td>
<td>Human IgG4</td>
<td>Constant region</td>
</tr>
</tbody>
</table>

Binding sites or epitopes are also given if they were specified by the manufacturer. aa, amino acids.

* Cross-reaction with skeletal TnI >50%.

LABELING OF ANTIBODIES WITH BIOTIN AND LANTHANIDE CHELATE

Antibodies were labeled with biotin or intrinsically fluorescent europium chelate as described previously (5). Capture antibodies were biotinylated with a 10- to 30-fold molar excess of biotin isothiocyanate (a gift from Jaana Rosenberg, Department of Biotechnology, University of Turku, Finland), and detection antibodies were labeled with a 25- to 35-fold molar excess of Eu-chelate. Biotinylated or Eu(III)-labeled MAbs were stabilized with BSA (1 g/L) and stored at 4 °C.
Characterization of Autoantibodies to Cardiac Troponin

IMMUNOASSAYS FOR MEASURING THE ANALYTICAL RECOVERY OF ITC COMPLEX
We determined the binding sites of cTnAAbs on the cTnI molecule by comparing the analytical recoveries of ITC in cTnAAb-positive samples to the recoveries in cTnAAb-negative samples. We measured the fluorescence signal from each unspiked and ITC-spiked serum sample with sandwich-type immunoassays using various cTnI-specific MAbs for capture and the same troponin C–specific MAb as a tracer. First, 300 ng biotinylated capture was immobilized to SA-coated microtiter wells in 25 μL Kaivogen buffer solution with approximately 1-h incubation at ambient room temperature. Meanwhile, ITC was added into serum aliquots to a final concentration of 30 μg/L cTnI and incubated for 1 h at 4 °C. After washings, 20 μL serum aliquots (ITC-spiked and unspiked of every serum sample) and 100 ng Eu-labeled 7B9 MAb in 20 μL incubation layer II (III, Radiometer/Innotrac Diagnostics Oy) were added into triplicate wells. The wells were incubated for 1 h at 36 °C, 900 rpm in a plate shaker (iEMS incubator/shaker, Thermo Electron/Labsystems, www.thermoscientific.com). The washed wells were then dried, and we measured the time-resolved fluorescence directly from the surface with a Victor ×4 Multilable Counter (Perkin-Elmer/Wallac, www.perkinelmer.com). Finally, we calculated sample specific recoveries for different capture epitopes by comparing the ITC-specific signal of each sample to the mean signal of cTnAAb-negative samples.

IMMUNOASSAYS FOR DETECTING HUMAN cTnAAbS
Serum samples were diluted 5-fold with ILII, and the fluorescence signal was measured from each diluted sample with and without ITC addition (30 μg/L cTnI). After binding the possible cTnAAb to ITC complex (1 h, >4 °C), 30 μL sample and 200 μL Kaivogen buffer solution supplemented with 10 mg/L native and 5 mg/L denatured mouse IgG (Meridian Life Science/BioDesign International, www.meridianlifescience.com) were added into triplicate SA wells preimmobilized with 100 ng of each biotinylated capture antibody (MF4, 4C2, and 7B9). The wells were incubated for 1 h at 36 °C, 900 rpm, and then washed. Subsequently, Eu-labeled detection antibodies (50–150 ng) were added into wells in 200 μL of Kaivogen buffer solution supplemented with mouse IgG. We used MAb 3D3 as a tracer in total IgG assay detecting all IgG subclasses and MAbs 2C11, 3C7, 5G12, and 5C7 in 4 subclass-specific assays detecting exclusively IgG subclasses 1–4, respectively. The wells were incubated for another 1 h at 36 °C, and finally the fluorescence was measured. Specific signals ≥100 counts were regarded as positive if the Student t-test gave a P value < 0.05 when the signals obtained from the same sample with and without added ITC were compared.

SAMPLES
The study protocols were approved by the local ethics committees. Informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki of 1975 as revised in 2006.

We performed epitope specificity studies with serum samples collected from apparently healthy volunteers (n = 20) at the Department of Biotechnology, University of Turku, between 2010 and 2011. Ten of these samples were known to be cTnAAb negative and 10 cTnAAb positive according to the previously published cTnAAb assay (10, 21). Briefly, cTnAAbS of the serum sample were first bound to ITC, and the formed ITC-cTnAAb complexes were subsequently captured on an SA-coated microtiter well preimmobilized with 150 ng biotinylated MAbs 4C2 and MF4. Finally, we detected captured cTnAAbS with 40 ng Eu-labeled 3D3 MAb. A sample was defined to be negative or positive as described above.

We analyzed total IgG and IgG subclasses in admission and 3-month follow-up serum samples from 28 chest pain patients from a substudy (10) of 81 non–ST-elevation ACS patients. Samples were collected between 1998 and 2000 from 9 different central hospitals in Finland for a placebo-controlled study on clarithromycin treatment of ACS patients, as described earlier (22). The samples were stored at −70 °C and had been thawed a few times before the cTnAAb measurements. We used the total IgG assay to categorize the patients into 2 study groups: the first group included 14 cTnAAb-negative patients, and the second group 14 cTnAAb-positive patients at 3-month follow-up.

STATISTICAL ANALYSIS
We analyzed differences in IgG-specific signals between sampling points with PASW Statistics 18 software (SPSS, www-01.ibm.com/software/analytics/spss) using the Wilcoxon signed ranks test. All cTnAAb-negative samples were given a value of 50 counts (the lowest positive signal divided by 2). P values < 0.05 were considered statistically significant.

Results
To evaluate the cTnAAb binding sites on the cTnI molecule, we measured ITC recovery with 19 different cTnI-specific capture antibodies using the same troponin C–specific antibody as a tracer. Although the mean ITC recovery in cTnAAb-positive samples for all 19 epitopes was 89%, mean recovery values for single cTnI epitopes ranged from 37% to 211% (Fig. 1A). The
Fig. 1. Analytical recoveries of ITC for different cTnI sites.
(A), Analytical recoveries in 10 cTnAAb-positive samples. Whiskers represent minimum and maximum signals; boxes represent 25th percentile, median, and 75th percentile; and squares represent means. (B), An example of site-specific recoveries of ITC in 3 cTnAAb-positive samples.
lowest mean recoveries were obtained for epitopes on the midfragment and N-terminal parts of the C-terminus. In addition, we found considerable variation in the individual recoveries of cTnAAb-positive samples: the minimum and maximum recoveries for single epitopes ranged from 4% to 92% and 78% to 309%, respectively. The comparison between the sitespecific recoveries of 3 cTnAAb-positive subjects in Fig. 1B demonstrates that interindividual variation in interference differs between epitopes.

To study the IgG subclasses of cTnAAbs, we analyzed the cTnAAbs with total IgG and 4 subclass-specific assays. All 14 chest pain patients chosen for the cTnAAb-negative group were cTnAAb negative by the total IgG assay at both sampling points. In the 14 patients chosen for the cTnAAb-positive group, 6 were positive at admission and 8 reached positivity at follow-up. The specific signals of these IgG-positive samples ranged from 105 to 69 502 counts (median 2 111 counts). As illustrated in Table 2, none of the samples in the cTnAAb-negative group were IgG1 or IgG2 positive, but 4 admission and 5 follow-up samples were weakly positive for IgG3 and/or IgG4 (specific signal 125–794 counts, median 242). Although all IgG subclasses 1–4 were observed in the cTnAAb-positive group (specific signal 101–115 579 counts, median 620), IgG4 had the highest prevalence and was detected in 8 admission and 11 follow-up samples. In addition, 3 admission and 10 follow-up samples from the cTnAAb-positive group were positive simultaneously for 2–4 subclasses (Table 3). According to all 5 IgG assays studied, the specific signals were generally higher at follow-up compared to the admission levels, indicating higher cTnAAb titers and/or improved binding affinity (Fig. 2). Differences between sampling points were statistically significant ($P < 0.05$), with all assays except the IgG3-specific assay ($P = 0.249$).

**Table 2. Prevalence (n) of cTnAAb-positive samples with total IgG and 4 subclass-specific assays in 2 study groups at admission and 3 months’ follow-up.**

<table>
<thead>
<tr>
<th>Assay specificity</th>
<th>cTnAAb negative (n = 14)</th>
<th>cTnAAb-positive (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Admission</td>
<td>3 months</td>
</tr>
<tr>
<td>Total IgG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgG1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgG2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgG3</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>IgG4</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 3. Number of samples with 1–4 observed IgG subclasses in the cTnAAb-positive cohort.**

<table>
<thead>
<tr>
<th>Number of observed IgG subclasses per sample</th>
<th>Admission</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**Discussion**

Currently the reasons and mechanisms for initiation and maintenance of cTnAAbs are unclear, and their possible clinical and diagnostic significance remains...
open. Although cTnAAbs have been established as an important analytical confounder in cTnl assays, detailed data on their molecular characteristics are scarce. Therefore, the aim of this study was to characterize the epitope specificity and IgG subclass distribution of these autoantibodies.

Our epitope specificity studies confirm the previously published observation (23) that the stable midfragment of the cTnl molecule is frequently targeted by cTnAAbs but also demonstrate that the cTnAAb interference extends beyond the midfragment, in particular toward the C-terminus. Although some epitopes may be less prone to cTnAAb-related interferences, our ITC recovery data for 10 cTnAAb-positive samples show that none of the studied epitopes completely escaped it. In addition, since the area of strongest interference varied between different individuals, the use of 3 or more carefully selected binding sites should be considered in future cTnl assays.

Besides being able to block the binding of assay antibodies, the observed recoveries clearly >100% imply that cTnAAbs may also stabilize some epitopes or enhance the affinity of assay antibodies against their binding sites. Nevertheless, our results from this study confirm our recently published conclusion that novel cTnl assays based on antibody configurations different from those derived by the IFCC-recommended midfragment approach improve cTnl detection in cTnAAb-positive samples and also prove our preliminary assumption that the cTnl-specific interference is even more heterogeneous than initially reported (4).

Eight chest pain patients of the cTnAAb-positive group became positive between sampling points. Additionally, specific signals with all 5 cTnAAb assays were generally higher 3 months after the index event. These data confirm that cardiac troponin leakage from cardiomyocytes during a primary ischemic injury may induce an autoimmune response leading to cTnAAb formation (6, 10). Alternatively, the leakage may serve as a booster of a previous immunization, which can both increase cTnAAb titers in blood and improve the affinity of formed autoantibodies.

In the cTnAAb-positive group, all IgG subclasses were detected and 46% of individual sera were positive for multiple subclasses. Because the frequency of cTnAAb-positive samples observed with certain subclass-specific assays is related to assay sensitivity, direct comparison between these frequencies is not possible. However, it is noteworthy that IgG4, which is generally the least frequent of the 4 IgG subclasses, was the most commonly detected cTnAAb in this study (found in 68% of samples in the cTnAAb-positive group). Because repeated antigenic stimulation is known to promote IgG4-type antibody formation, the appearance of IgG4-type cTnAAbs suggests that longer-term cardiac troponin leakage may have occurred in cardiac patients before the acute event. Also interesting is that IgG4 is frequently considered to be a benign and nonpathogenic antibody with even positive effects in some allergic reactions (24). Both of these points may be linked to the observation that as a result of the half-antibody exchange reaction (Fab-arm exchange), most IgG4-type antibodies are bispecific antibodies with two different antigen-binding sites that make the molecules functionally monovalent for a given antigen (25). Thus the high prevalence of IgG4-type cTnAAbs may also explain why our attempts to develop bridge-type immunoassays that use the same antigen as a capture and a tracer for cTnAAb detection have not succeeded (results not shown). Another feature of IgG4-type antibodies is that they may have a high affinity for various animal IgGs, such as mouse IgG, via their constant regions rather than their antigen-binding sites (26, 27). If cardiac troponin–specific IgG4s bind to animal IgGs used as immunoassay reagents, then in addition to blocking specific cardiac troponin epitopes, they could potentially interfere with cardiac troponin immunoassays nonspecifically.

This study was limited in investigating only the IgG-type antibodies; although IgM-type antibodies are predominant in a primary immune response, IgM response was not studied. The discrepancy between total IgG and subclass-specific assay results illustrates the challenges in autoantibody assays. First, different affinities of tracer antibodies lead to varying assay sensitivity, which cannot be determined due to the lack of defined standards. Second, as demonstrated with our epitope specificity studies, cTnAAbs form a heterogeneous population in which precise epitope specificity and binding affinity against cardiac troponin may vary widely. In addition, the use of different assay formats makes direct comparison between different reports impossible. Although many published cTnAAb assays use directly coated cTnl or cTnT surfaces, an advantage in our assays is that we correct the results for the nonspecific binding of other antibodies by measuring the sample-specific background, which seem to fluctuate substantially between individuals (21). On the other hand, because we have used ITC as a target molecule in our cTnAAb assays, we cannot differentiate cTnl- and cTnT-specific autoantibody positivity.

In conclusion, because cTnAAbs are common even in apparently healthy individuals and the cTnAAb formation may be triggered or boosted in acute cardiac events, their analytical interference effects should be acknowledged in the design of future cTnl assays. The midfragment of the cTnl molecule is usually the only
Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honorary: None declared.
Research Funding: T. Savukoski, DIA-NET, the Graduate School of Advanced Diagnostic Technologies and Applications.
Expert Testimony: None declared.
Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We gratefully acknowledge Pirjo Laaksonen for excellent technical assistance at the Department of Biotechnology, University of Turku.

References


Characterization of Autoantibodies to Cardiac Troponin

Authors: K. Pettersson, S. Eriksson, S. Wittfoth, E. Engstrom, T. Savukoski

In this study, the authors investigated the characterization of autoantibodies to cardiac troponin (cTnI). The authors confirmed that autoantibodies are present in healthy blood donors and are associated with dilated cardiomyopathy. The research detailed the interference of these autoantibodies in cardiac troponin I immunoassays, highlighting the importance of understanding their role in clinical diagnostics. The study also underscores the need for further research into the nature of these autoantibodies and their potential clinical implications.

The authors concluded that the presence of autoantibodies to cardiac troponin I is significant in the context of dilated cardiomyopathy and other cardiac conditions, emphasizing the importance of developing robust immunoassays to accurately measure cardiac troponin levels in clinical practice.